TRIM24-REGULATED ESTROGEN RESPONSE IS DEPENDENT ON SPECIFIC HISTONE MODIFICATIONS IN BREAST CANCER CELLS

Teresa T. Yiu

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TRIM24-REGULATED ESTROGEN RESPONSE IS DEPENDENT ON SPECIFIC HISTONE MODIFICATIONS IN BREAST CANCER CELLS

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TRIM24-REGULATED ESTROGEN RESPONSE IS DEPENDENT ON SPECIFIC HISTONE MODIFICATIONS IN BREAST CANCER CELLS

A DISSERTATION

Presented to the Faculty of
The University of Texas
Health Science Center at Houston
and
The University of Texas
M.D. Anderson Cancer Center
Graduate School of Biomedical Sciences
in Partial Fulfillment
of the Requirements
for the Degree of

DOCTOR OF PHILOSOPHY

By
Teresa Tingting Yiu, B.S.
Houston, Texas
December, 2012
DEDICATION

This thesis is dedicated to my beloved father, in memory of the sacrifice of his life to cancer in 2008, to whom I promised to persevere at the pursuance of this degree and dedicate my career to cancer research.

I thank the Creator for giving me the burden to help the unfortunate ones and the opportunity to take flight and solve this complicated human disease at M.D. Anderson Cancer Center, a world-renowned cancer institute.

Because of the unconditional love, endless support and encouragement from my family, friends, advisor, committees, and lab-mates, I have sustained till this point, and am ready to start a whole new chapter of my life, to be filled with exciting research discoveries in making this deadly disease history.
ACKNOWLEDGEMENT

First I would like to acknowledge the Center for Cancer Epigenetics at M.D. Anderson Cancer Center, especially Director Dr. Sharon Dent, for funding my first year's education. I am also thankful for the generous contribution from NIH T32 Training Grant and the American Legion Auxiliary Fellowship.

I am extremely thankful for my advisor, Dr. Michelle Barton, for the opportunity to learn and mature in experimental knowledge, critical thinking and communication. She has opened up many opportunities in my research, inspired and assisted me tremendously in the past three years. I am thankful for her patience, encouragement, and the freedom to pursue my research interest in her laboratory. Most importantly, because of her support, I am able to have a balanced life between work and family.

I would like to acknowledge members of my present and past committees, for their patience, guidance, scientific discussion, intuitive comments and time commitment: Drs. Sharon Dent, Gary Gallick, Guillermina Lozano, Pierre McCrea, Gary Gallick, Xiaobing Shi, Jessica Tyler, Shinoko Takata, and Bin Wang. I especially thank Dr. Gary Gallick for his encouragement and inspiration, which greatly helped me to persist in pursuing science; Drs. Pierre McCrea and Bin Wang for their guidance in fulfilling my candidacy exam requirements; Dr. Xiaobing Shi for sharing his ideas and experimental skills when I was exploring the field of epigenetics. I would also like to thank present and past members of Barton laboratory, who have assisted in my experiments, inspired my research, and encouraged me, especially when my goals seem impossible to accomplish.
TRIM24-REGULATED ESTROGEN RESPONSE IS DEPENDENT ON SPECIFIC HISTONE MODIFICATIONS IN BREAST CANCER CELLS

Publication No. __________

Teresa Tingting Yiu, B.S.
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In this dissertation, I discovered that function of TRIM24 as a co-activator of ERα-mediated transcriptional activation is dependent on specific histone modifications in tumorigenic human breast cancer-derived MCF7 cells. In the first part, I proved that TRIM24-PHD finger domain, which recognizes unmethylated histone H3 lysine K4 (H3K4me0), is critical for ERα-regulated transcription. Therefore, when LSD1-mediated demethylation of H3K4 is inhibited, activation of TRIM24-regulated ERα target genes is greatly impaired. Importantly, I demonstrated that TRIM24 and LSD1 are cyclically recruited to estrogen responsive elements (EREs) in a time-dependent manner upon estrogen induction, and depletion of their expression exert corresponding time-dependent effect on target gene activation. I also identified that phosphorylation of histone H3 threonine T6 disrupts TRIM24 from binding to the chromatin and from activating ERα-regulated targets. In the second part, I revealed that TRIM24 depletion has additive effect to LSD1 inhibitor- and Tamoxifen-mediated reduction in survival and proliferation in breast cancer cells.
TABLE OF CONTENTS

DEDICATION ........................................................................................................ iii
ACKNOWLEDGEMENT ........................................................................................ iv
TABLE OF CONTENTS ......................................................................................... vi
LIST OF FIGURES .............................................................................................. xi
LIST OF ABBREVIATIONS .................................................................................... xv

CHAPTER 1: GENERAL INTRODUCTION .............................................................. 1
  1.1.1. Epigenetics control literally all DNA-templated processes in the cell ......1
  1.1.2. Histone acetylation opens up chromatin for transcriptional activation … 3
  1.1.3. Histone methylation and transcription activation/repression ............... 4
  1.1.4. Histone phosphorylation cooperates with nearby PTMs .................... 6
  1.1.5. The histone code hypothesis ................................................................. 6
  1.1.6. Histone binding modules decode the histone language .................... 7
  1.1.7. The RBCC protein family member TRIM24 ......................................11
  1.1.8. TRIM24 regulates nuclear receptor-mediated signaling pathways ...... 13
  1.1.9. TRIM24 associates with chromatins and remodeling proteins ..........14
  1.1.10. Histone demethylase 1 (LSD1) specifically demethylates H3K4me1/2 ........................................................................................................15
  1.1.11. LSD1 mediates transcription repression ......................................... 18
  1.1.12. LSD1 plays important roles during development and differentiation...18
  1.2. AIMS OF THIS WORK ............................................................................. 20

CHAPTER 2: ACTIVATION OF TRIM24-REGULATED ERα TARGET GENES IS DEPENDENT ON LSD1-MEDIATED H3K4ME2 DEMETHYLATION ....... 22

  2.1. INTRODUCTION ......................................................................................... 22
    2.1.1. Estrogen receptor-alpha (ERα) is the master transcriptional regulator..22
    2.1.2. ERα and the transcriptional machinery ..............................................23
2.1.3. Histone modifying enzymes as ERα co-regulators ......................... 25
2.1.4. Chromatin remodeling and estrogen response ............................. 26
2.1.5. Potential involvement of histone chaperones in ERα regulation .... 29
2.1.6. Timely cyclical recruitment of ERα and its co-factors ................. 29
2.1.7. TRIM24 functions as an ERα co-activator through chromatin
    recognition .................................................................................. 31
2.1.8. TRIM24 simultaneously recognizes two histone signatures on H3.... 34
2.1.9. TRIM24 preferentially binds to regions depleted of H3K4me2 ........ 35
2.1.10. LSD1 as a transcription co-activator in androgen and
    estrogen receptors-mediated signaling ......................................... 37
2.1.11. H3T6ph inhibits H3K4 demethylation mediated by LSD1 and
    chromatin binding by H3K4me0 reader proteins .......................... 38
2.1.12. *Hypothesis*: Function of TRIM24 is dependent on LSD1-mediated
    H3K4 demethylation ..................................................................... 39

2.2. MATERIALS AND METHODS .......................................................... 41
2.2.1. Cell culture .............................................................................. 41
2.2.2. Mutagenesis ........................................................................... 41
2.2.3. Transient DNA plasmid transfection ........................................ 41
2.2.4. Transient knockdown by siRNAs ............................................ 42
2.2.5. RNA extraction, cDNA, and real-time-RT-PCR ......................... 42
2.2.6. Chromatin immunoprecipitation (ChIP) .................................. 43
2.2.7. GST-tagged protein expression and purification ....................... 46
2.2.8. Biotinylated peptide pulldown assay ....................................... 47

2.3. RESULTS ....................................................................................... 48
2.3.1. Estrogen triggers immediate dynamic histone modifications ........ 48
2.3.2. TRIM24 expression is essential for timely estrogen response ........ 55
2.3.3. Recruitment of LSD1 and TRIM24 and changes of H3K4 methylation at GREB1 ERE upon estrogen induction .................. 64
2.3.4. Recruitment of LSD1 and TRIM24 and changes of H3K4 methylation at PR ERE upon estrogen induction .................. 68
2.3.5. Recruitment of LSD1 and TRIM24 and changes of H3K4 methylation at pS2 ERE upon estrogen induction .................. 71
2.3.6. LSD1 enzymatic activity is critical for ERα-mediated transcription .... 75
2.3.7. Chromatin-binding ability of TRIM24 is dependent on the enzymatic activity of LSD1 .......................... 89
2.3.8. H3T6ph disrupts TRIM24 from binding to histone peptide ............ 107
2.3.9. H3T6ph and its potential role in ERα target gene activation .......... 111

2.4. FUTURE DIRECTIONS AND PRELIMINARY RESULTS .................. 119
2.4.1. Does depletion of LSD1 also exert time-specific effect? .......... 119
2.4.2. Does TRIM24 and LSD1 physically interact and when? .......... 119
2.4.3. Role of H3T6 phosphorylation in regulating estrogen response ..... 120

2.5. DISCUSSIONS ................................................. 121
2.5.1. Immediate E2-induced changes of chromatin structure at enhancers ............................................... 121
2.5.2. Cyclical recruitment of LSD1 and concurrent changes in H3K4me .. 122
2.5.3. TRIM24 binding not always concurrent with H3K4me2
demethylation ................................................. 123
2.5.4. Inhibition of LSD1 does not affect H3K4me3 or H3K9me2 .......... 124
2.5.5. Establishment of the role of H3T6ph in ERα-regulated transcription ............................................... 124

viii
CHAPTER 3: BIOLOGICAL SIGNIFICANCE OF TRIM24- AND LSD1-MEDIATED ER-ALPHA CO-ACTIVATION IN BREAST CANCER CELLS ........................................................................................................ 126

3.1. INTRODUCTION ........................................................................................................ 126

3.1.1. Epigenetics and cancers .................................................................................. 126

3.1.2. Implication of histone methylation in oncogenesis ................................. 127

3.1.3. Aberrant expression of TRIM24 is correlated with tumorigenesis .... 130

3.1.4. Possible roles of TRIM24 in breast cancer transformation and cell cycle regulation .................................................................................................................. 131

3.1.5. Roles of LSD1 in tumorigenesis ................................................................. 132

3.1.6. Hypothesis: Functions of TRIM24 for the survival and proliferation of breast cancer cells in dependent on the enzymatic activity of LSD1 ........................................................................................................ 138

3.2. MATERIAL AND METHODS .............................................................................. 140

3.2.1. Clonogenic assay ....................................................................................... 140

3.2.2. Statistical analysis ..................................................................................... 140

3.3. RESULTS ............................................................................................................. 141

3.3.1. Activated ERα is required for the survival and proliferation of MCF7 ................................................................................................................................. 142

3.3.2. Depletion of TRIM24 affects survival and proliferation of MCF7 .... 143

3.3.3. Inhibition of LSD1 by TCP affects survival and proliferation of MCF7 ................................................................................................................................. 143

3.3.4. Knockdown of TRIM24 is highly additive to TCP- and 4-OHT-induced inhibition in colony formation ................................................................. 149

3.4. DISCUSSIONS AND FUTURE DIRECTIONS ............................................. 154

3.4.1. TRIM24 affects breast cancer cell survival and proliferation ....... 154
3.4.2. Correlation of TCP effectiveness and TRIM24 expression? ........ 154
3.4.3. HDAC inhibitors and TRIM24 knockdown? ............................. 155
3.4.5. Biological functions of LSD1 and TRIM24 in vivo? ..................... 156

CHAPTER 4: CONCLUSION .................................................................. 157
REFERENCES .................................................................................... 160
VITA ...................................................................................................... 190
LIST OF FIGURES

Figure 1-1: Overview of epigenetic regulator mechanisms ............................. 2
Figure 1-2: Covalent histone modifications mediated by epigenetic enzymes .... 5
Figure 1-3: Readers of histone modifications .................................................. 9
Figure 1-4: TRIM24 protein domains ................................................................. 12
Figure 1-5: LSD1 protein domains ................................................................. 16
Figure 1-6: Postulated chemical reactions for LSD1-catalyzed demethylation of H3K4me2 ................................................................. 17

Figure 2-1: Protein complexes involved in estrogen-induced activation of pS2 gene ................................................................................................. 24
Figure 2-2: Coactivators of estrogen receptor-alpha ..................................... 28
Figure 2-3: ERα and TRIM24 are recruited together to EREs upon estrogen induction .............................................................................................. 32
Figure 2-4: Depletion of TRIM24 decreases ERα binding to ERE and estrogen-activated gene induction ............................................................... 33
Figure 2-5: TRIM24 binds to regions depleted of H3K4me2 ....................... 36
Figure 2-6: Total H3 decreases immediately upon estrogen treatment ........ 49
Figure 2-7: Changes of H3K23ac levels upon estrogen treatment ............... 50
Figure 2-8: Changes of H3K27ac levels upon estrogen treatment ............... 51
Figure 2-9: Recruitment of ERα upon estrogen treatment ......................... 52
Figure 2-10: Changes of H3K4me2 levels upon estrogen treatment ............ 53
Figure 2-11: Changes of H3K4me3 levels upon estrogen treatment ............ 54
Figure 2-12: Ectopic expression of TRIM24 in MCF7 depleted of endogenous TRIM24 ..................................................................................... 56
Figure 2-13: TRIM24 expression allows estrogen response at lower levels of hormone ................................................................. 57
Figure 2-14: Western blot analysis revealed decreased TRIM24 protein level mediated by siRNA in MCF7 cells ................................................................. 58
Figure 2-15: Knockdown of LSD1 by siRNAs .......................................................... 59
Figure 2-16: Effects of siTRIM24 or siLSD1 on ERα target gene activation ...... 60
Figure 2-17: Effects of siTRIM24 or siLSD1 on ERα target gene activation ...... 62
Figure 2-18: Effects of siTRIM24 or siLSD1 on non-E₂ response gene

BCAS4 .................................................................................................................. 63

Figure 2-19: Cyclical recruitments of LSD1 and TRIM24 to GREB1 distal
ERE site .................................................................................................................. 65
Figure 2-20: Dynamic changes in H3K4 methylation levels at GREB1 distal
ERE site .................................................................................................................. 67

Figure 2-21: Cyclical recruitments of LSD1 and TRIM24 to PR ERE site........ 70
Figure 2-22: Dynamic changes in H3K4 methylation levels at PR ERE site..... 71
Figure 2-23: Cyclical recruitments of LSD1 and TRIM24 to pS2 ERE site....... 72
Figure 2-24: Dynamic changes in H3K4 methylation levels at pS2 ERE site..... 74
Figure 2-25: Effects of LSD1 inhibitor TCP on ERα target gene activation ...... 75
Figure 2-26: Pilot study of LSD1 inhibitors and their effects on ERα target
gene activation ........................................................................................................ 81

Figure 2-27: Effects of potent LSD1 inhibitors on LSD1 protein expression
in MCF7 cells ......................................................................................................... 83
Figure 2-28: Effects of potent LSD1 inhibitors on global H3K4me2 in
MCF7 cells ............................................................................................................. 84

Figure 2-29: Effects of potent LSD1 inhibitor AH124 on ERα target gene
activation ............................................................................................................... 85
Figure 2-30: Effects of potent LSD1 inhibitors Chem778 and Chem779
on ERα target gene activation ............................................................................. 87
Figure 2-31: LSD1 inhibitor Tranylcypromine (TCP) leads to changes in
H3K4 methylation ................................................................................................. 91
Figure 2-32: LSD1 inhibitor Tranylcypromine (TCP) impairs recruitment of
TRIM24 and ERα ............................................................................................... 93
Figure 2-33: LSD1 inhibitor Tranylcypromine (TCP) does not affect H3K9 methylation ................................................................. 95
Figure 2-34: LSD1 inhibitor Tranylcypromine (TCP) leads to changes in H3K4 methylation ............................................................... 96
Figure 2-35: LSD1 inhibitor Tranylcypromine (TCP) impairs recruitment of TRIM24 and ERα ............................................................... 98
Figure 2-36: LSD1 inhibitor Tranylcypromine (TCP) leads to re-methylation of H3K4 ................................................................. 100
Figure 2-37: LSD1 inhibitor Tranylcypromine (TCP) impairs TRIM24 recruitment ........................................................................... 102
Figure 2-38: LSD1 inhibitor Tranylcypromine (TCP) impairs ERα recruitment ........................................................................... 104
Figure 2-39: Summary of changes in histone modifications and recruitment of TRIM24 and ERα in the presence of Tranylcypromine (TCP) .......... 106
Figure 2-40: Purification of GST-only and GST-tagged TRIM24 recombinant proteins ................................................................. 108
Figure 2-41: H3T6 phosphorylation and/or H3K4 methylation hinder TRIM24 from binding to H3 ................................................................. 110
Figure 2-42: Specificity of H3T6ph antibody ......................................................... 112
Figure 2-43: Changes of H3T6 phosphorylation upon estrogen treatment .... 113
Figure 2-44: Inhibition of H3T6-specific protein kinase C (PKC) by BisI

Leads to dephosphorylation of H3T6 at GREG1, PR, pS2 and IGFBP4 EREs .............................................................................. 115
Figure 2-45: Effects of Bis I on ERα target gene induction ......................... 117
Figure 3-1: H3K4 methylation is tightly associated with cancer development .. 129
Figure 3-2: Global TRIM24 target genes upon E2 treatment ....................... 134
Figure 3-3: TRIM24 expression is highly enriched in E₂-treated cells during G2/M transition ................................................................. 135

Figure 3-4: TRIM24 binding to ERα target genes during cell cycle .............. 136

Figure 3-5: 4-hydroxy-tamoxifen (4-OHT)-induced reduction of colony
Formation in MCF7 but not MDA-MB-231 cells ......................... 142

Figure 3-6: Depletion of TRIM24 has additive effect in Tamoxifen-inhibited
ERα target gene activation ............................................................... 145

Figure 3-7: Representative images of Tet-treated colonies in the presence
Of the indicated treatment ............................................................... 146

Figure 3-8: Depletion of TRIM24 expression leads inhibit the survival of
MCF7 breast cancer cells, and is highly additive to
4-hydroxy-tamoxifen (4-OHT)-induced survival inhibition .................. 147

Figure 3-9: Decreased TRIM24 expression sensitizes MCF7 cells lower
dosage of TCP-mediated reduction in colonies .............................. 150

Figure 3-10: Knockdown of TRIM24 is highly additive to TCP- and 4-OHT-
Induced inhibition in colony formation .......................................... 152
LIST OF ABBREVIATIONS

4-OHT  4-hydroxy-Tamoxifen
AML  Acute myeloid leukemia
AR  Androgen receptor
Bromo  Bromodomain
CBP  CREB-binding protein
ChIP  Chromatin immunoprecipitation
ChIP-Seq  ChIP-sequencing
COREST  REST corepressor
CREB  cAMP response element-binding
CTD  C-terminal domain of RNAP II
DHT  Dihydrotestosterone
E₂  17β estradiol, estrogen
EMS  Myeloproliferative syndrome
EMT  Epithelial-mesenchymal transition
ERα  Estrogen receptor alpha
ERβ  Estrogen receptor beta
ERE  Estrogen-responsive DNA elements
FAD  Flavin adenine dinucleotide
GRIP1  Glucocorticoid receptor-interacting protein 1
H3K23ac  Acetylated H3K23
H3K27ac  Acetylated H3K27
H3K4me0  Unmethylated H3K4
H3K4me1  Mono-methylated H3K4
H3K4me2  Di-methylated H3K4
H3K4me3  Tri-methylated H3K4
<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>H3K9me2</td>
<td>Di-methylated H3K9</td>
</tr>
<tr>
<td>H3T6ph</td>
<td>Phosphorylated H3T6</td>
</tr>
<tr>
<td>HAT</td>
<td>Histone acetyltransferase</td>
</tr>
<tr>
<td>hESCs</td>
<td>Human embryonic stem cells</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
</tr>
<tr>
<td>HMEC</td>
<td>Human mammary epithelial cells</td>
</tr>
<tr>
<td>HMT</td>
<td>Histone methyltransferase</td>
</tr>
<tr>
<td>HDM</td>
<td>Histone demethylase</td>
</tr>
<tr>
<td>HP1α</td>
<td>Heterochromatin protein 1 alpha</td>
</tr>
<tr>
<td>ING2</td>
<td>Inhibitor of growth 2</td>
</tr>
<tr>
<td>ISWI</td>
<td>Imitation switch chromatin remodeling complex</td>
</tr>
<tr>
<td>ITC</td>
<td>Isothermal titration calorimetry</td>
</tr>
<tr>
<td>K_D</td>
<td>Dissociation constant</td>
</tr>
<tr>
<td>KDM5B</td>
<td>Lysine-specific demethylase 5B</td>
</tr>
<tr>
<td>KDM5C</td>
<td>Lysine-specific demethylase 5C</td>
</tr>
<tr>
<td>LSD1</td>
<td>Lysine demethylase 1</td>
</tr>
<tr>
<td>MLL</td>
<td>Mixed-lineage leukemia</td>
</tr>
<tr>
<td>MYC</td>
<td>Myelocytomatosis viral oncogene homolog</td>
</tr>
<tr>
<td>NR</td>
<td>Nuclear receptor</td>
</tr>
<tr>
<td>NUP98</td>
<td>Nucleoporin 98</td>
</tr>
<tr>
<td>OGG1</td>
<td>8-oxoguanine-DNA glycosylase 1</td>
</tr>
<tr>
<td>PHD</td>
<td>Plant homeo domain</td>
</tr>
<tr>
<td>PIC</td>
<td>Pre-initiation complex</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PTM</td>
<td>Post-translational modification</td>
</tr>
<tr>
<td>RBP2</td>
<td>Retinol-binding protein 2</td>
</tr>
<tr>
<td>REST</td>
<td>Repressor element 1-silencing transcription factor</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Name</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>RAR</td>
<td>Retinoic acid receptor alpha</td>
</tr>
<tr>
<td>RNAP II</td>
<td>RNA polymerase II</td>
</tr>
<tr>
<td>RXR</td>
<td>Retinoid X receptor</td>
</tr>
<tr>
<td>RING</td>
<td>Really interesting new gene</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>SIRT1</td>
<td>Sirtuin 1</td>
</tr>
<tr>
<td>SWIRM</td>
<td>SWI13p, Rsc8p and Moira</td>
</tr>
<tr>
<td>SWI/SNF</td>
<td>Switch/sucrose non-fermenting chromatin remodeling complex</td>
</tr>
<tr>
<td>TAM</td>
<td>Tamoxifen</td>
</tr>
<tr>
<td>TBP</td>
<td>TATA-box containing protein</td>
</tr>
<tr>
<td>TCP</td>
<td>Tranylcypromine, a LSD1 inhibitor</td>
</tr>
<tr>
<td>TET</td>
<td>Tetracyclin</td>
</tr>
<tr>
<td>TF</td>
<td>Transcription factors</td>
</tr>
<tr>
<td>TRIM24</td>
<td>Tripartite motif containing 24</td>
</tr>
<tr>
<td>TSA</td>
<td>Trichostatin A</td>
</tr>
<tr>
<td>VDR</td>
<td>Vitamin D3 receptor</td>
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</table>
CHAPTER 1: GENERAL INTRODUCTION

1.1.1. Epigenetics control literally all DNA-templated processes in the cell

Eukaryotic DNA is packaged by an octamer of highly conserved histone proteins (two H2A/H2B dimers and one H3/H4 tetramer) into the basic unit called the nucleosome [1]. Repeating unit of nucleosomes are connected by the linker histone (H1) to generate nucleosomal arrays, and then further organized into higher-order chromatin structure (illustrated in Figure 1-1). These structures are highly plastic and governs the accessibility of DNA through epigenetic processes such as ATP-catalyzed remodeling and repositioning of the nucleosomes [2,3], covalent modifications of residues on the tail or globular domains of histone proteins [4], as well as methylation of 5’ position of the cytosine ring in CpG dinucleotides. Orchestration of these epigenetic regulatory mechanisms controls literally all DNA-templated cellular processes, such as transcription, DNA replication, DNA repair, recombination and others [5].

This thesis focuses on the post-translational modifications (PTMs) decorating the N-terminal tails of the core histone H3 and their effects on gene transcription such as H3K4 methylation, H3 acetylation, and H3T6 phosphorylation. Essentially, specific residues on histone tails are targeted by a wide array of histone-modifying enzymes, which either add or remove PTMs from different histone residues. To date, numerous PTMs have been identified, such as methylation, acetylation, phosphorylation, ubiquitination and others [6,7], which result in either an open (euchromatin) or closed chromatin structure (heterochromatin), leading to gene activation or repression, respectively.
Figure 1-1: Overview of epigenetic regulatory mechanisms. Reprinted by permission from Macmillan Publishers Ltd: *Nature Reviews Drug Discovery* 11: 384-400, copyright (2012) [8].
1.1.2. Histone acetylation opens up chromatin for transcriptional activation

Histone acetylation is probably the most studied of the PTMs, and the functions in transcriptional activation are widely appreciated [9]. Earlier observation suggested that acetylated chromatin regions are associated with active gene transcription [10]. In fact, hyper-acetylation creates an accessible chromatin conformation and thus enhances the exposure of DNA to restriction enzymes and transcription factors to allow transcription [11,12,13]. Removal of the positive charge on the acetylated histone tails disrupts the stability between histones and the negatively charged DNA, as well as the inter-nucleosomal interactions [14]. Another hypothesis is that histone acetylation may be part of the combinatorial modifications that lead to downstream events via binding of other epigenetic regulatory proteins [15]. In fact, adjacent phosphorylation and methylation can also regulate histone acetyltransferase (HAT) activity [16,17]. The enzymes involved (histone acetyltransferases, HATs; histone deacetylases, HDACs) are not only responsible for the steady-state balance of histone acetylation, but are also present in different regulatory complexes and have distinct biological functions [18].
1.1.3. Histone methylation and transcription activation/repression

In contrast to acetylation, histone methylation is associated with both transcriptional activation and repression, in a context-specific manner [19]. Histone methylation occurs on both lysine (K) and arginine (R) residues [19, 20, 21]. Particularly, lysine methylation on different sites (H3K4, H3K9, H3K27, H3K79, and H4K20), and to different degrees (mono-, di-, or tri-methylation) [22], contributes to the complexity of transcription in regulation. Histone lysine methylation is dynamically mediated through histone methyltransferases (HMTs), and the opposing enzymes, histone demethylases (HDMs). SET-domain containing HMTs [23, 24], especially in H3K4 methylation, include MLL1-5, SET1/7/9, ASH1, and SMYD3 (summarized in Figure 1-2) [25]. Histone lysine demethylase 1 (LSD1) was the first HDM identified and is specific for the demethylation of monomethylated and dimethylated H3K4 (H3K4me1/2) [4, 16]. LSD1 plays critical roles in several cellular processes such as regulation of transcription, development and differentiation (to be further discussed below).

In addition to LSD1, more HDMs have been identified from members of the Jimonji-containing protein family. H3K4-specific HDMs also include retinol-binding protein 2 (RBP2/JARID1A/KDM5A), which demethylates H3K4me2/3 and lysine-specific demethylase 5B (KMD5B/PLU-1/JARID1B) and lysine-specific demethylase 5C (KDM5C/SMCX/JARID1C), both of which enzymatically target H3K4me3 (summarized in Figure 1-2) [25].
Figure 1-2: Covalent histone modifications mediated by epigenetic enzymes. Reprinted from Trends in Biochemical Sciences, 36, S. Kato, A. Yokoyama, R. Fujiki, Nuclear receptor coregulators merge transcriptional coregulation with epigenetic regulation, p.272-281, Copyright (2011) [25].
1.1.4. Histone phosphorylation cooperates with nearby PTMs

Phosphorylation of histones is especially important in cell cycle regulation [26,27]. However, emerging evidence also suggests its functions in transcriptional regulation [28]. For example, phosphorylation on Histone H3 threonine T3 (H3T3ph) interrupts the recognition of H3K4 by other chromatin binding modules due to the close proximity of T3 and K4 on H3 [29]. Notably, H3T3ph and H3K4me1 often associate with regions of repressed transcription [30]. Therefore, it is possible that H3T3 phosphorylation creates electrostatic interference for H3K4 recognition, leading to silent chromatin. Another example is H3T6ph, which also antagonizes the binding of H3K4-specific reader module and demethylation mediated by LSD1 and JARID1B [31]. On the other hand, H3T11ph enhances JMJD2C-mediated demethylation of H3K9me3 [32]. Taken together, histone phosphorylation underlies a complex language of histone modifications, yet its effects on combinatorial modifications await further investigations.

1.1.5. The histone code hypothesis

The histone code hypothesis proposes that combinatorial or sequential histone PTMs can be decoded by different reader proteins or protein modules to amplify a cascade of downstream responses [5,15]. The hypothesis is appealing because it may explain how distinct patterns of histone marks achieve context-specific diversity on transcriptional regulation. For example, tri-methylation on H3K4 (H3K4me3) recruits chromatin-remodeling complexes, and is considered
an active mark for transcription. Although methylated H3K4 is generally associated with euchromatin and transcriptional activation [8,25,33,34], further studies support the concept that cellular context determines the outcome of histone methylation. In response to DNA damage, robust recognition of H3K4me3 by the PHD domain of ING2 (inhibitor of growth 2, a tumor suppressor protein) mediates gene repression by serving as a bridging module with mSin3a–HDAC1 complex at the promoters [35]. Therefore, specific effector proteins, acting as a reader module for specific histone modifications, determine the outcome of dynamic and highly contextual gene regulation. In fact, a recent study investigated how chromatin reader modules interact with histone H3 decorated by combinatorial modifications on different residues in close proximity. This finding showed that combinatorial PTMs on the same histone tail affect the binding affinity for effector proteins, leading to divergent downstream “readouts” [29].

1.1.6. Histone binding modules decode the histone language

The histone code hypothesis postulates the importance of effector (or chromatin binding modules) in determining the biological outcomes of single or combinatorial histone PTMs [5,15], which, in the last decade, has led to extensive discoveries in their interaction with specifically modified histone peptides [36]. These reader/binding modules are highly evolved and are able to recognize distinct histone marks. For example, proteins with bromodomains recognize acetylated lysines, whereas (un)methylated lysines can be bound by proteins containing chromodomains, double chromo, double Tudor domains,
MBT (malignant brain tumor) 1 repeats, PHD (plant homeo domain) fingers, cysteine-rich ADD domain, WD40 repeats and others. Notably, relatively fewer readers for phosphorylated histone peptides have been identified, which include 14-3-3 and tandem BRCT (BRCA1 C terminus) -containing proteins [36,37].

For the recognition of methylation status on H3K4, unmethyl-H3K4 (H3K4me0) can by read by PHD-finger readers AIRE [38] and BHC80 [39], WD40-reader WDR5/9 [40,41] and ADD-reader DNMT3L/3A [42,43]; while methylated-H3K4 (H3K4me) by chromo-reader CHD1 [44,45], PHD readers RAG2 [46], ING2 [41], BPTF [47], TAF3 [48], PHF2 [49], ING4 [50], YNG1 [35,51] and PHF8 [52], Tudor-readers JMJD2A [53] and Sgf29 [54], Zf-CW reader ZCWPW1 [55] and others. Readers for other histone modifications are summarized in Figure 1-3. The majority of the epigenetic binding modules can recognize multiple histone marks and represent an active area of research. However, how the histone code is decoded also largely depends on the complicated network between epigenetic readers as well as modifying enzymes in response to combinatorial histone PTMs, in specific context and cell types [36,56].
**Figure 1-3: Readers of histone modifications.** Reprinted by permission from Macmillan Publishers Ltd: *Cell Research* **21**:564-578, copyright (2011) [37].

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<th>PTMs</th>
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Figure 1-3 (Continued): Readers of histone modifications. Reprinted by permission from Macmillan Publishers Ltd: *Cell Research* **21**:564-578, copyright (2011) [37]

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1.1.7. The RBCC protein family member TRIM24

Our lab discovered new function of TRIM24 (Tripartite Motif containing 24) as an epigenetic reader, which simultaneously recognizes two histone marks, H3K4me0K23ac [57]. The TRIM/RBCC protein family is characterized by the presence of a conserved, N-terminal tripartite motif, containing a Really Interesting New Gene (RING) domain, two B-boxes, and a coiled-coil domain, while the carboxyl-terminal domains are variable [58,59]. TRIM24, also known as TIF1α (Transcription Intermediary Factor 1 Alpha), belongs to the TIF1 sub-family, and contains a consensus LXXLL nuclear receptor interaction motif, a tandem Plant Homeo Domain (PHD) domain and a bromodomain (Bromo) at the C-terminus (Figure 1-4). TRIM24 is a multi-functional protein: through its N-terminal RING domain, TRIM24 functions as an E3 ligase of p53, and targets p53 protein for post-translational proteasomal degradation; upon ligand stimulation, TRIM24-LXXLL motif interacts with, and acts as a potent co-regulator of multiple nuclear receptors (NRs) [60], including RAR (retinoic acid receptor alpha), retinoid X receptor (RXR) [61], Vitamin D3 receptor (VDR), Androgen Receptor (AR), progesterone receptor (PR) and ERα (estrogen receptor alpha) [57,60,62,63,64], as well as NR co-activators coactivator-associated arginine methyltransferase 1 (CARM1) and glucocorticoid receptor-interacting protein 1 (GRIP1) [65]. TRIM24-Bromo also associates with the chromatins through bromodomain–DNA and bromodomain–nucleosome interactions [66].
1.1.8. TRIM24 regulates nuclear receptor-mediated signaling pathways

TRIM24 promotes ligand-dependent transcriptional activation by RAR, RXR, and VDR in vitro, suggesting that TRIM24 may function as a nuclear receptor co-activator. However, in mice lacking Trim24, RARα- and VDR-repressed genes are re-expressed [67,68,69,70], implicating that Trim24 acts as a co-repressor in RAR- and VDR-mediated signaling in vivo. In addition, Trim24 loss leads to overactivation of interferon (IFN)/STAT pathway. Notably, Trim24 and RAR bind to the retinoid acid (RA)-responsive element of the Stat1 promoter and mediate ligand-dependent repression [71,72], indicating that Trim24 is a co-repressor of the IFN/STAT signaling pathway in mice. Moreover, the Drosophila homolog of TIF1 proteins, Bonus, interacts with nuclear receptor beta-FTZ-F1 and represses its target gene transcription in vivo [73,74].

However, in vitro evidence supports the co-activation function of TRIM24. In prostate cancer cells, TRIM24 promotes AR-mediated transactivation in response to dihydrotestosterone (DHT), through functional interaction and synergy with histone acetyltransferase TIP60, as well as bromodomain containing 7 (BRD7), a negative regulator for cell proliferation and growth [75,76]. TRIM24 also physically interacts with co-activators CARM1 and GRIP1, whereas TRIM24-depletion attenuates GRIP- and AR-mediated transactivation [57,65].

In addition, TRIM24 also play critical roles in the activation of ERα-mediated gene regulation in breast cancer cells. Our lab uses chromatin immunoprecipitation (ChIP)- sequencing (ChIP-seq) analysis and reveals that TRIM24 is recruited by ERα to hundreds of estrogen-response elements (EREs)
in response to estrogen (E$_2$) stimulation. TRIM24 and ER$\alpha$ physically interact with each other on the chromatins, in an estrogen-dependent manner. When TRIM24 is depleted by shRNA, binding of ER$\alpha$ to, and induction of, estrogen-responsive target genes is reduced.

**1.1.9. TRIM24 associates with chromatins and remodeling proteins**

Earlier studies show that mouse heterochromatin protein 1 alpha (mHP1$\alpha$) physically interacts with Trim24 [1,60], which is dependent on the HP1-binding region and PHD-Bromo domain of Trim24. However, transcription repression mediated by *Drosophila* homolog of TIF1 proteins, Bonus, requires its RBCC motif, but is not dependent on HP1 binding [2,3,74]. In addition, Bonus is not physically associated with *Drosophila* HP1 proteins. Strikingly, in early mouse embryos Trim24 is highly expressed and co-localizes with the euchromatin of interphase nuclei [4,77]. In addition, Trim24 is involved in the regulation of gene expression during the first wave of transcription activation in mouse embryo development, where Trim24 is localized to the site of active transcription enriched with the mouse chromatin remodeling proteins BRG-1 and SNF2H [6,7,78]. Remarkably, Trim24 depletion leads to mis-regulation of gene expression in the zygote via SNF2H. Taken together, these observations indicate that Trim24 regulates gene activation during the first wave of mouse embryo development and cell differentiation.
1.1.10. Histone demethylase 1 (LSD1) specifically demethylates H3K4me1/2

Histone lysine methylation is dynamically mediated through histone methyltransferases (HMTs) and the opposing enzymes, histone demethylases (HDMs), in response to cellular signals. LSD1 is the first identified HDM, which functions as a transcriptional regulator by catalyzing the demethylation of H3K4me1/2 [9,70,72]. LSD1 possess a SWIRM (SWI3p, Rsc8p and Moira) domain, a FAD (flavin adenine dinucleotide)-binding motif, and a C-terminal amine oxidase domain (Figure 1-5); all of these are structurally indispensible for the demethylation of specific histone lysine substrates. In particular, SWIRM domain is commonly found in histone-interacting proteins. The SWIRM domain of LSD1 forms a structural interface with the amine oxidase domain to assist in substrate binding. Through its FAD-binding motif, LSD1 uses FAD as a cofactor to act on the methylated H3K4 side chain [10,79,80]. The flavin-dependent reaction results in unmodified lysines, and the reduction of FAD to FADH$_2$, which is re-oxidized by oxygen and forms hydrogen peroxide H$_2$O$_2$ as a by-product. The imine intermediate is then further demethylated via hydrolysis, together with the release of formaldehyde (Figure 1-6). However, LSD1 is unable to act on tri-methylated H3K4 due to electrostatic limitation.
Figure 1-6: Postulated chemical reactions for LSD1-catalyzed demethylation of H3K4me2. Reprinted from Cell, 119, Y. Shi, F. Lan, C. Matson, P. Mulligan, J.R. Whetstine, P.A. Cole, R.A. Casero, Histone demethylation mediated by the nuclear amine oxidase homolog LSD1, 941–953, Copyright (2004), with permission from Elsevier [72].
1.1.11. LSD1 mediates transcription repression

Recombinant LSD1 can demethylate H3K4me1/2 on histone peptides or free histone substrates in vitro, but not on nucleosomal substrates [11,12,13,81,82], suggesting that associated factors are required for LSD1-mediated demethylation. In fact, LSD1 is associated with repressor element 1-silencing transcription factor (REST) corepressor (CoREST), histone deacetylases HDAC1/2, and BHC80 (also known as PHD finger protein 21A, PHF21A, a PHD finger protein) for the repression of neuronal genes in non-neuronal cells [14,39]. BHC80 inhibits the enzymatic activity of LSD1, while CoREST functions in the opposite way by allowing LSD1 to demethylate nucleosomal substrates and protecting LSD1 from proteasomal degradation.

In addition, LSD1 also interacts with the orphan nuclear receptor TLX (homolog of the Drosophila tailless gene), which are both recruited to the PTEN gene at regions depleted of H3K4me2 and deacetylated of H3. Knockdown of LSD1 results in de-repression of endogenous PTEN expression and inhibition of cell proliferation [15,83]. Moreover, direct interaction between LSD1, additional sex comblike protein 1 (ASXL1), and HP1α suggests that LSD1 cooperates with ASXL1 in RAR-mediated repression through HP1α [16,17,84].

1.1.12. LSD1 plays important roles during development and differentiation

Several lines of evidence suggested that LSD1 is functionally critical in the processes of development. For example, temporal and spatial expression of
mouse LSD1 is tightly correlated with demethylation of H3K4 during male germ cell differentiation [17,85]. Notably, Lsd1 is required for pituitary terminal cell-type differentiation through previous regulation of transcriptional activation and repression of developmentally essential genes [5,15,86]. In addition, Lsd1 has been shown to be critical for gastrulation during mouse embryogenesis, possibly through demethylating and stabilizing DNA methyltransferase Dnmt1 protein, and thus maintaining global DNA methylation during embryogenesis [29,87]. Particularly, DNMT1 recruits LSD1 to target gene promoters and regulates DNMT1 target gene expression in human colon cancer cell line [88]. The LSD1-CoREST-HDAC corepressor core also physically interacts with Gfi-1/1b and regulates hematopoietic differentiation by demethylating H3K4 at the promoters of Gfi targets [89]. The association with the transcriptional repressor B lymphocyte-induced maturation protein-1 (Blimp-1) leads to the binding of Lsd1 to Blimp1 target sites and Blimp-1-mediated silencing of mature B-cell genes in plasma cell differentiation [90].

LSD1 is also involved in stem cell differentiation. Through its interaction with CoREST, LSD1 demethylates H3K4me1/2 at a subset of developmentally essential genes that contain bivalent domains of both H3K4me2/3 and H3K27me3 marks in human embryonic stem cells (hESCs) [91]. LSD1 regulates the pattern of H3K4 and H3K27 methylation, leading to the precise balance between self-renewal and differentiation in hESCs. In addition, the inhibition of LSD1 enzymatic activity prevents the proliferation of pluripotent cancer stem cells [92]. Interestingly, mouse Lsd1 is recruited to and is required for
decommissioning enhancers of the pluripotency genes during differentiation. Notably, depletion of LSD1 results in incomplete chromatin demethylation and failure of mESC differentiation in new cell states.

The function and structure of LSD1/CoREST corepressor complex is evolutionally conserved in vertebrates and invertebrates [93]. Particularly, sirtuin 1 (SIRT1) and LSD1 physically interact and cooperatively regulation gene repression by mediating H3K16 deacetylation and H3K4 demethylation respectively. Mutations in dSirt1 and dLsd1 (Drosophila Sirt1 and Lsd1) genetically interact with the Notch pathway in Drosophila. In particular, SIRT1-LSD1 expression is required for NOTCH target repression [94]. In addition, an inactivating mutation of dLsd1 (Drosophila Lsd1) disrupts H3K4 methylation and expression of a subset of target genes, resulting in tissue-specific defects (sterility especially in females) and reduction of animal viability in a gender-specific manner during Drosophila development [94]. In addition, studies on SPR-5, the C. elegans ortholog of human LSD1, showed that LSD1 possibly controls the reprogramming of epigenetic memory in the germline. Notably, spr-5 mutants exhibit defects in H3K4me2 demethylation in the primordial germ cells, resulting in transgenerational misregulation of spermatogenesis-associated gene expression and progressive sterility.

1.2. AIMS OF THIS WORK

The histone code hypothesis postulates that histone modifying enzymes “write” or “erase” histone marks, while histone binding modules “read”, decode
and execute these marks to effectively trigger downstream responses [5,15]. Here I focus my thesis on estrogen receptor-alpha (ERα), the master transcriptional regulator in breast cancer [95], and determine the significance of erasure and recognition of H3K4 methylation on estrogen-responsive elements (EREs) over a time course of LSD1- and TRIM24-regulated estrogen response. Chapter 2 focuses on the co-activation of ERα-mediated transcription by LSD1 and TRIM24, the binding events and dynamics of histone modifications during the time course. I will also discuss how TRIM24 binding and estrogen response is influence by H3T6 phosphorylation. Chapter 3 focuses the biological functions mediated by LSD1 and TRIM24 and how they affect survival and proliferation in breast cancer cells. The work presented in the dissertation provides a comprehensive analysis of the kinetics of demethylated histones through enzymatic activities and recognition by epigenetic reader proteins, and the molecular events eading to effective histone demethylation, a prerequisite for the chromatin association of TRIM24 and activated ERα-mediated transcription. The findings on the inhibitory effects mediated by depletling or inhibiting TRIM24 and LSD1 in the proliferation breast cancer cells may give insights to future development of combinatorial therapeutics in breast cancer.

Introduction of ERα-mediated signaling, ERα co-activators, involvement of TRIM24 and LSD1 in hormone receptor is presented in section 2.1; introduction of disease-related functions of TRIM24 and LSD1 is presented in section 3.1.
CHAPTER 2: **ACTIVATION OF TRIM24-REGULATED ERα TARGET GENES**

**IS DEPENDENT ON LSD1-MEDIATED H3K4ME2 DEMETHYLATION**


**2.1. INTRODUCTION**

**2.1.1. Estrogen receptor-alpha (ERα) is the master transcriptional regulator**

Estrogen receptors (ERα and ERβ) belong to the ligand-dependent nuclear receptors (NRs), activated by the binding of 17β-estradiol (E2), a predominantly naturally occurring estrogen in human. This dissertation focuses on the ERα subtype because it is the master transcriptional regulator that influences most of the physiological processes, such as the growth and maintenance of the reproductive tract, central nervous system and skeletons, and disruptions of ERα functions in the hormone-responsive tissues often lead to the pathological development of cancers, such as breast cancer [57,60,62,63,64,95,96,97]. Ligand-activated ERα immediately undergo conformational change [65,98] and directly binds to sequence-specific DNA elements (termed estrogen-responsive elements; EREs), upstream of target gene promoters, to mediate massive changes in the transcriptome [66,99,100,101]. This process requires the recruitment of transcription factors as well as a plethora of co-regulator proteins. In fact, hundreds of ERα co-regulators have been identified to date [102,103], most of them exist in multi-subunit complexes, and contribute to our understanding of the orchestral molecular events upon estrogen stimulation.
2.1.2. ERα and the transcriptional machinery

In eukaryotic cells, the transduction of cellular and environmental signals often lead to gene transcription, whose activation or repression is regulated through a series of temporal-specific recruitments of transcription factors (TFs) and co-regulators on EREs (Figure 2-1). The model of transcription initiation postulates that during the assembly of pre-initiation complex (PIC), TATA-box binding protein (TBP, a subunit of transcription factor TFIID) is first recruited to the TATA box and is stabilized by TFIIA [104]. TFIIB is then associated with PIC for the conformational remodeling of PIC and for the selection of transcription initiation sites, where RNA polymerase II (RNAP II) is loaded to activate transcription [105,106,107]. TRAP/mediator complex is recruited and structurally remodeled to co-activate the phosphorylation of C-terminal domain of RNAP II (CTD/Rbp1, the largest subunit of RNAP II) [108,109,110], and then exchanged by elongation complexes [111], for transcription to initiate. Components of the transcriptional machinery, such as TFIIB, TFIIE, and TFIIF, as well as TAFs and the TBP of TFIID, interact with ERα and are involved in ERα-mediated transcription activation [112,113]. A closer look at the kinetic profile of transcription machinery reveals that every cyclical engagement of TFIIB is aligned with ERα [73], suggesting that ERα recruitment associates with the structural remodeling of PIC by TFIIB. Importantly, ERα-driven cycles lead to the sequential and cyclical recruitment of TRAP/mediator (Med7 and TRAP220), cdk7 (essential component of the transcription factor TFIIH), followed by activated RNAP II and transcriptional elongators (Elp1 and Elp3) [73].
In fact, DNA accessibility is largely dependent on the nucleosomal architecture, which determines subsequently whether transcription is to be initiated. Enhanced ERα binding is achieved by epigenetic machinery that modifies the histone-DNA interface of the surrounding chromatin architecture. Through covalent histone modifications, chromatin remodeling, and histone-octamer exchange by histone chaperones, recruitment or dismissal of specific regulatory proteins occur in a context- and temporal-dependent manner [25,114,115].

2.1.3. Histone modifying enzymes as ERα co-regulators

Recent works demonstrated the potential role of histone-modifying enzymes as ERα co-regulators [116,117]. Histone acetyltransferase p300/CBP is the first epigenetic regulator that links epigenetics to transcription co-regulation. p300/CBP relaxes the nucleosomal DNA to allow for TF binding and is always associated with transcription activation. HATs not only co-activate ERα, but also other classes of TFs, in order to enhance activated transcription [118]. For example, the cAMP response element-binding (CREB)-binding protein (CBP)/p300 and the GCN5 complex, TFTC/SAGA [TATA-binding protein (TBP)-free TBP associated factors (TAF)-containing complex/Spt–Ada–Gcn5 acetyltransferase] are both HATs that have been shown to co-activate ERα-mediated transactivation [119]. Certain HDACs are also associated with ERα, possibly for the termination of activated transcription [120,121].
The histone code hypothesizes that crosstalk between histone PTMs orchestrates dynamic and context-specific reorganization of chromatin, an essential process for transcriptional regulation [5,15,122]. For example, it is suggested that histone methylation on specific residues is more upstream than acetylation in order to direct the chromatin state for gene activation/repression [123]. Notably, the roles of histone methylation/demethylation in ERα co-regulation are more dynamic [124]. HMTs/HDMs can be associated with either activation or repression, in a histone residue-, DNA sequence- and context-specific manner [33]. ERα co-activators are not only limited to HATs/HDACs and HMTs/HDMs, but also include histone kinases and histone phosphatases, ubiquitin, SUMO ligases and others. Modifiers of other histone PTMs functioning as ERα co-regulators are shown in Figure 2-2.

2.1.4. Chromatin remodeling and estrogen response

Chromatin remodelers play essential function in the process of chromatin reorganization, in an ATP-dependent manner [125,126]. By influencing histone-DNA interactions and sometimes through nucleosome sliding, this reversible process exposes naked DNA to a wide array of TFs and indirectly regulates transcription. This process can be mediated by three types of chromatin remodelers: Switch/Sucrose Non-Fermenting (SWI/SNF)-, Imitation Switch (ISWI)-, and Chromdomain Helicase DNA Binding Protein 4 (CHD4/MI2)-complexes [127]. Both SWI/SNF and ISWI can either activate or represses chromatin [125,128], while CHD4/MI2 is involved in chromatin inactivation [129].
Notably, the core subunits of these remodeler complexes can function as transcription coregulators. For example, BAF57, a subunit of the SWI/SNF complex, co-activates ERα-regulated transcription [130]. In addition, the association between ERα and these remodelers is believed to remodel the nucleosomes surrounding hormone-responsive DNA elements, and is indispensable for the binding of other ER co-regulators.
Figure 2-2. Co-activators of estrogen receptor-alpha. Reprinted with permission from Cold Spring Harbor Laboratory Press, Genes and Development 20: 1405-1428, copyright (2006) [75].
2.1.5. Potential involvement of histone chaperones in ERα regulation

Effects of histone chaperones in transcriptional regulation also gained researchers’ attention recently. By the regulation of chromatin reorganization through the eviction and reassembly of histones during replication, repair and transcription, chaperon proteins re-organize nucleosomal DNA to permit the accessibility of sequence-specific TFs and NR co-regulators during gene activation [131]. For examples, Drosophila DEK (dDEK) co-localizes with nuclear ecdysone receptor (EcR) in the salivary gland. Importantly, phosphorylation of Drosophila DEK (dDEK) induces complex formation with casein kinase 2; and this complex is known to associate with active histone marks. dDEK functions as a histone chaperon and facilitates H3.3 assembly [132]. Therefore histone chaperones such as dDEK may regulate gene activation and represent a distinct class of nuclear receptor co-regulators; but this potential still awaits further validation.

2.1.6. Timely and cyclical recruitment of ERα and its co-factors

Studies in MCF7 human breast cancer cells suggested that activation of ERα target genes exhibit distinct patterns of estrogen-mediated response, in a time-dependent manner [100,101]. It is therefore reasonable for estrogen to induce cyclical and sequential binding of ERα and its co-regulators. One study of the canonical pS2 ERE in MCF7 human breast cancer cells led to a model where ERα binds to EREs in 3 distinct cycle of protein recruitment to chromatin (protein complexes involved illustrated in Figure 2-1) [73]. During the first “transcriptionally undproductive” cycle, initial engagement of the SWI/SNF
component BRG1 remodels the chromatin, and then H3K14 becomes acetylated and H4R3 becomes dimethylated, concurrent with the recruitment of acetyltransferases Tip60/p300 and arginine methyltransferase PRMT1, respectively. Transcription machinery regulators TATA-box binding protein (TBP, a subunit of transcription factor TFIID), TFIIA, mediator/TAF130, and acetyltransferase GCN5 are also recruited, all of which occur before the recruitment of activated RNA polymerase II [73]. During the second and third “transcriptionally productive” cycles, ERα is recruited every 40-50 minutes [73] and this recycling is dependent on proteasomal-mediated turnover of ERα [73,133]. Notably, every ERα recruitment coincides with the acetylation of H3K14 and H4K16, as well as the di-methylation of H3R17, and this process precedes the recruitment of activated RNA polymerase II. Moreover, timely recycling of p160 factors, histone methyltransferases (HMTs), and histone acetyltransferases is also observed on the pS2 ERE.

Studies of histone methylation suggest that it is associated with either activation or repression of ERα-mediated response, in a histone residue-, DNA sequence-, and context-specific manner [31,33,124,134]. However, whether regulation of histone lysine methylation and demethylation is critical in the stepwise process of ERα-mediated transcriptional activation remains to be explored. Specifically, a comprehensive view of the kinetics of histone lysine demethylation through enzymatic activities and the recognition of demethylated histones by epigenetic reader proteins awaits further investigation, and thus is the central focus of this study.
2.1.7. TRIM24 functions as an ERα co-activator through chromatin recognition

Our lab recently identified an ERα co-activator, namely TRIM24. ChIP analysis showed that TRIM24 is co-recruited with ERα upon estrogen treatment to GREB1 ERE sites (Figure 2-3A). Notably, ERα and TRIM24 are recruited as a complex, as revealed by sequential ChIP experiment (Figure 2-3B). TRIM24 depletion reduces recruitment of ERα to GREB1 ERE (Figure 2-4B) and lead to down-regulation of estrogen-induced GREB1 activation at t = 3hr and t = 6hr (Figure 2-4A, left). A detailed investigation of TRIM24 PHD-Bromo crystal structure establishes the simultaneous interaction of PHD-Bromo with unmethylated H3K4 (H3K4me0) and acetylated H3K23 (Figure 1-4B) [57]. I performed mutagenesis and generated TRIM24-PHD point mutation (C840W) and Dr. Tsai showed that reintroduction of WT-TRIM24, but not TRIM24-C840W, is able to fully rescue TRIM24 function in shTRIM24 cells.
Figure 2-3. ERα and TRIM24 are recruited together to EREs upon estrogen induction. (A) ChIP experiment showing both ERα (left) and TRIM24 (right) recruitment to GREB1 distal and proximal EREs upon 15 min and 6 hours of estrogen (E₂) treatment. (B) Sequential ChIP showing that ERα and TRIM24 recruitment as a complex after 6 hours of E₂ activation. Reprinted by permission from Macmillan Publishers Ltd: Nature 468:927-932, copyright (2009) [57].
Figure 2-4. Depletion of TRIM24 decreases ERα binding to ERE and estrogen-activated gene induction. (A) qPCR: (left) depletion of TRIM24 by shRNA reduces E₂-induced activation of GREB1 at indicated time points; (right) re-introduction of wildtype (WT) but not PHD-finger mutant (C840W) rescues TRIM24-coactivated GREB1 induction. (B) ChIP: Knockdown of TRIM24 leads to decreased ERα recruitment to GREB1 distal ERE. Reprinted by permission from Macmillan Publishers Ltd: Nature 468:927-932, copyright (2009) [57].
2.1.8. TRIM24 simultaneously recognizes two histone signatures on H3

Sequence alignment shows that TRIM24 PHD finger is highly similar to BHC80, especially the conserved residues critical for BHC80-H3K4me0 interaction. Binding of TRIM24 PHD-Bromo is abolished by H3K4 mono-, di-, or tri-methylation. In fact, TRIM24 PHD-Bromo binds to unmodified H3(1-15)K4 with a dissociation constant ($K_D$) of 8.6mM, as measured by isothermal titration calorimetry (ITC)-based binding assay. Even with the addition of one methyl group on H3K4, TRIM24 PHD-Bromo loses its binding ($K_D = 41$mM), the affinity is even weaker for di-methylated H3K4 peptide ($K_D = 198$mM), and with tri-methylation on H3K4 the interaction is totally abolished ($K_D > 400$mM). These observations suggest that TRIM24 PHD-Bromo specifically binds to unmethylated H3K4. Simultaneous with H3K4me0 recognition by TRIM24-PHD, TRIM24 bromodomain interacts with acetylated H3(13-21)K23ac peptide with a $K_D$ of 8.8mM. Acetylation of H3K23 enhances the binding of TRIM24 to unmethylated H3K4. Interestingly, TRIM24 PHD-Bromo is tolerable with H3K4 tri-methylation in the presence of H3K23 acetylation (H3K4me3K23ac), suggesting that the combinatorial recognition of TRIM24 is dynamic and requires further investigation in the cellular context.
2.1.9. **TRIM24 preferentially binds to regions depleted of H3K4me2**

ChIP sequencing analyses of TRIM24 and H3K4me2 reveal that TRIM24 preferentially binds to genome-wide regions depleted of H3K4me2 [57]. As an example, Figure 2-5A shows TRIM24 binding and H3K4me2 occupancy at the estrogen responsive element (ERE) of an ERα target gene *IGFBP4*. TRIM24 is recruited to *IGFBP4*-ERE in response to estrogen stimulation. At the same ERE, demethylation of H3K4me2 is also prominent upon E2. Figure 2-5B shows the global binding profile of TRIM24 in regions relative to H3K4me2 level. Corresponding number of tags sequenced for H3K4me2 occupancy is represented graphically on the y-axis, in relation to the distance to TRIM24 binding site (in terms of base pair, bp) on the x-axis (Figure 2-5B). It is clear that global demethylation in response to E2 is observed, and TRIM24 preferentially binds to regions of low H3K4me2 occupancy. These observations, together with the peptide binding and ITC assays mentioned above, suggested that H3K4me2 must be demethylated in order for TRIM24 to bind to the chromatins, and targeting histone demethylases that mediate H3K4me2 demethylation may regulate the functions of TRIM24.
Figure 2-5. TRIM24 binds to regions depleted of H3K4me2. (A) TRIM24 binding and H3K4me2 occupancy at IGFBP4 ERE locus. (B) H3K4me2 averaged levels at TRIM24 binding. Reprinted by permission from Macmillan Publishers Ltd: Nature 468:927-932, copyright (2009) [57].
2.1.10. LSD1 as a transcription co-activator in androgen and estrogen receptors-mediated signaling

Among the four H3K4-specific demethylases, I focus on LSD1 in this study because it is the only enzyme capable of the demethylation from H3K4me2 to H3K4me1 and then to H3K4me0 [9,70,72], a preferential substrate for TRIM24 recognition, leading to TRIM24-regulated co-activation of ERα. Although LSD1 plays an important role in mediating gene repression under specific circumstances and in certain tissues, in hormone-responsive tissues, evidence showed that LSD1 mainly functions as a co-activator in nuclear receptor-mediated transcription activation. For example, estrogen induction leads to demethylation of H3K4me2 and H3K9me2/3 at ERα target genes pS2 and GREB1 EREs [124]. LSD1 interacts with ERα and co-activates estrogen-mediated expression of these genes [124]. However, whether LSD1 functions as an ERα by depleting H3K4 methylation has not been addressed. Another study of the ERα pioneer factor FOXA1 (Forkhead Box A1) showed that FOXA1 binding sites are enriched with H3K4me1/2 [135]. Notably, when LSD1 is overexpressed, H3K4me2 decreases while H3K9me2 remains unchanged, but FOXA1 recruitment is impaired [135]. These observations strongly suggested a role of LSD1 in ERα-regulated transcription through its ability to demethylate H3K4me2.

On the other hand, LSD1-mediated demethylation also generates the by-product hydrogen peroxide (H₂O₂), which has been shown in MCF7 cells to recruit 8-oxo-guanine–DNA glycosylase 1 (OGG1) and topoisomerase IIβ
(TopoIIβ), triggering chromatin looping for activated ERα binding [136]. In prostate cancer LNCaP cells, LSD1 interacts with androgen receptor (AR), binds to and co-activates AR target genes [134]. However, in both studies, LSD1 has been suggested to demethylate H3K9, a biochemically non-preferable substrate, instead of H3K4.

The enzymatic specificity of LSD1 has been debatable for awhile until a recent study showed that LSD1 interacts with H3K9me3-specific demethylase JMJD2C in prostate cancer cells, where JMJD2C also functions as a co-activator of AR [137]. Therefore, the change in H3K9 methylation when LSD1 is depleted could possibly due to the loss of interaction with JMJD2C and other H3K9 demethylase(s), or even gain of H3K9 methyltransferase(s) recruitment. Although the conclusion from this study still emphasized the ability of LSD1 to demethylate mono- and di-methylated H3K9, and switch its substrate specificity with methyl-H3K4, how and whether substrate switch of LSD1 is possible and what unknown cofactors may be involved still remain unknown.

2.1.11. H3T6ph inhibits H3K4 demethylation mediated by LSD1 and chromatin binding by H3K4me0 reader proteins

LSD1-mediated demethylation can be regulated by several mechanisms [138,139,140]; one newly identified regulation is by phosphorylation of H3T6 (H3T6ph). Mass spectrometry analyses revealed that the presence of H3T6ph on H3K4me2 peptide inhibits LSD1-mediated demethylation [31]. H3T6ph is mediated by PKC family kinases PKCa, βI, and βII. In the absence of PKCβI and
H3T6ph, LSD1 is able to demethylate H3K4me2 on core histones or nucleosomes in demethylation assays. However, when PKCβI is added, LSD1 fails to mediate H3K4me2 demethylation. The same histone mark also influences the histone recognition of several PHD-finger reader proteins, such as BHC80 and AIRE [29], as well as ARTX-ADD [141], which specifically bind to unmethylated H3K4. Therefore, it is plausible to postulate that functions of H3K4me0 reader TRIM24 in estrogen response may be intrinsically regulated by LSD1-mediated H3K4me2 demethylation and H3T6 phosphorylation.

2.1.12. **Hypothesis: Function of TRIM24 is dependent on LSD1-mediated H3K4 demethylation**

Despite the many functions mediated by TRIM24 and LSD1, and their respective roles in estrogen response, no study has focused on the potential cooperation of TRIM24 and LSD1 in mediating ERα target gene activation through changes and recognition of specific histone modifications. Through its recognition of unmethylated H3K4, TRIM24 functions as a chromatin reader and an ERα co-activator. However, it is unknown whether LSD1-mediated demethylation of H3K4me1/2 leads a favorable histone substrate for TRIM24 binding and orchestrated ERα-mediated transcriptional activation. Therefore, I hypothesize that the function of TRIM24 as an ERα co-activator is dependent on LSD1-mediated H3K4 demethylation. To test the hypothesis, I have formulated the following specific aims and test them using several approaches in human tumor-derived MCF7 breast cancer cells:
• Establish the time-dependent profile of ERα target gene activation when TRIM24 or LSD1 is depleted

• Establish a cyclical recruitment profile of TRIM24 and LSD1, as well as dynamic H3K4 methylation over a time course of E₂ stimulation

• Screen for potent LSD1 inhibitors and their effects on H3K4me2 on EREs and ERα-mediated transcription activation

• Determine whether inhibition of LSD1 prevents TRIM24 from binding to EREs on the chromatins

• Investigate how H3T6 affects TRIM24-regulated ERα-mediated transcription

In summary, the results presented in this chapter suggested that TRIM24 and LSD1 are cyclically recruited to EREs in response to E₂ stimulation. Estrogen induction mediates dynamic H3K4 methylation/demethylation events over a time course. Moreover, inhibition of LSD1 enzymatic activity results in re-methylated of H3K4me2, decreased binding of TRIM24 and ERα, leading to down-regulation of TRIM24-regulated ERα-mediated transcription activation. Finally, H3T6ph impairs TRIM24 binding to histone H3 and may play a critical role in ERα-mediated transactivation.
2.2. MATERIALS AND METHODS

2.2.1. Cell culture

MCF7 cells are obtained from ATCC and cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), as previously described [57,68]. For hormone depletion, cells are changed to charcoal-stripped hormone-free medium (Gibco) supplemented with 10% charcoal dextran-treated FBS (Hyclone) for 96h. Estrogen induction is performed in the presence of 17β-estradiol (Sigma) for the indicated times. Ethanol is used as a vehicle control.

2.2.2 Mutagenesis

Site-specific point mutations are introduced to wildtype and FLAG-tagged TRIM24 plasmid using QuickChange® Site-Directed Mutagenesis Kit (Stratagene), according to manufacturer’s suggestions. Each transformation reaction is then plated on LB-ampicillin agar plates and incubated at 37°C overnight. DNA isolated from E. coli culture is purified using DNA Miniprep Kit (Qiagene). Sequences of specific point mutation have been confirmed by M.D. Anderson DNA Analysis Core Facility.

2.2.3. Transient DNA plasmid transfection

MCF7 cells cultured in 6-well plates in the presence of hormone-depleted medium are transfected with FLAG-TRIM24 using Effectene (Qiagen), according to manufacturer’s instructions. Briefly, 2 µg of DNA plasmid is resuspended in
300µL EC Buffer, together with 16µL Enhancer and vortexed for 1 sec. After 5 min of incubation at room temperature (RT), 60µL of Effectene Transfection Reagent is added to the mixture, and vortexed for 10 sec. At the end of 10 min incubation at RT, medium is added to reach 500 µL total and pipetted evenly onto the cells. Cells are treated with estrogen or control, as indicated. RNA is harvested 48 hr after transfection.

2.2.4. Transient knockdown by siRNAs

MCF7 cells cultured at 6-well plates are transfected with siControl, siTRIM24, or siLSD1 (ON-TARGETplus SMARTpool, Dharmacon) using Lipofectamine™ 2000 (Invitrogen), according to manufacturer’s instructions. Briefly, cells are changed to Pen/Strep-free medium 2 to 3 hr prior to transfection. For each transfection, 100 pmol of siRNA and 5 µL Lipofectamine™ 2000 are first individually resuspended into 250 µL medium and incubated at RT separately for 5 min. Then, siRNA and Lipofectamine™ 2000 are mixed together and further incubated at RT for 20 min. Cells covered with 250 µL medium are then transfected with the siRNA-Lipofectamine mixture for 4 to 6 hr. Medium is changed and cells are cultured for a total of 72 hr before harvest. Estrogen treatment is added prior to harvest for indicated time points.

2.2.5. RNA extraction, cDNA, and real-time RT-PCR

RNA in each 6-well plate is isolated using Trizol reagent (Invitrogen), according to manufacturer’s suggestions. 3 µg of RNA are used to synthesize
cDNA using RT-PCR kit (Invitrogen), according to manufacturer’s suggestions. Each real time PT-PCR reaction mix, containing 2µL of dilutes cNDA (1:10 dilution), 5µL SYBR Green Reaction Mix (Applied Biosystems), 0.25µL forward primer (20µM), 0.25µL reverse primer (20µM), and 2.5µL sterile water, is set up in a 96-well plate and performed in a 7500 Fast Real Time PCR instrument (Applied Biosystems). Primer sequences are listed below:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
</tr>
</thead>
<tbody>
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</tr>
<tr>
<td>PR</td>
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<tr>
<td>pS2</td>
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</tr>
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<td>CCTGGCCCGGGCTGATGGA</td>
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<tr>
<td>TRIM24</td>
<td>TATCTCCAGAGGCAGTTG</td>
<td>CTCACAGTACAGCTTCAG</td>
</tr>
<tr>
<td>LSD1</td>
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</tr>
<tr>
<td>GAPDH</td>
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<td>GAAGATGGTGATGGGATTTC</td>
</tr>
</tbody>
</table>

2.2.6. Chromatin immunoprecipitation (ChIP)

MCF7 cells are cultured in 150 mm² plates for ChIP experiments as previously described⁵⁸,⁷⁰. Essentially, after 96 h of hormone depletion, cells are treated with indicated treatment or corresponding control, in addition to 20nM 17β-estradiol (Sigma) or ethanol, for indicated time course. Upon harvest, cells
are cross-linked with 556 µL 37% formaldehyde (in 20 mL medium) and rotated at RT for 15 min. Cross-linking is stopped with 6.8 mL of glycine (0.5M stock) and rotated at RT for an additional 10 min. The media on the plates are then removed and washed with chilled sterile PBS twice before scrapping in 5 mL PBS (with 1X PMSF). Cells pellets are spun down 2K rpm for 5 min at 4°C. After the supernatant has been discarded, cells are lyzed with 1 mL Cell Lysis Buffer (5mM PIPES pH 8.0, 85mM KCl, 0.5% NP-40, fresh protease inhibitors) and incubated on ice for 15 min. Then the cell lysates are collected after centrifugation at 5K rpm for 5 min and resuspended in 300 µL Nuclear Lysis Buffer (50mM Tris pH 8.1, 10mM EDTA, 1% SDS, and fresh protease inhibitors). 100mg glass beads (Sigma) are added to the samples. Sonication is performed using a bioruptor (Diagenode, Bioruptor Twin #UCD-400) at high input for 36 min (in a 30 sec ON, 30 sec off mode). After sonication, the tubes are spun at 14K for 15 min at 4°C. Supernatant is transferred to a new tube to check for fragment size. For ChIP experiments, lysates are divided and diluted using ChIP Lysis Buffer (150mM NaCl, 25mM Tris pH 7.5, 5mM EDTA, 1% TritonX 100, 01% SDS, 0.5% Deoxycholate, and fresh protease inhibitors). After IgG preclearing (2.5 µg for 1 hr at 4°C), immunoprecipitation is performed overnight (O/N) with specific antibodies: ERα (F-10, Santa Cruz), TRIM24 (ProteinTech), LSD1 (Abcam), histone H3 (Abcam), H3K4me1 (Abcam), H3K4me2 (Active Motif), H3K4me3 (Active Motif), H3T6ph (Abcam), H3K9me2 (Abcam) or normal sheep IgG (Upstate/Millipore). The next day, pre-washed Protein A Sepharose beads (GE Health) are incubated with antibody/protein bound complexes for 2 hr at 4°C.
Then, Protein A beads are washed once with RIPA Buffer (50mM Tris pH 8.0, 150mM NaCl, 0.1% SDS, 0.5% Deoxycholate, 1% NP-40, and 1mM EDTA), once with High Salt Buffer (50mM Tris pH 8.0, 500mM NaCl, 0.1% SDS, 0.5% Deoxycholate, 1% NP-40, and 1mM EDTA), once with LiCl Wash (50mM Tris pH 8.0, 1mM EDTA, 250mM LiCl, 1% NP-40, and 0.5% Deoxycholate), and twice with TE Buffer, each wash for 10 min at 4°C. The input and the ChIP samples are resuspended into 300µL TE Buffer and incubated with 1.5µL RNaseA (10mg/mL stock) for 30 min at 37°C and then with 15µL SDS (10% stock) and 7.5µL ProteaseK (10mg/mL stock) for 2 hr at 55°C.

The crosslinks are reversed by incubating the samples at 65°C O/N. The next day, Protein/antibody bound DNA fragments are extracted with 300µL Phenol/Chloroform twice and 300µL Chloroform once. Precipitation is performed using 30 µL NaOAc (3M stock), 600 µL ethanol (100% stock) and 25 µL glycogen (1mg/mL stock) for 1 hr at -80°C. qPCR analyses are performed to analyze specific antibody- and protein-bound DNA using SYBR Green Reaction Mix (Applied Biosystems) in a 7500 Fast Real Time PCR instrument (Applied Biosystems). Sequences of ChIP primers are listed below:
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<thead>
<tr>
<th>Region</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
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<td>GGTTTTAAGCAGCCAGCAG</td>
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<tr>
<td>GREB1-proximal</td>
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<tr>
<td>ERE</td>
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</tr>
<tr>
<td>GREB1</td>
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</tr>
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</tr>
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</tr>
<tr>
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<td>AGACAGGCTCAGGTCAAGA</td>
</tr>
<tr>
<td>IGFBP4-ERE</td>
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</tr>
<tr>
<td>GAPDH</td>
<td>GAAGGTGAAGGTCGGAGTC</td>
<td>GAAGATGGTGATGGGATTTC</td>
</tr>
</tbody>
</table>

**2.2.7. GST-tagged protein expression and purification**

GST-only and GST-tagged TRIM24 recombinant proteins are expressed using BL21-Al strains of *E. coli* (Invitrogen) and cultured in 2X LB media in the presence of 100 µg/mL Ampicillin at 37°C. When the overnight culture reaches OD<sub>600</sub> of 0.5 to 0.6, it is induced with final concentration 0.2% L-arabinose (Sigma) and 100 µM ZnCl (for PHD zinc finger expression), and subsequently cultured at RT for 24 hr. The cell pellets are collected by spinning at 4K rpm for
15 min at 4°C and snap-freeze at -80°C. The frozen pellets are resuspended in cold lysis buffer (50mM Tris pH 7.5, 150mM NaCl, 0.05% NP-40, 1X PMSF and protease inhibitors) in the presence of final concentration 0.5mg/mL lysozyme solution and sonicated at an output of 18% for 40 sec (in a 1 sec on, 1 sec off manner) on ice. The supernatant is collected by centrifugation at 12K rpm for 15 min at 4°C, and incubated with washed 50% slurry mix of glutathione (GST) beads (AmerSham) O/N at 4°C. The GST-beads are washed three times with lysis buffer, then once with elution buffer (100mM Tris pH 8.0) at 4°C for 5 min. Finally, the GST-proteins are eluted with elution buffer containing 10mM fresh glutathione. Concentration is measured using Bradford protein assay at A280 and analyzed by Coomassie blue staining of SDS-PAGE gel, in comparison to BSA standards. Final concentration of 10% glycerol is added to the recombinant proteins for long-term storage at -80°C.

2.2.8. Biotinylated peptide pulldown assay

GST-RBP2 (PHD1 or PHD2) recombinant proteins are obtained from Dr. Xiaobing Shi’s laboratory, GST-JMJD2A-Double Tudor Domain (DTD) from Dr. Mark Bedford’s laboratory and GST-LSD1 from Dr. Yang Shi’s laboratory. Peptides are biotin-labeled and custom-made by peptide synthesis facility at Yale University.

In each binding assay, 2 µg of GST-tagged recombinant proteins and 1 µg of biotinylated histone peptides (1mg/mL) are incubated together in 500µL NTP binding buffer (50mM Tris PH 7.5, 200mM NaCl, 0.1% NP-40) O/N at 4°C. For
input, no peptide is added. On the next day, 20 µL of washed 50% slurry of Streptavidin beads (GE Heath) are added into each binding assay and rotated at 4°C for 1 hr. The beads are washed with NTP binding buffer and rotated at 4°C for 10 min. Flowthrough (FT) is saved. After three washes, the beads are resuspended in 60 µL of 2X SDS loading dye, boiled and loaded on a 10% SDS-PAGE gel, together with 10% input and FT for each binding assay. The peptide-bound GST proteins are detected by GST-antibody (Cell Signaling, 1:1000).

2.3. RESULTS

2.3.1. Estrogen triggers immediate dynamic histone modifications

The addition of an ERα agonist, 17β-estradiol (estrogen, E₂), immediate triggers changes in the chromatin architecture. First, total H3 at ERα target genes, GREB1 and PR estrogen responsive elements (EREs), decreases as early as 15 min of E₂ treatment, and the low levels of H3 persist until 1 hr after E2 induction (Figure 2-6). The decrease in total suggests that the chromatin may employ a more “open” conformation for the recruitment of co-activator proteins and transcription machinery for ER-regulated gene activation. Consistent with this notion, active histone marks such as H3K23ac (Figure 2-7) and H3K27ac (Figure 2-8) are also enriched at GREB1 and PR EREs and promoters. Consistently, ERα is recruited to GREB1 and PR EREs at \( t = 15 \) min and \( t = 1 \) hr. Notably, dynamic H3K4me2/3 levels are also observed at these time points, suggesting that H3K4 methylation may be involved in the regulation of ERα-mediated transcription activity.
Figure 2-6. Total H3 decreases immediately upon estrogen treatment.
qChIP: H3 occupancy at (A) GREB1 and (B) PR EREs and promoters at untreated (Control) or estrogen (E2) treated for 15 min or 1 hr.

A

qChIP : H3
GREB1

B

qChIP : H3
PR
Figure 2-7. Changes of H3K23ac levels upon estrogen treatment. qChIP: H3K23ac levels, normalized with H3, at (A) GREB1 and (B) PR EREs and promoters at untreated (Control) or estrogen (E\textsubscript{2}) treated for 15 min or 1 hr.

A

![Graph showing changes in H3K23ac levels for GREB1](image)

B

![Graph showing changes in H3K23ac levels for PR](image)
Figure 2-8. Changes of H3K27ac levels upon estrogen treatment. qChIP: H3K27ac levels, normalized with H3, at (A) GREB1 and (B) PR EREs and promoters at untreated (Control) or estrogen (E₂) treated for 15 min or 1 hr.

A

qChIP : H3K27ac/H3
GREB1

relative fold

DISTAL-ERE  PROXIMAL-ERE  +54

Control
E₂ 15min
E₂ 1hr

B

qChIP : H3K27ac/H3
PR

relative fold

-221kb  -205kb  +4kb

Control
E₂ 15min
E₂ 1hr
Figure 2-9. Recruitment of ERα upon estrogen treatment. qChIP: ERα recruitment at (A) GREB1 and (B) PR EREs and promoters at untreated (Control) or estrogen (E2) treated for 15 min or 1 hr.

A

qChIP : ERα
GREB1

relative fold

0.0
0.5
1.0
1.5
2.0
2.5

Distal-ERE
Proximal-ERE
+54

Control
E2 15min
E2 1hr

B

qChIP : ERα
PR

relative fold

0.0
0.5
1.0
1.5
2.0

-221kb
-205kb
+4kb

Control
E2 15min
E2 1hr
Figure 2-10. Changes of H3K4me2 levels upon estrogen treatment. qChIP: H3K4me2 levels, normalized with H3, at (A) GREB1 and (B) PR EREs and promoters at untreated (Control) or estrogen (E₂) treated for 15 min or 1 hr.

A

qChIP : H3K4me2/H3
GREB1

<table>
<thead>
<tr>
<th>relative fold</th>
<th>Control</th>
<th>E₂ 15min</th>
<th>E₂ 1hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distal-ERE</td>
<td></td>
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<tr>
<td>Proximal-ERE</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>+54</td>
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</tr>
</tbody>
</table>

B

qChIP : H3K4me2/H3
PR

<table>
<thead>
<tr>
<th>relative fold</th>
<th>Control</th>
<th>E₂ 15min</th>
<th>E₂ 1hr</th>
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<tbody>
<tr>
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Figure 2-11. Changes of H3K4me3 levels upon estrogen treatment. qChIP: H3K4me3 levels, normalized with H3, at (A) GREB1 and (B) PR EREs and promoters at untreated (Control) or estrogen (E₂) treated for 15 min or 1 hr.

A

qChIP : H3K4me3/H3
GREB1

relative fold

Distal-ERE  Proximal-ERE  +54

Control  E₂ 15min  E₂ 1hr

B

qChIP : H3K4me3/H3
PR

relative fold

-221kb  -205kb  +4kb

Control  E₂ 15min  E₂ 1hr

A
2.3.2. **TRIM24 expression is essential for timely estrogen response**

TRIM24 and ERα co-occupy nearly 1600 genes when ERα is activated by estrogen (E$_2$) [57]. Among these genes, I first focused on *GREB1* and studied the mechanism of TRIM24-regulated transcription. Previous study by Dr. Tsai suggested that TRIM24 depletion reduces E$_2$-induced GREB1 activation at $t = 3$hr and $t = 6$hr. Here I re-introduced WT-TRIM24 or EGFP (plasmid control) into shTRIM24 MCF7 cells (48 hr transfection) and treated with different doses of estrogen (E$_2$) before assaying *GREB1* activation with qPCR. When I compared *GREB1* induction in shControl and shTRIM24 cells, TRIM24 expression is essential for response at lower level of estrogen (Figure 2-13, E$_2$ = 10$^{-10}$M). Rescue of WT-TRIM24 expression (Figure 2-12) is able to fully rescue estrogen response at sub-nanomolar of E$_2$ (Figure 2-13). When TRIM24 is depleted (Figure 2-14) and *GREB1* transactivation assayed over a time course of $t = 0$hr, 1hr, 2hr, 3hr, 4hr, and 5hr, it suggested that TRIM24 expression is essential for timely estrogen response (Figure 2-16A). Taken together, TRIM24 expression allows timely activation of *GREB1* expression at lower levels of E$_2$. In fact, depletion of TRIM24 or LSD1 also reduces estrogen-induced *PR* activation (Figure 2-16B). However, only LSD1, but not TRIM24 regulates *IGFBP4* transactivation (Figure 2-17). As a control, neither TRIM24 nor LSD1 knockdown affects non-estrogen responsive gene *BCAS4* (Figure 2-18). Therefore, LSD1 and TRIM24 may cooperatively regulate the time-dependent activation on a subset of ERα target genes.
Figure 2-12. Ectopic expression of TRIM24 in MCF7 depleted of endogenous TRIM24. qPCR analyses of cDNA prepared from TRIM24 knockdown cells transfected with exogenous TRIM24-WT or EGFP control vector for 48 hours. Cells are pretreated with vehicle, or indicated concentration of Estrogen (E₂), and/or 4-hydroxy-Tamoxifen (Tam) before assaying for TRIM24 expression. RNA levels are normalized to GAPDH; value from EGFP-transfected cells is set as one. Averaged results from duplicates; error bars = SEM.

Overexpression of TRIM24
Figure 2-13. TRIM24 expression allows estrogen response at lower levels of hormone. qPCR analyses of cDNA prepared from shControl or shTRIM24 cells transfected with exogenous TRIM24-WT or EGFP control vector for 48 hours. Cells are pretreated with vehicle, or indicated concentration of Estrogen (E₂) before assaying for GREB1 induction. RNA levels are normalized to GAPDH; untreated shControl MCF7 is set as one. Averaged results from duplicates; error bars = SEM.
Figure 2-14. Western blot analysis revealed decreased TRIM24 protein level mediated by siRNA in MCF7 cells. (A) Western blot showing knockdown efficiency of TRIM24 by siRNA (for 72 hours) at indicated time of estrogen (E2) treatment. (B) Quantification of TRIM24 expression as normalized to β-ACTIN control in siControl and siTRIM24 cells.
Figure 2-15. Knockdown of LSD1 by siRNAs. qPCR analyses of cDNA prepared from MCF7 cells transfected with siControl or siLSD1 for 48 hr and treated with 10 nM estrogen at the indicated time points before assaying for LSD1 expression. RNA levels are normalized to GAPDH; siControl at each time point is set as one. Average results from triplicates; error bars = SEM.

qPCR : LSD1

![Graph showing qPCR results for LSD1 expression over time]

- **siControl**
- **siLSD1**

relative fold

minutes of E₂ treatment
Figure 2-16A. Effects of siTRIM24 or siLSD1 on ERα target gene activation.

qPCR analyses of cDNA prepared from MCF7 cells transfected with siControl, siTRIM24, or siLSD1 for 48 hr and treated with 10 nM estrogen at the indicated time points before assaying for (A) GREB1, (B) PR expression. RNA levels are normalized to GAPDH; vehicle-treated MCF7 is set as one. Average results from triplicates; error bars = SEM (Student t test: *p-value<0.05).
Figure 2-16B. Effects of siTRIM24 or siLSD1 on ERα target gene activation. qPCR analyses of cDNA prepared from MCF7 cells transfected with siControl, siTRIM24, or siLSD1 for 48 hr and treated with of 10 nM estrogen at the indicated time points before assaying for (A) GREB1, (B) PR expression. RNA levels are normalized to GAPDH; vehicle-treated MCF7 is set as one. Average results from triplicates; error bars = SEM (Student t test: *p-value<0.05; *** p-value<0.001).
**Figure 2-17. Effects of siTRIM24 or siLSD1 on ERα target gene activation.**

qPCR analyses of cDNA prepared from MCF7 cells transfected with siControl, siTRIM24, or siLSD1 for 48 hr and treated with 10 nM estrogen at the indicated time points before assaying for *IGFBP4* expression. RNA levels are normalized to *GAPDH*; vehicle-treated MCF7 is set as one. Average results from triplicates; error bars = SEM.
Figure 2-18. Effects of siTRIM24 or siLSD1 on non-E\(_2\) response gene BCAS4. qPCR analyses of cDNA prepared from MCF7 cells transfected with siControl, siTRIM24, or siLSD1 for 48 hr and treated with 10 nM estrogen at the indicated time points before assaying for BCAS4 expression. RNA levels are normalized to GAPDH; vehicle-treated MCF7 is set as one. Average results from triplicates; error bars = SEM (Student t test: *p-value<0.05).
2.3.3. Recruitment of LSD1 and TRIM24 and changes of H3K4 methylation at GREB1 ERE upon estrogen induction

Although global binding of TRIM24 preferentially concentrates on demethylated regions, no change of H3K4me2 was observed at GREB1 ERE sites after 6 hours of E2 treatment [57]. Therefore, I decided to expand our profile and perform ChIP analyses and examined the snapshots of ERα, LSD1, and TRIM24 recruitment on the chromatin at t = 15 min, 30 min, 90 min, 150 min, 180 min, 210 min, 240 min, 270 min, and 300 min after E2. In hormonally synchronized MCF7 cells, estrogen-induced transcriptional activation of GREB1 occurs as early as 60 min and continues to up-regulate until 240 min (Figure 2-16A). At 300 min, ERα-mediated activated GREB1 expression levels off (Figure 2-16A). I also aimed to detect any methylation/demethylation events of histone H3 lysine 4 (H3K4) at these time points.

Upon estrogen stimulation, LSD1 and TRIM24 are naturally recruited to GREB1 distal ERE in non-identical cyclical patterns (Figure 2-19). The engagement of LSD1 occurs every 90 min while TRIM24 is bound at 15 min, 90 min, and 240 min. As for ERα, estrogen-induced recruitment is initiated at 15 min, which persists and fluctuates until 210 min, followed by a sharp peak at 240 min, then maintains at low levels from 270 min to 300 min. Previous study suggested that total H3 methylation is cyclical [73]. Here, I provided a detailed profile of mono-, di-, and tri-methylated H3K4 (H3K4me1/2/3) after E2 treatment and detected dynamic methylation/demethylation events at specific time points.
Figure 2-19. Cyclical recruitments of LSD1 and TRIM24 to GREB1 distal ERE site. Percent of maximum ChIP signals for recruitments of LSD1, TRIM24 and ERα to GREB1 distal ERE at indicated time points of estrogen (E2) treatment.
The pre-E₂ recruitment of LSD1 has been previously reported. Here, I observed high level of H3K4me2 and co-occupancy of LSD1 before E₂ induction (Figures 2-19 and 2-20). As soon as 15 min post-E₂, LSD1 immediately disengages GREB1 ERE and the reason still remains unclear. On the chromatin, the loss of H3K4me2/1 is accompanied by gain of H3K4me3, suggesting that unknown lysine methyltransferases(s) may be involved. Notably, both TRIM24 and ERα are recruited at t = 15 min (Figure 2-19), the previously described “transcriptionally unproductive cycle” [73].

From 15 min to 30 min, LSD1 is re-engaged on GREB1 ERE; ERα accumulates while TRIM24 cycles off the chromatin (Figure 2-19). At the same time, rapid demethylation events from H3K4me3 to H3K4me2, as well as from H3K4me2 to H3K4me1, are observed. Because LSD1 is unable to demethylate trimethyl-H3K4, I suspected that another unknown lysine demethylases(s) might also be present on the chromatin at this interval.

The first synchronized recruitments of ERα, LSD1 and TRIM24 occur from 30 min to 180 min (Figure 2-19). Consistent with the observation, knockdown of LSD1 or TRIM24 during this period leads to down-regulation of GREB1 induction (Figure 2-16A). During this period, the successive enrichment of H3K4me2 is concurrent with gradual loss of H3K4me3/1, possibly through demethylation of H3K4me3 and re-methylation of H3K4me1.
Figure 2-20. Dynamic changes in H3K4 methylation levels at \textit{GREB1} distal ERE site. Percent of maximum ChIP signals for H3K4me1, H3K4me2, and H3K4me3, normalized with total H3, to \textit{GREB1} distal ERE at indicated time points of estrogen (E\textsubscript{2}) treatment.

![Graph showing dynamic changes in H3K4 methylation levels at GREB1 distal ERE site.](image-url)
The second cycle and maximum recruitment of LSD1 occurs at $t = 180$ min, when TRIM24 and ERα are accumulating on the chromatin (Figure 2-19). From 180 min to 210 min, immediate loss of H3K4me2 is accompanied by gain of TRIM24 recruitment, while ERα partially cycles off the chromatin.

From 210 to 240 min, TRIM24 continues to accumulate and ERα cycles back to GREB1 ERE. Highest magnitude of TRIM24 and ERα binding is observed at 240 min, while minimal level of LSD1 is detectable. From 240 min to 300 min, LSD1, TRIM24 and ERα are gradually dis-engaged from the chromatin, while H3K4me2 continues to increase.

2.3.4. Recruitment of LSD1 and TRIM24 and changes of H3K4 methylation at PR ERE upon estrogen induction

The recruitment profile of LSD1 and TRIM24 to PR ERE (Figure 2-21) is similar but not identical to GREB1 (Figure 2-19). Similar to GREB1, the cycling time for LSD1 is 90 min while TRIM24 engagement peaks at $t = 150$min, 210min, and 300min (Figure 2-21), suggesting that there are intrinsic differences of LSD1 and TRIM24 recruitment in a gene-specific manner. For ERα, estrogen-induced recruitment occurs as early as $t = 15$ min, which persists and continues to be enriched until $t = 180$ min, and maintains at detectable levels from 210 min to 300 min (Figure 2-21). Essentially, pre-E2 recruitment of LSD1 is also observed on PR ERE. Disengagement of LSD1 at $t = 15$min (Figure 2-21) is accompanied by loss of H3K4me2 and gain of H3K4me1/3 (Figure 2-22), suggesting dynamic changes in chromatin architecture during immediate estrogen response. From 30 min to 150 min, H3K4me1/2 dominant while H3K4me3 is kept at barely
detectable level (Figure 2-22). During the same period, ERα, LSD1, and TRIM24 are co-recruited to *PR* ERE (Figure 2-21). Notably, the second cycle and maximum recruitment of LSD1 occurs at $t = 180$ min, preceding that of TRIM24 at $t = 210$ min (Figure 2-21). From 180 min to 210 min, the loss of H3K4me2 is concurrent with gain of H3K4me1, suggesting that demethylation of H3K4me2 is actively ongoing (Figure 2-22). Interestingly, TRIM24 has a third recruitment peak at $t = 300$ min on *PR* ERE (Figure 2-19), which is not observed on *GREB1* ERE (Figure 2-21).
Figure 2-21. Cyclical recruitments of LSD1 and TRIM24 to PR ERE site.
Percent of maximum ChIP signals for recruitments of LSD1, TRIM24 and ERα to PR ERE (205kb upstream of promoter) at indicated time points of estrogen (E₂) treatment.

qChIP : PR -205kb

0 30 60 90 120 150 180 210 240 270 300
minutes after E₂

0 20 40 60 80 100
percent of maximum ChIP signal

LSD1
TRIM24
ERα
Figure 2-22. Dynamic changes in H3K4 methylation levels at PR ERE site.
Percent of maximum ChIP signals for H3K4me1, H3K4me2, and H3K4me3, normalized with total H3, to PR ERE (205kb upstream of promoter) at indicated time points of estrogen (E₂) treatment.

qChIP : PR -205kb

0 30 60 90 120 150 180 210 240 270 300
0 20 40 60 80 100
percent of maximum ChIP signal
minutes after E₂
2.3.5. Recruitment of LSD1 and TRIM24 and changes of H3K4 methylation at pS2 ERE upon estrogen induction

As for pS2 ERE, LSD1 also has a recruitment cycle of 90min and pre-occupied the chromatin before estrogen stimulation. The disengagement of LSD1 at $t = 15$ min (Figure 2-23) is accompanied by loss of H3K4me2 and gain of H3K4me3 (Figure 2-24), suggesting that methylation of H3K4me2 to H3K4me3 is activated. From 15 min to 30 min, loss of H3K4me3 is concurrent with gain of H3K4me1/2 (Figure 2-24), implying demethylation of H3K4me3 is ongoing. Again, the first synchronized recruitment of LSD1, TRIM24, and ERα occur from 30min 150min (Figure 2-23). From 30 min to 60 min, H3K4me2 is accumulating while H3K4me1/3 is gradually lost (Figure 2-24). From 60 min to 150min, occupancies of H3K4me1/2/3 are all decreasing (Figure 2-24). The second synchronized peak for LSD1, TRIM24, and ERα is from 150 min to 240 min (Figure 2-23), which is not observed in neither GREB1 (Figure 2-19) nor PR EREs (Figure 2-21). During this time, H3K4me2 is first being accumulated, and then H3K4me3 is enriched as H3K4me2 is gradually weakening (Figure 2-24). The third synchronized peak for LSD1 and ERα at $t = 270$ min. From 270 min to 300 min, LSD1 and ERα are disengaged from the chromatins and H3K4me2/3 marks are accumulating. During this time, TRIM24 recruitment is still lagging behind.
Figure 2-23. Cyclical recruitments of LSD1 and TRIM24 to pS2 proximal ERE site. Percent of maximum ChIP signals for recruitments of LSD1, TRIM24 and ERα to pS2 proximal ERE at indicated time points of estrogen (E₂) treatment.
Figure 2-24. Dynamic changes in H3K4 methylation levels at pS2 proximal ERE site. Percent of maximum ChIP signals for H3K4me1, H3K4me2, and H3K4me3, normalized with total H3, to pS2 proximal ERE at indicated time points of estrogen (E2) treatment.
2.3.6. LSD1 enzymatic activity is critical for ERα-mediated transcription

In 2006, LSD was discovered to share close homology with monoamine oxidases (MAO), and MAO inhibitors, often used as antidepressants, can effectively inhibit the enzymatic activity of LSD1 [140]. Among these inhibitors, Tranylcypromine (TCP) is the only one that specifically inhibits LSD1 for the demethylation of H3K4 but not H3K9. After consulting with the author Dr. Min Gyu Lee, we were advised to pretreat cells with 2 µM TCP for 24 hr for optimal inhibitory effects. Using this condition, I tracked estrogen-induced ERα target gene activation at \( t = 0 \) hr, 1hr, 2hr, 3hr, 4hr or 5hr after estrogen stimulation. First, TCP treatment does not affect LSD1 RNA level (Figure 2-25A). However, TCP-treated cells responds slowly to estrogen, compared to vehicle control (Figure 2-25) and the most significant down-regulation of \( GREB1 \) gene occurs at \( t = 3 \) hr (Figure 2-25B), \( PR \) gene at \( t = 3 \) hr (Figure 2-25C) and \( IGFBP4 \) gene at \( t = 2 \) hr (Figure 2-25D). However, TCP treatment does not affect estrogen-irresponsible gene \( BCAS4 \) (Figure 2-25E). This is the first use of TCP to inhibit LSD1 in breast cancer cells and the first evidence that TCP can affect estrogen response, similar to siLSD1 in ERα target genes at specific time points (Figures 2-16 and 2-17).
Figure 2-25 (A-B). Effects of LSD1 inhibitor TCP on ERα target gene activation. qPCR analyses of cDNA prepared from MCF7 cells pretreated with vehicle (DMSO) or 100 µM AH124 for 24 hr and treated with of 10 nM estrogen at the indicated time points before assaying for (A) LSD1, (B) GREB1, (C) PR, (D) IGFBP4, or (E) BCAS4 expression. RNA levels are normalized to GAPDH; vehicle-treated MCF7 is set as one. Average results from triplicates; error bars = SEM (Student t test: *p-value<0.05).
Figure 2-25 (C-D). Effects of LSD1 inhibitor TCP on ERα target gene activation. qPCR analyses of cDNA prepared from MCF7 cells pretreated with vehicle (DMSO) or 100 µM AH124 for 24 hr and treated with of 10 nM estrogen at the indicated time points before assaying for (A) LSD1, (B) GREB1, (C) PR, (D) IGFBP4, or (E) BCAS4 expression. RNA levels are normalized to GAPDH; vehicle-treated MCF7 is set as one. Average results from triplicates; error bars = SEM (Student t test: *p-value<0.05).

C

qPCR: PR

D

qPCR: IGFBP4
Figure 2-25 (E). Effects of LSD1 inhibitor TCP on ERα target gene activation. qPCR analyses of cDNA prepared from MCF7 cells pretreated with vehicle (DMSO) or 100 µM AH124 for 24 hr and treated with of 10 nM estrogen at the indicated time points before assaying for (A) LSD1, (B) GREB1, (C) PR, (D) IGFBP4, or (E) BCAS4 expression. RNA levels are normalized to GAPDH; vehicle-treated MCF7 is set as one. Average results from triplicates; error bars = SEM (Student t test: *p-value<0.05).
As more and more of LSD1 functions are implicated in cancer biology, recently there is a growing interest in generating specific LSD1 inhibitors for treating cancer cells. In collaboration with Dr. Manfred Jung (Institute of Pharmaceutical Sciences, University of Freiburg, Freiburg, German), I obtained several biochemically verified but unpublished LSD1 inhibitors: AH3, AH124, AH71, and MS30. I pre-treated MCF7 cells with these inhibitors (100 µM, the recommended dosage) and induced with 10 nM E2 for 3 hr. qPCR analyses demonstrated that these potent LSD1 inhibitors are able to down-regulate ERα target genes GREB1 (Figure 2-26A), PR (Figure 2-26B), IGFBP4 (Figure 2-26C), but not the control gene BCAS4 (Figure 2-26D), similar to TCP treatment. Protein level of LSD1 before and after estrogen treatment does not change in DMSO (vehicle)-, AH3, or AH124-treated cells (Figure 2-27). Global H3K4me2 (by Western blot) slightly increases in the presence of AH124 and MS30 (Figure 2-28). I selected AH124 to perform a detailed time course of estrogen treatment. Preliminary results from qPCR analyses demonstrated that AH124 down-regulates ERα target genes at a wider range of time points (Figure 2-29), while LSD1 RNA expression does not change in the presence or absence of AH124 or E2. Taken together, I have validated the ability of AH124 and MS30 to down-regulate ERα target gene activation and to increase global H3K4me2 levels in MCF7 cells.

Similarly, chemicals that share similar structure to the above LSD1 inhibitors, such as Chem778 and Chem779 (from Chembridge Inc.), are tested in this screening (Figure 2-30). The addition of Chem778 or Chem779 down-
regulates estrogen-induced expression of \textit{GREB1} at $t = 180$ min and 240 min (Figure 2-30A); PR at $t = 120$ min, 180 min, 240 min, and 300 min (Figure 2-30B); IGFBP4 at $t = 180$ min and 240 min (Figure 2-30C); and \textit{pS2} at $t = 240$ min (Figure 2-30D). These observations suggested that Chem788 and Chem779 can potentially be potent LSD1 inhibitors, but future biochemical validations are required. Results obtained from these experiments showed that potent and potential LSD1 inhibitors can also effectively down-regulate estrogen-induced ER\textsubscript{α} target gene activation in MCF7 cells.
Figure 2-26 (A-B). Pilot study of LSD1 inhibitors and their effects on ERα target gene activation. qPCR analyses of cDNA prepared from MCF7 cells pretreated with vehicle (DMSO), 100µM of AH3, AH124, Chem778, Chem779 or Chem782 for 24 hr, and treated with 10 nM estrogen for 3 hr before assaying for (A) GREB1, (B) PR, (C) pS2, or (D) IGFBP4 expression. RNA levels are normalized to GAPDH; vehicle-treated MCF7 is set as one. Average results from duplicates; error bars = SEM.
Figure 2-26 (C-D). Pilot study of LSD1 inhibitors and their effects on ERα target gene activation. qPCR analyses of cDNA prepared from MCF7 cells pretreated with vehicle (DMSO), 100µM of AH3, AH124, Chem778, Chem779 or Chem782 for 24 hr, and treated with 10 nM estrogen for 3 hr before assaying for (A) GREB1, (B) PR, (C) pS2, or (D) IGFBP4 expression. RNA levels are normalized to GAPDH; vehicle-treated MCF7 is set as one. Average results from duplicates; error bars = SEM.
Figure 2-27. Effects of potent LSD1 inhibitors on LSD1 protein expression in MCF7 cells. Cell lysates were prepared from MCF7 cells pretreated with vehicle (DMSO), 100 µM AH3 or AH124 for 24 hr and treated with ethanol control (E0) or 10 nM estrogen for 3 hr (E3). Western blot analysis reveals LSD1 protein level and TUBULIN (loading control).
Figure 2-28. Effects of potent LSD1 inhibitors on global H3K4me2 in MCF7 cells. Cell lysates were prepared from MCF7 cells pretreated with vehicle (DMSO), AH124 (100 µM or 200 µM), or MS30 (30 µM or 100 µM) for 24 hr. Western blot analysis reveals global H3K4me2 level and H3 loading control (at short and long exposure).
Figure 2-29 (A-B). Effects of potent LSD1 inhibitor AH124 on ERα target gene activation. qPCR analyses of cDNA prepared from MCF7 cells pretreated with vehicle (DMSO) or 100 µM AH124 for 24 hr and treated with of 10 nM estrogen at the indicated time points before assaying for (A) LSD1, (B) GREB1, (C) IGFBP4, or (D) pS2 expression. RNA levels are normalized to GAPDH; vehicle-treated MCF7 is set as one. Gene induction in the presence of TCP is shown as a control.

A

qPCR: LSD1

B

qPCR: GREB1
Figure 2-29 (C-D). Effects of potent LSD1 inhibitor AH124 on ERα target gene activation. qPCR analyses of cDNA prepared from MCF7 cells pretreated with vehicle (DMSO) or 100 µM AH124 for 24 hr and treated with 10 nM estrogen at the indicated time points before assaying for (A) LSD1, (B) GREB1, (C) IGFBP4, or (D) pS2 expression. RNA levels are normalized to GAPDH; vehicle-treated MCF7 is set as one. Gene induction in the presence of TCP is shown as a control.

C

**qPCR: PR**

![Graph showing PR gene expression](image)

D

**qPCR: IGFBP4**

![Graph showing IGFBP4 gene expression](image)
Figure 2-30 (A-B). Effects of potential LSD1 inhibitors Chem778 and Chem779 on ERα target gene activation. qPCR analyses of cDNA prepared from MCF7 cells pretreated with vehicle, 100µM Chem778 or Chem779 for 24 hr and treated with 10 nM estrogen at the indicated time points before assaying for (A) GREB1, (B) PR, (C) IGFBP4, or (D) pS2 expression. RNA levels are normalized to GAPDH; vehicle-treated MCF7 is set as one. Gene induction in the presence of TCP is shown as a control. Average results from duplicates; error bars = SEM.

A

qPCR : GREB1

B

qPCR : PR
Figure 2-30 (C-D). Effects of potential LSD1 inhibitors Chem778 and Chem779 on ERα target gene activation. qPCR analyses of cDNA prepared from MCF7 cells pretreated with vehicle, 100µM Chem778 or Chem779 for 24 hr and treated with 10 nM estrogen at the indicated time points before assaying for (A) GREB1, (B) PR, (C) IGFBP4, or (D) pS2 expression. RNA levels are normalized to GAPDH; vehicle-treated MCF7 is set as one. Gene induction in the presence of TCP is shown as a control. Average results from duplicates; error bars = SEM.

C

**qPCR : IGFBP4**

![IGFBP4 qPCR graph](image)

D

**qPCR : pS2**

![pS2 qPCR graph](image)
2.3.7. Chromatin-binding ability of TRIM24 is dependent on the enzymatic activity of LSD1

The effects of LSD1 inhibitors on ERα gene activation led to the question of how these inhibitors work mechanistically and whether TRIM24 is involved. The kinetic recruitment of transcription factors (Figures 2-19, 2-21, and 2-23) and dynamic H3K4 methylation (Figures 2-20, 2-22, and 2-24) represents valuable information in guiding the time-specific ChIP experiments in the presence of Tranylcypromine (TCP, an inhibitor of LSD1). For GREB1, the maximum recruitment of LSD1 occurs at 180 min post-E2 (Figure 2-21), so I sought to determine how LSD1 affects the accumulation of TRIM24 and ERα on the chromatin at this time point. When I pre-treated MCF7 cells with 1 µM of TCP, estrogen-induced transcriptional activation of GREB1 is down-regulated, most significantly at $t = 180$ min. (Figure 2-16A). Consistently, at $t = 180$ min, ChIP assays reveal that TCP treatment leads to re-methylation of H3K4me2 (Figure 2-31C), but not H3K4me3 (Figure 2-31D), at GREB1 EREs. I also observed a reduction in H3K4me1 in the presence of TCP at $t = 180$ min (Figure 2-31B), possibly due to the inhibition of demethylation from H3K4me2 to H3K4me1. Importantly, inhibition of LSD1 by TCP does not affect H3K9me2 levels at GREB1 distal ERE (Figure 2-33), suggesting that TCP only induces changes in H3K4 (a preferable substrate of LSD1) but not H3K9 methylation (a biochemically non-preferred LSD1 substrate). Notably, gain of H3K4me2 is concurrent with decreased binding of TRIM24 (Figure 2-32A) and ERα (Figure 2-32B), as well as LSD1 (Figure 2-32C) to the chromatin. Similar effects of TCP
on H3K4me2 occupancy (Figure 2-36), TRIM24 (Figure 2-37) and ERα (Figure 2-38) recruitment are also observed in PR, pS2, and IGFBP4 ERE sites, but not at unspecific site GAPDH (negative control). A summary of TCP-induced changes in histone modifications and recruitments of TRIM24 and ERα is presented in Figure 2-39. These observations confirmed the gene- and time-specific dependence on chromatin structure for unique TRIM24 and ERα binding events.
Figure 2-31 (A-B). LSD1 inhibitor Tranylcypromine (TCP) leads to changes in H3K4 methylation. ChIP assays for (A) total H3, (B) H3K4me1, (C) H3K4me2, (D) H3K4me3 at GREB1 distal and proximal ERE sites with the indicated treatment: vehicle (ethanol), estrogen (E\textsubscript{2}), or E\textsubscript{2} + TCP. Cells are pre-treated with 2 \( \mu \)M TCP for 24 hr and treated with 20 nM E\textsubscript{2} for 3 hr. Average results from triplicates; error bars = SEM.

A.

![qChIP : H3 GREB1](image)

B.

![qChIP : H3K4me1 GREB1](image)
Figure 2-31 (C-D). LSD1 inhibitor Tranylcypromine (TCP) leads to changes in H3K4 methylation. ChIP assays for (A) total H3, (B) H3K4me1, (C) H3K4me2, (D) H3K4me3 at GREB1 distal and proximal ERE sites with the indicated treatment: vehicle (ethanol), estrogen (E₂), or E₂ + TCP. Cells are pre-treated with 2 μM TCP for 24 hr and treated with 20 nM E₂ for 3 hr. Average results from triplicates; error bars = SEM.

C.

qChIP : H3K4me2
GREB1

D.

qChIP : H3K4me3
GREB1
Figure 2-32 (A-B). LSD1 inhibitor Tranylcypromine (TCP) impairs recruitment of TRIM24 and ERα. ChIP assays for (A) TRIM24, (B) ERα, and (C) LSD1 at GREB1 distal and proximal ERE sites with the indicated treatment: vehicle (ethanol), estrogen (E₂), or E₂ + TCP. Cells are pre-treated with 2 µM TCP for 24 hr and treated with 20 nM E₂ for 3 hr. Average results from triplicates; error bars = SEM.

A.

qChIP: TRIM24
GREB1

percentage of input

Vehicle
E₂
E₂ + TCP
distal ERE proximal ERE

B.

qChIP: ERα
GREB1

percentage of input

Vehicle
E₂
E₂ + TCP
distal ERE proximal ERE
Figure 2-32C. LSD1 inhibitor Tranylcypromine (TCP) impairs recruitment of TRIM24 and ERα. ChIP assays for (A) TRIM24, (B) ERα, and (C) LSD1 at GREB1 distal and proximal ERE sites with the indicated treatment: vehicle (ethanol), estrogen (E2), or E2 + TCP. Cells are pre-treated with 2 µM TCP for 24 hr and treated with 20 nM E2 for 3 hr. Average results from triplicates; error bars = SEM.

C.
Figure 2-33. LSD1 inhibitor Tranylcypromine (TCP) does not affect H3K9 methylation. ChIP assays for H3K9me (normalized with total H3) at GREB1, PR, pS2, and IGFBP4 ERE sites with the indicated treatment: vehicle (ethanol), estrogen (E2), or E2 + TCP. Cells are pre-treated with 2 µM TCP for 24 hr and treated with 20 nM E2. Average results from duplicates; error bars = SEM.
Figure 2-34 (A-B). LSD1 inhibitor Tranylcypromine (TCP) leads to changes in H3K4 methylation. ChIP assays for (A) total H3, (B) H3K4me1, and (C) H3K4me2 at GREB1 distal and proximal ERE sites with the indicated treatments: vehicle (ethanol), estrogen (E2), or E2 + TCP. Cells are pre-treated with 2 μM TCP for 24 hr and treated with 20 nM E2 for 5 hr. Average results from triplicates; error bars = SEM.

A.

qChIP : H3
GREB1 distal ERE

Vehicle
E2 5hr
E2 5hr + TCP

Percentage of input

Vehicle
E2 5hr
E2 5hr + TCP

B.

qChIP : H3K4me1
GREB1 distal ERE

Vehicle
E2 5hr
E2 5hr + TCP

Relative fold over H3

Vehicle
E2 5hr
E2 5hr + TCP
Figure 2-34C. LSD1 inhibitor Tranylcypromine (TCP) leads to changes in H3K4 methylation. ChIP assays for (A) total H3, (B) H3K4me1, and (C) H3K4me2 at GREB1 distal and proximal ERE sites with the indicated treatment: vehicle (ethanol), estrogen (E2), or E2 + TCP. Cells are pre-treated with 2 µM TCP for 24 hr and treated with 20 nM E2 for 5 hr. Average results from triplicates; error bars = SEM.

C.

qChIP: H3K4me2
GREB1 distal ERE

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>E2 5hr</th>
<th>E2 5hr + TCP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative fold</td>
<td>1.0</td>
<td>0.5</td>
<td>2.0</td>
</tr>
<tr>
<td>Over H3</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Vehicle
E2 5hr
E2 5hr + TCP
Figure 2-35 (A-B). LSD1 inhibitor Tranylcypromine (TCP) impairs recruitment of TRIM24 and ERα. ChIP assays for (A) TRIM24, (B) ERα, and (C) LSD1 at GREB1 distal and proximal ERE sites with the indicated treatment: vehicle (ethanol), estrogen (E₂), or E₂ + TCP. Cells are pre-treated with 2 µM TCP for 24 hr and treated with 20 nM E₂ for 5 hr.

A. 

qChIP : TRIM24  
GREB1 distal ERE

B. 

qChIP : ERα  
GREB1 distal ERE
Figure 2-35C. LSD1 inhibitor Tranylcypromine (TCP) impairs recruitment of TRIM24 and ERα. ChIP assays for (A) TRIM24, (B) ERα, and (C) LSD1 at GREB1 distal and proximal ERE sites with the indicated treatment: vehicle (ethanol), estrogen (E₂), or E₂ + TCP. Cells are pre-treated with 2 µM TCP for 24 hr and treated with 10 nM E₂ for 5 hr.

C.

qChIP : LSD1
GREB1 distal ERE

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>E₂ 5hr</th>
<th>E₂ 5hr + TCP</th>
</tr>
</thead>
<tbody>
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<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>0.02</td>
<td>0.08</td>
<td>0.08</td>
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<tr>
<td>E₂ 5hr</td>
<td>0.02</td>
<td>0.08</td>
<td>0.08</td>
</tr>
<tr>
<td>E₂ 5hr + TCP</td>
<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
</tr>
</tbody>
</table>

[Graph showing qChIP results for LSD1 at GREB1 distal ERE sites with different treatments.]
Figure 2-36 (A-B). LSD1 inhibitor Tranylcypromine (TCP) leads to remethylation of H3K4me2. ChIP assays for H3K4me2 occupancy (normalized with total H3) at (A) PR, (B) pS2, and (C) IGFBP4 ERE sites, or negative control (D) GAPDH, with the indicated treatment: vehicle (ethanol), estrogen (E2), or E2 + TCP. Cells are pre-treated with 2 µM TCP for 24 hr and treated with 20 nM E2. Average results from duplicates; error bars = SEM.

A.

B.
Figure 2-36 (C-D). LSD1 inhibitor Tranylcypromine (TCP) leads to re-methylation of H3K4me2. ChIP assays for H3K4me2 occupancy (normalized with total H3) at (A) PR, (B) pS2, and (C) IGFBP4 ERE sites, or negative control (D) GAPDH, with the indicated treatment: vehicle (ethanol), estrogen (E₂), or E₂ + TCP. Cells are pre-treated with 2 µM TCP for 24 hr and treated with 20 nM E₂. Average results from duplicates; error bars = SEM.

C.

D.
Figure 2-37 (A-B). LSD1 inhibitor Tranylcypromine (TCP) impairs TRIM24 recruitment. ChIP assays for TRIM24 recruitment (normalized with input) at (A) PR, (B) pS2, and (C) IGFBP4 ERE sites, or negative control (D) GAPDH, with the indicated treatment: vehicle (ethanol), estrogen (E2), or E2 + TCP. Cells are pre-treated with 2 µM TCP for 24 hr and treated with 20 nM E2. Average results from duplicates; error bars = SEM.

A.

B.
Figure 2-37 (C-D). LSD1 inhibitor Tranylcypromine (TCP) impairs TRIM24 recruitment. ChIP assays for TRIM24 recruitment (normalized with input) at (A) PR, (B) pS2, and (C) IGFBP4 ERE sites, or negative control (D) GAPDH, with the indicated treatment: vehicle (ethanol), estrogen (E₂), or E₂ + TCP. Cells are pre-treated with 2 µM TCP for 24 hr and treated with 20 nM E₂. Average results from duplicates; error bars = SEM.

C.

![qChIP: TRIM24 IGFBP4](image)

D.

![qChIP: TRIM24 GAPDH](image)
**Figure 2-38 (A-B). LSD1 inhibitor Tranylcypromine (TCP) impairs ERα recruitment.** ChIP assays for ERα binding (normalized with input) at (A) PR, (B) pS2, and (C) IGFBP4 ERE sites, or negative control (D) GAPDH, with the indicated treatment: vehicle (ethanol), estrogen (E₂), or E₂ + TCP. Cells are pre-treated with 2 μM TCP for 24 hr and treated with 20 nM E₂. Average results from duplicates; error bars = SEM.

A.

**qChIP : ERα**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percentage of input</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
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</tr>
<tr>
<td>E₂</td>
<td>0.4</td>
</tr>
<tr>
<td>E₂ + TCP</td>
<td>0.1</td>
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</table>

B.

**qChIP : ERα**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percentage of input</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>0.0</td>
</tr>
<tr>
<td>+ E₂</td>
<td>0.5</td>
</tr>
<tr>
<td>+ E₂ &amp; TCP</td>
<td>0.1</td>
</tr>
</tbody>
</table>
Figure 2-38 (C-D). LSD1 inhibitor Tranylcypromine (TCP) impairs ERα recruitment. ChIP assays for ERα binding (normalized with input) at (A) PR, (B) pS2, and (C) IGFBP4 ERE sites, or negative control (D) GAPDH, with the indicated treatment: vehicle (ethanol), estrogen (E₂), or E₂ + TCP. Cells are pre-treated with 2 µM TCP for 24 hr and treated with 20 nM E₂. Average results from duplicates; error bars = SEM.

C.

D.
Figure 2-39. Summary of changes in histone modifications and recruitment of TRIM24 and ERα in the presence of Tranylcypromine (TCP). Summarized results for ChIP assays from Figures 2-31 to 2-36.

<table>
<thead>
<tr>
<th>Transcription factor binding or histone modification</th>
<th>Changes in the presence of TCP</th>
</tr>
</thead>
<tbody>
<tr>
<td>H3K4me1</td>
<td>No change</td>
</tr>
<tr>
<td><strong>H3K4me2</strong></td>
<td><strong>Increase</strong></td>
</tr>
<tr>
<td>H3K4me3</td>
<td>No change</td>
</tr>
<tr>
<td>H3K9me2</td>
<td>No change</td>
</tr>
<tr>
<td><strong>TRIM24</strong></td>
<td><strong>Decrease</strong></td>
</tr>
<tr>
<td><strong>ERα</strong></td>
<td><strong>Decrease</strong></td>
</tr>
<tr>
<td>LSD1</td>
<td>Decrease</td>
</tr>
</tbody>
</table>
2.3.8. H3T6ph disrupts TRIM24 from binding to histone peptide

LSD1-mediated demethylation of H3K4me2 is dependent on H3T6 dephosphorylation [31] and H3T6 phosphorylation inhibits H3K4me0 readers BHC80- and AIRE-PHD from binding to H3 [29]. Since chromatin binding of TRIM24 follows LSD1 pre-recruitment and H3K4me2 demethylation (Figure 2-19), and TRIM24 preferentially recognizes H3K4me0, it is reasonable that H3T6ph will disrupt the interaction between TRIM24 and unmodified H3.

To determine whether H3T6ph has any effects on TRIM24 binding, I performed biotinylated peptide pulldown assays to study the biophysical interaction between TRIM24 and modified histone peptides. I used purified GST-tagged TRIM24 recombinant protein (Figure 2-40) in the assays, as well as GST-only, GST-RBP2 (PHD1 or PHD2), GST-JMJD2A-Double Tudor Domain (DTD) as controls. I also included full-length LSD1 for reference. These GST-proteins are individually incubated with commercially available histone peptides: unmodified H3 (1-21), H3K4me2, H3T6ph, or H3K4me2T6ph. The pulldown assays shown in Figure 2-41 suggested that GST-only protein does not interact with any peptides (negative controls) and confirmed that methylated H3K4 disrupts the binding of TRIM24-PB and RBP2-PHD1 (lane 3, H3K4me2), which preferentially recognize unmodified H3K4 (lane 2, H3_{1-21}). As a control, I also examined readers that specifically bind to methyl-H3K4, such as RBP2-PHD3 and JMJD2A-DTD. These H3K4me readers are also pulled down with methylated-H3K4 (lane 3, H3K4me2), but not unmodified H3 (lane 2, H3_{1-21}). The
Figure 2-40. Purification of GST-only and GST-tagged TRIM24 recombinant proteins. Coomassie staining shows the purity and approximate concentration of GST-only and GST-tagged TRIM24-PHD-Bromo (T24-PB), TRIM24-PHD (T24-PH), and TRIM24-Bromo (T24-BR), as compared to BSA controls.
quality control confirms that these GST-proteins and histone peptides are functional.

Next, the question is whether peptides phosphorylated at H3T6 disrupts the interaction with the readers. Both TRIM24-PB and RBP2-PHD1 lose binding in the presence of H3T6ph (Figure 2-41, lane 4). As for H3K4-methyl readers, RBP2-PHD3 and JMJD2A-DTD, H3T6ph only inhibits the binding of RBP2-PHD3 to H3K4me2, but does not affect JMJD2A-DTD (Figure 2-41, lane5). Therefore, H3T6 phosphorylation selectively disrupts binding of some but not all readers. Importantly, TRIM24 fails to bind to histone H3 (residues 1-21) in the presence of H3K4me2 (Figure 2-41, lane3), H3T6oh (lane 4), or both (lane 5, H3K4me2T6ph). Taken together, the inhibitory mark H3T6 is possibly significant for the biological functions of TRIM24 and RBP2.
Figure 2-41. H3T6 phosphorylation and/or H3K4 methylation hinder TRIM24 from binding to H3. Biotinylated peptide pulldown assay: 2 µg of GST-only, GST-tagged TRIM24-PHD/Bromo (PB), GST-RBP2-PHD1, GST-RBP2-PHD3, GST-JMJD1A-Double Tudor Domain (DTD) or full-length LSD1 (GST-LSD1-full) is incubated with 1 µg biotinylated-labeled histone peptides: unmodified H3 (1-21), H3K4me2, H3T6ph, or H3K4me2T6ph. The complex is pulled down by Streptavidin beads, and shown here is a Western blot probed with GST-antibody. 10% input is used as a positive control.
2.3.9. H3T6ph and its potential role in ERα target gene activation

If H3T6 phosphorylation is critical for ERα-mediated transcription activation, I expect that upon estrogen stimulation, dephosphorylation will occur to allow for H3K4 demethylation by LSD1 and chromatin binding of TRIM24. Using an antibody specific for H3T6ph (Figure 2-42), I sought to determine the potential roles of H3T6 in ERα target gene activation. Although estrogen treatment does not change the global H3T6 phosphorylation level in MCF7 cells (data not shown), it triggers dynamic H3T6ph in a gene-specific manner. For example, upon E₂ stimulation, phosphorylation of H3T6 at GREB1 EREs exhibits a cyclical pattern from 0 hr to 3 hr, and dephosphorylation occurs from 1 hr to 2 hr (Figure 2-43 A-B). The pattern is slightly different at PR EREs, where H3T6ph slightly increases at $t = 1$ hr and then dramatically decreases at $t = 2$ hr and $t = 3$ hr (Figure 2-43 C-D). Taken together, dephosphorylation of H3T6 does not happen immediately after E₂ stimulate, but occurs from 1 hr to 2 hr, at GREB1 and PR EREs.

PKC kinases PKC-α, -β1 and βII have been previously shown to be specific for H3T6 phosphorylation [31]. To determine how ERα-regulated transcription will be affected by H3T6 phosphorylation, I performed a pilot study using a potent PKC inhibitor, bis-indolylmaleimide I (Bis I), that inhibits the kinase activity of α/β/γ isoforms. Bis I treatment dramatically dephosphorylates H3T6 at the EREs of target genes GREB1, PR, pS2 and IGFBP4, compared to DMSO control (Figure 2-44) but the effects on estrogen-induced transcription is gene- and time-specific.
Figure 2-42. Specificity of H3T6ph antibody. Dot blot analysis is performed to determine the specificity of H3T6 antibody (Abcam) on recognizing H3T6 phosphorylation on different peptides: unmodified H3 (1-21), H3K4me2, H3T6ph, and H3K4me2T6ph. A five-fold dilution of BSA is used.
**Figure 2-43 (A-B). Changes of H3T6 phosphorylation upon estrogen treatment.** qChIP analyses of H3T6ph occupancy on GREB1 EREs in the indicated time course of 10nM estrogen (E$_2$) normalized with (A) input or (H3). Average numbers from triplicates. Error bars = SEM.

**A**

**H3T6ph**

qChIP: GREB1

- **EtOH**
- **E$_2$ 1hr**
- **E$_2$ 2hr**
- **E$_2$ 3hr**

% of Input

- distal ERE
- proximal ERE
- +54kb

**B**

**H3T6/H3**

qChIP: GREB1

- **EtOH**
- **E$_2$ 1hr**
- **E$_2$ 2hr**
- **E$_2$ 3hr**

Relative fold over H3

- distal ERE
- proximal ERE
- +54kb
Figure 2-43 (C-D). Changes of H3T6 phosphorylation upon estrogen treatment. qChIP analyses of H3T6ph occupancy on PR EREs in the indicated time course of 10nM estrogen (E2) normalized with (A) input or (H3). Average numbers from triplicates. Error bars = SEM.

C

H3T6ph
quChIP: PR

% of Input

EtOH
E2 1hr
E2 2hr
E2 3hr

D

H3T6ph/H3
quChIP: PR

relative fold over H3

EtOH
E2 1hr
E2 2hr
E2 3hr
Figure 2-44. Inhibition of H3T6-specific protein kinase C (PKC) by BisI leads to dephosphorylation of H3T6 at GREB1, PR, pS2 and IGFBP4 EREs. qChIPs normalized by (A) percent of input or (B) relative fold over H3 for H3T6ph occupancy in vehicle (DMSO)-treated or 1µM bis-indolylmaleimide I (BisI) for 1 hour at GREB1, PR, pS2 and IGFBP4 EREs. Average numbers from duplicates. Error bars = SEM.
Dephosphorylation of H3T6 at GREB1 and PR EREs occurs between 1 hr and 2 hr after E2 treatment (Figure 2-43). Constantly, BisI up-regulates GREB1 and PR expression at $t = 1\text{hr}$ and $t = 2\text{hr}$ (Figure 2-45 A-B). For pS2 gene, the up-regulation occurs later at $t = 5\text{ hr}$ (Figure 2-45C), whereas the activation of IGFBP4 is not affected at all (Figure 2-45D). These observations demonstrated that H3T6ph might potentially regulate ERα-mediated transcription in a subset of target genes.
Figure 2-45 (A-B). Effects of BisI on ERα target gene induction. qPCR analyses of estrogen-induced activation of (A) GREB1, (B) PR, (C) pS2, and (D) IGFBP4 in vehicle (DMSO)- or bis-indolylmaleimide I (BisI)-treated cells. Treatment of 1 µM BisI for one hour in addition to the indicated treatment time of 10nM estrogen. RNA levels are normalized to GAPDH; vehicle-treated MCF7 is set as one. Average numbers from duplicates. Error bars = SEM.
Figure 2-45 (C-D). Effects of BisI on ERα target gene induction. qPCR analyses of estrogen-induced activation of (A) GREB1, (B) PR, (C) pS2, and (D) IGFBP4 in vehicle (DMSO)- or bis-indolylmaleimide I (Bisl)-treated cells. Treatment of 1 µM BisI for one hour in addition to the indicated treatment time of 10nM estrogen. RNA levels are normalized to GAPDH; vehicle-treated MCF7 is set as one. Average numbers from duplicates. Error bars = SEM.

C

pS2 RNA

D

IGFBP4 RNA
2.4. FUTURE DIRECTIONS AND PRELIMINARY RESULTS

2.4.1. Does depletion of LSD1 also exert time-specific effect?

This study established a detailed recruitment profile of ERα and several co-activators, as well as dynamic H3K4me occupancy on EREs, which is useful when functional assays are designed to determine the efficiency of an epigenetic drug. For example, on GREB1 ERE, dramatic inhibitory effect of TCP for is observed for TRIM24 and ERα recruitment when cells have been treated with estrogen for 180 min (Figure 2-32 A and B), because LSD1 recruitment peaks at \( t = 180 \) min (Figure 2-17). However, at \( t = 300 \) min, when LSD1 is disengaged from the chromatin and H3K4me2 accumulates (Figure 2-17), preliminary results showed that the addition of TCP further increases the level of H3K4me2 on GREB1 EREs (Figure 2-34C). However, engagement of TRIM24 (Figure 2-35A) and ERα (Figure 2-35B) only slightly decreases. For future studies, the profile can be expanded by depleting LSD1 (using siRNA or shRNA construct) and perform ChIP assays over a detailed time course. These findings will provide a comprehensive analysis when the designing functional assays in future investigation of LSD1 inhibitors.

2.4.2. Does TRIM24 and LSD1 physically interact and when?

My results demonstrated that TRIM24 and LSD1 are co-recruited at specific time points, but at other time, LSD1 recruitment precedes TRIM24. Intriguingly, unpublished mass spectrometry data from our lab revealed a possible interaction between TRIM24 and LSD1 in several cell lines, suggesting
that these two proteins may physically interact with each other at a given time course. Preliminary data suggested that at $t = 0$ and $t = 180$min, endogenous TRIM24 and LSD1 are not immuneprecipitated together in the whole cell lysates (data not shown). However, further studies should address whether their interaction is present only at specific time course of estrogen. Another question is whether the interaction only happens on the chromatins. Re-ChIP assays at specific $E_2$ induction time points (tested in the kinetic ChIP experiments) will be an excellent approach to address this.

2.4.3. **Role of H3T6 phosphorylation in regulating estrogen response?**

My findings suggested that H3T6 phosphorylation possibly play a critical role in mediating estrogen response. One important observation is that H3T6ph shows dynamic and gene-specific changes at EREs at specific time points in MCF7 cells. Essentially, pulldown assays showed that H3T6 phosphorylation abolishes the interaction between TRIM24 and histone H3 peptide, while demethylation assays by others showed that H3T6ph inhibits LSD1-mediated demethylation of H3K4me2 [31]. These observations suggested that H3T6 phosphorylation is probably upstream of LSD1 and TRIM24 in regulating the chromatins for efficient and timely estrogen response. Constant with the findings by Metzger et al. in prostate cancer cells LNCaP [31], I observed phosphorylation of H3T6 at GREB1 EREs and promoter at $t = 1$hr. However, both dephosphorylation of H3T6 is observed at GREB1 and $PR$ between 1hr to 2hr of hormone induction. Responses at these time points were not reported in previous
study by Metzger et al. Future studies should establish the kinetic profile of H3T6ph over a time course and compare it with H3K4me2 occupancy, as well as recruitments of LSD1, ERα and TRIM24. Using kinase inhibitors or PKC knockdown, detailed ChIP analyses should be performed to determine how phosphorylation of H3T6 affects H3K4me2 demethylation and recruitment of TRIM24.

2.5. DISCUSSIONS

2.5.1. Immediate E2-induced changes of chromatin structure at enhancers

ERα is known to regulate gene expression patterns through facilitation of the transcription machinery as well as a plethora of co-regulators. Epigenetic regulation represents an essential mechanism that determines the accessibility of DNA through changes of the surrounding chromatin structure. In this thesis, I showed that estrogen stimulation immediately triggers an open form of chromatin, enriched with H3K23ac and H3K27ac on the ERE sites. These immediate changes on the chromatin is consistent with the previously termed “transcriptionally unproductive cycle” of ERα, marked by chromatin remodeler, HATs and HMTs [73].

In contrast to the previously reported cyclical pattern of total H3 methylation [73], in this study I have demonstrated a dynamic H3K4 methylation profile. Intriguingly, I consistently observed immediate estrogen-induced gain of H3K4me3 on several of the ERE sites examined, which contrasts with the
commonly accepted view that H3K4me3 is often associated with transcription start sites of actively transcribed genes [34,142,143] but not with enhancer regions [73,144]. However, other reports have also suggested H3K4me3 enrichment at enhancer regions [34,142,143,145,146]. My experimental results strongly suggest that monomethylation, dimethylation, and trimethylation of H3K4 are present at specific time points upon estrogen stimulation at estrogen-responsive enhancer regions. I also demonstrated that H3K4me3 loss is concurrent with H3K4me2 gain, which shortly precedes H3K4me1 enrichment (Figures 2-20, 2-22, and 2-24). Therefore, my results suggest that estrogen immediately induces H3K4me3 methylation, and then H3K4me3 is enzymatically demethylated to H3K4me1/2 at the ERE sites examined. However, it remains to be determined which H3K4 methyltransferase(s) mediate H3K4me3 methylation and whether other H3K4 demethylases are also involved.

2.5.2. Cyclical recruitment of LSD1 and concurrent changes in H3K4me

Here I demonstrated that LSD1 and TRIM24 co-regulate transcriptional activation of a subset of ERα target gene in a time-dependent manner. I also showed that LSD1 and TRIM24 are cyclically recruited to EREs, constantly with previous findings of other ERα co-regulators [73]. For the ERE sites examined here, LSD1 has a recruitment cycle of 90min and the first recruitment of LSD1 always coincides with ERα and TRIM24. LSD1 binding is negatively correlated with H3K4me2 level during each cycle. Importantly, LSD1 binding precedes TRIM24 at the time points studied here. When LSD1 is enzymatically inhibited by
TCP, H3K4me2 is re-methylated and TRIM24 binding is impaired, suggesting that demethylated chromatin mediated by LSD1 is essential for subsequent recruitment of TRIM24. My results established the mechanism of how LSD1 regulates TRIM24-mediated functions through H3K4me2 demethylation, and further confirmed our previous findings that TRIM24 specifically recognizes unmethylated-H3K4, and global binding of TRIM24 preferentially concentrates in genomic regions depleted of H3K4me2 [57].

2.5.3. TRIM24 binding not always concurrent with H3K4me2 demethylation

TRIM24 is recruited to the chromatin, along with ERα, as early as \( t = 15 \text{min} \), during which H3K4me3 is at the highest. Previous isothermal titration calorimetry (ITC) analyses suggested that the presence of tri-methylated H3K4 completely abolished TRIM24 interaction with H3 [57]. Note, however, that TRIM24 PHD-Bromo can still bind to H3K4me3K23ac [57], suggesting that in certain cellular contexts, recognition of H3K23ac by TRIM24-Bromo can allow TRIM24 to retain its affinity to H3 even when H3K4 is methylated. Accordingly, I also observed H3K23ac enrichment at \( t = 15 \text{min} \), when TRIM24 is initially recruited to EREs. These observations point to a more complex model of histone language, in which the dual epigenetic reader TRIM24 can determine which histone mark to recognize in a context-specific manner. Intriguingly, a previous study from my lab showed that TRIM24 PHD-Bromo also binds to H3K9me1/2/3 and H3K9ac [57]. How H3K9 methylation and acetylation changes through the time course examined here remains unknown. A related question is how TRIM24 distinguishes between methylated and acetylated H3K9, or between methylated...
H3K4 and H3K9, which adds to the complexity of the histone code recognized by TRIM24.

2.5.4. Inhibition of LSD1 does not affect H3K4me3 or H3K9me2

In this thesis I demonstrated the addition of TCP to estrogen-induced cells result in re-methylation of H3K4me2, but no change in H3K4me1, H3K4me3, or H3K9me3 at several EREs examined here. These observations suggested that the demethylation of H3K4me2, and possibly H3K4me1, is inhibited in the presence of TCP, while this inhibitor is not specific for H3K4me3 or H3K9me2 demethylation. This observation is constantly with previous findings that overexpression of LSD1 in MCF7 cells only lead to H3K4me2 decrease but no change in H3K9me2. Taken together, this evidence strongly suggested that in MCF7 cells, neither H3K4me3 nor H3K9me2 is a substrate for LSD1, which specifically targets H3K4me1/2 [4,16].

2.5.5. Establishment of the role of H3T6ph in ERα-regulated transcription

This work also identified a previously unknown inhibitory histone mark for TRIM24-PHD, namely, phosphorylated H3T6. Interestingly, I found that this histone mark also interferes the H3K4 recognition by RBP2-PHD, a histone demethylase involved in estrogen response. Metzger et al. also showed that H3T6ph inhibits demethylation of H3K4me1/2 by LSD1 and H3K4me2/3 by JARID1B [31]. I also showed that inhibition of H3T6 kinase up-regulated ERα-regulated gene activation in a subset of target genes. Collectively, H3T6
phosphorylation may influence a number of readers and histone demethylases to regulation transcription.
CHAPTER 3: BIOLOGICAL SIGNIFICANCE OF TRIM24- AND LSD1-
MEDIATED ER-ALPHA CO-ACTIVATION IN BREAST CANCER CELLS


3.1. INTRODUCTION

3.1.1. Epigenetics and cancers

Both genetic mutations and epigenetic misregulation contribute to tumor initiation and progression [147]. Recently, epigenetic regulation represents a promising new area for translational research due to its reversible nature. Briefly, epigenetic factors can modulate the disease states by 1) genetic mutation or aberrant expression, and/or 2) altering the gene expression profiles in cooperation with upstream cellular and environmental signals [8,148]. In order to further our understanding, the tissue-specific cancer epigenomes are being established by compiling the expression of dysregulated epigenetic regulator proteins, as well as altered global and local histone modifications [149,150]. The development of small molecule inhibitors also aims to alter the disease states (such as cancer) through manipulating the underlying epigenetic events [8,151,152]. For example, a number of HDAC (histone deacetylase) inhibitors are already in the clinic to treat cancers and other diseases [8]. On the other hand, a growing list of literatures also reported the possibility of drugging epigenetic factors, which include lysine demethylases, bromodomain-containing reader
proteins, methyl-lysine readers, and others [8]. Essentially, epigenetic inhibitors can possibly target the “undruggable” oncoproteins by altering the chromatin interactions. For example, BRD4 (bromodomain-containing protein 4) reader protein specifically recognizes acetylated lysines and modulates transcription of target genes of MYC (myelocytomatosis viral oncogene homolog, oncogenic transcription factor) through its chromatin interaction [153,154]. Importantly, inhibition of BRD4 is able to reduce MYC binding to its target loci as well as down-regulation of MYC gene transcription [155,156]. Taken together, elucidation of more in-depth knowledge of epigenetic regulatory mechanisms may contribute to the development of effective cancer therapeutics.

3.1.2. Implication of histone methylation in oncogenesis

Epigenetic factors responsible to “write”, “read”, or erase” the histone marks in response to cellular and environmental signals are important for normal cellular processes. Therefore, aberration of these regulatory proteins is often associated with the development of various cancers (summarized in Figure 3-1) [157]. For example, MLL (mixed-lineage leukemia) gene encodes H3K4-specific methyltransferases [158,159] and is frequently rearranged in acute myeloid (AML) or lymphoid leukemias [160,161]. The most common MLL rearrangement form, MLL-PTD [161,162], retains the C-terminal H3K4me- catalytic domain [160] and in the mouse it has been shown to cause aberrant up-regulation of H3K4 methylation at the HOXA loci [163,164]. Apart from epigenetic “writers”, “readers” such as PHD-finger-containing JARID1A, also fuses with NUP98 (nucleoporin 98)
in leukemias [165]. Intriguingly, mutation of single amino acid in JAIRID1A-PHD responsible for H3K4me2/3 recognition is critical for oncogenic potential in leukemias [166]. Moreover, demethylases such as LSD1 (to be further discussed below) and Jumonji-family proteins have also been implicated in cancers. For example, JARID1B is overexpressed in both breast and prostate cancer [167,168]. JARID1B plays critical roles in facilitating G1/S [167] and mitotic spindle checkpoints [169]. JARID1B also represses metallothionein genes and tumor suppressor genes such as BRCA1 and caeolin1 by demethylating H3K4me2/3 [167,169]. Taken together, epigenetic regulators have functional significance in oncogenesis and are worth for further investigation.

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<td></td>
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<tr>
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<td>MLL</td>
<td>Rearrangement of MLL commonly found in myeloid and lymphoblastic leukaemia</td>
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<td></td>
<td>MLL2</td>
<td>Somatic mutation of MLL2 found in renal cell carcinoma</td>
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<tr>
<td>Reader</td>
<td>ING1, ING2, ING3, ING4 and ING5</td>
<td>Loss-of-function mutations of putative tumour suppressor genes ING1–5, including somatic mutation, allelic loss, downregulation of expression and aberrant cytoplasmic sequestration, associate with various solid tumors. A subset of ING2 somatic mutations interferes with binding to H3K4me3 specifically</td>
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<td>PHF23</td>
<td>Owing to chromosomal translocation, the H3K4me3-binding PHD finger of PHF23 is fused to NUP98 in myeloid leukaemia. It has been shown that H3K4me3 binding is crucial for leukaemogenesis induced by NUP98–PHF23 oncoproteins</td>
</tr>
<tr>
<td></td>
<td>PYGO2</td>
<td>PYGO2, a component of the β-catenin signalling pathway, is crucial for self-renewal of mammary progenitor cells. Pygo levels are high in malignant breast tumours and low in non-malignant breast cells</td>
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<tr>
<td>Eraser</td>
<td>LSD1</td>
<td>LSD1, a component of NuRD–Mi-2 repressive complexes, demethylates H3K4me2/1 and suppresses the invasiveness and metastasis of breast cancer cells. LSD1 is downregulated in breast carcinoma tissues</td>
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<td>JARID1A</td>
<td>Similar to PHF23, the PHD finger of JARID1A is fused to NUP98 in a subset of myeloid leukaemia, forming an oncoprotein NUP98–JARID1A. H3K4me3 binding by the JARID1A PHD finger is crucial for leukaemogenesis</td>
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<td>Overexpression of JARID1B was found in advanced breast and prostate cancers</td>
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<td>Recurrent inactivating mutation of JARID1C was detected in around 3% of renal carcinoma</td>
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<td>Upregulation of JHDM1B or a related gene JHDM1A is commonly found in retrovirus-induced rat T cell lymphomas</td>
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3.1.3. Aberrant expression of TRIM24 is correlated with tumorigenesis

Previous studies have suggested a functional role of TRIM24 in cancers. For example, TRIM24 has been shown to be an oncogenic fusion partner via chromosomal translocation in acute promyelocytic leukemia [169], papillary thyroid carcinomas [170], myeloproliferative syndrome (EMS) [60,171] and liver cancer [60,172]. Particularly, TRIM24 PHD-Bromo domain is fused to the tyrosine kinase domain in EMS. In this fuse protein, TRIM24 PHD-Bromo facilitates the dimerization and constitutive activation of FGFR1, resulting in cellular transformation [171]. However, it is still unclear whether TRIM24-FGFR1 confers tumor formation.

In addition, our lab has reported that TRIM24 is a negative regulator of p53 and knockdown of TRIM24 by small interfering RNAs (siRNAs) induces apoptosis, which can be rescued by the depletion of p53. Indeed, TRIM24 depletion in human breast cancers causes p53-dependent apoptosis [173]. In addition, TRIM24 is also a co-activator of ERα [57], indicating that aberrant expression of TRIM24 may promote tumorigenesis through disruption in p53-mediated tumor suppression and over-activation of ERα-regulated cellular processes.

Moreover, our lab published a cohort study of breast cancer patients and revealed that high levels of TRIM24 protein are tightly associated with poor survival [57], which has been further confirmed by another recent publication [174], which suggested that aberrant over-expression of TRIM24 can be used as a prognostic factor in breast tumorigenesis. In fact, TRIM24 is highly expressed
in several breast cancer cell lines, as revealed by Western blot analysis (performed by Dr. Khandan Keyomarsi’s Laboratory, data not shown). Taken together, these observations raised the question of whether TRIM24 contributes to the early stages of tumorigenesis in breast cancer.

3.1.4. Possible roles of TRIM24 in breast cancer transformation and cell cycle regulation

Unpublished data from our laboratory revealed that TRIM24 protein expression progressively increases as human mammary epithelial cells (HMECs) transform from nonmalignant to hyperplastic, and then to invasive, metastatic phenotype (data not shown), suggesting that TRIM24 may function as a pivotal driver of mammary tumorigenesis. Interestingly, studies complied in Oncomine Database (URL: http://www.oncomine.org) revealed that high levels of TRIM24 RNA levels are even more prominent in high-grade ERα-positive breast carcinomas, suggesting that TRIM24 may also promote mammary tumorigenesis partially through ERα-regulated functions.

In addition, TRIM24 may also be involved in cell cycle regulation. I obtained cell cycle-synchronized RNA lysates from Dr. Khandan Keyomarsi’s Laboratory and performed qPCR analysis on TRIM24 expression. I found that TRIM24 expression (Figure 3-3) and its binding to ERα target genes GREB1 and pS2 (Figure 3-4) are highly enriched during G2/M phase. Moreover, bioinformatics analysis performed by Kadir Atdemir in our lab also suggested that TRIM24 binds to approximate 300 genes in the category of cell cycle (Figure 3-
2). Preliminary data by our group also suggest that TRIM24 plays a critical role in G2/M transition in cultured breast cancer cells (data not shown), implying that TRIM24 may tightly control cycle cell progression.

3.1.5. Roles of LSD1 in tumorigenesis

LSD1 is over-expressed in various human cancers, including breast, prostate, colorectal, gastric, lung, bladder cancer, neuroblastoma, chondrosarcoma, Ewing’s sarcoma, osteosarcoma, rhabdomyosarcoma and others [31,175,176,177,178,179,180,181]. Many reports have supported the role of LSD1 in driving cell proliferation due to its ability to demethylate histones and non-histone proteins. One example is the tumor suppressor p53, which interacts with LSD1. LSD1 demethylates p53, represses p53-mediated transcription and inhibits apoptosis [177]. LSD1 also physically interacts with and demethylate MYPT1, a known RB regulator [182]. LSD1-mediated demethylation of MYPT targets MPT1 for proteosomal degradation, increases phosphorylated (activated) RB1 and E2F activity in cancer cells [182]. On the other hand, LSD1 is localizes to centrosomes and spindle poles during metaphase and telophase of the cell cycle [183]. LSD1 also positively regulates BUBR1 (Bub-1 related kinase) and MAD2 (mitotic arrest deficient 2-like protein) expression [183]. Take together, LSD1 play an essential role in chromosomal segregation during mitosis possibly through transcription regulation of BUBR1 and MAD2 [183]. Moreover, LSD1 plays critical roles in EMT (epithelial-mesenchymal transition) by upregulating E-cadherin and EMT-driven cell migration [184,185]. Notably, oncoprotein MYC
binds and recruits LSD1 to the chromatin, while knockdown of LSD1 inhibits transcription of MYC target genes and leads to H3K4me2 remethylation on these gene loci [186]. In a mouse model mimicking human AML, LSD1 is essential for blocking differentiation and inducing proliferation of immature blast cells [187]. LSD1 co-localizes with genes bound by MLL fusion MLL-AF9 and correlates with decreased H3K4me2 at these gene loci. Notably, pharmacologic inhibition of LSD1 enzymatic activity phenocopies LSD1 knockdown in vitro and in vivo. These observations suggested that LSD1 may be selectively targeted in MLL leukemia and other cancers.
Figure 3-2. Global TRIM24 target genes upon E₂ treatment. Genes within 10kb of TRIM24 binding were assessed by Kadir Atdemir using Ingenuity Pathway Analysis (IPA, http://ingenuity.com) and the following categories were enriched: transcription, metabolic process, biosynthetic process, cell cycle, kinase activity, signal transduction, development, and chromosome organization.
Figure 3-3. TRIM24 expression is highly enriched in E$_2$-treated cells during G2/M transition. Cell cycle-synchronized RNA lysates obtained from Dr. Keyomarsi’s laboratory were used to make cDNA and perform qPCR to access TRIM24 expression through cell cycle. The corresponding cell cycle phases were marked under the time after Levostatin release. RNA levels are normalized to GAPDH; vehicle-treated MCF7 is set as one. Average numbers from triplicates. Error bars = SEM.
Figure 3-4A. TRIM24 binding to ERα target genes during cell cycle. Synchronized cell lysates obtained from Dr. Keyomarsi’s laboratory were used to perform ChIP experiments. Binding of TRIM24 on (A) GREB1 distal ERE and (B) pS2 ERE were quantified by qPCR analyses. The corresponding cell cycle phases were marked under the time after Levostatin release. Average numbers from duplicates. Error bars = SEM.
Figure 3-4B. TRIM24 binding to ERα target genes during cell cycle. Synchronized cell lysates obtained from Dr. Keyomarsi’s laboratory were used to perform ChIP experiments. Binding of TRIM24 on (A) GREB1 distal ERE and (B) pS2 ERE were quantified by qPCR analyses. The corresponding cell cycle phases were marked under the time after Levostatin release. Average numbers from duplicates. Error bars = SEM.
3.1.6. **Hypothesis:** Function of TRIM24 for the survival and proliferation of breast cancer cells is dependent on the enzymatic activity of LSD1

Several lines of published and preliminary evidence imply that TRIM24 may regulate mammary tumorigenesis by controlling ERα-mediated transcriptional activation of cancer-promoting genes and regulating cell cycle progression. Similarly, LSD1 is also overexpressed in breast cancer and drives cellular proliferation through several mechanisms. Chapter 2 established the interplay between LSD1 and TRIM24 in chromatin regulation, therefore, I hypothesize that *function of TRIM24 for the survival and proliferation of breast cancer cells is dependent on the enzymatic activity of LSD1*. To test the hypothesize, I have formulated the following specific aims and tested them using clonogenic assays in cultured cells:

- Confirm the growth inhibitory effects of 4-hydroxy-tamoxifen (4-OHT) in wildtype cells (MCF7 parental cells)
- Determine whether TRIM24 depletion leads to reduced survival and proliferation, and whether 4-OHT (Tamoxifen) exerts additive inhibition
- Determine whether inhibition of LSD1 by TCP exerts a dose-dependent reduction in survival and proliferation in MCF7 cells
- Determine whether TRIM24 depletion has additive effects to Tamoxifen- and TCP-mediated reduction in colony formation
In summary, the results presented in this chapter suggested that 4-OHT or TRIM24 depletion alone effectively reduced the formation of colony, indicating that the ability of MCF7 cells to survive and proliferate during the indicated time course of culture is impaired. Moreover, combination of TRIM24 depletion and 4-OHT and/or TCP treatment results in highly additive effects in the survival and proliferation of MCF7 cells.
3.2. MATERIALS AND METHODS

3.2.1. Clonogenic assay

Clonogenic assays performed in parental wildtype MCF7 cells where 500 cells are seeded in DMEM medium supplemented with 10% FBS and 1% Pen/Strep solution in 60mm² tissue culture dishes for 14 days, in the presence of the indicated treatment.

For Tetracyclin-inducible shTRIM24 and shControl MCF cells, 1000 cells are seeded. In these cells, in addition to the indicated treatment, 500ng of Tetracyclin (Tet) are added for shRNA induction. Medium is replaced every three days with fresh Tetracycline (Tet), estrogen (E₂), Tranylcypromine (TCP), and/or 4-hydroxy-Tamoxifen (4-OHT). After 14 days in culture, colonies are fixed and stained with 1X crystal violet dissolved in ethanol. Colonies of ≥ 50 cells are counted. Experiments performed in at least three biological replicates.

3.2.2. Statistical analysis

GraphPad Prism software 5.0 is utilized to calculate the averaged value and error bars (based on standard error of mean, SEM) of independent experiments in biological triplicates. The two-tailed paired student t test p-value is used to calculate the statistical significant when comparing the differences between two indicated groups. P-values of less than 0.05 are considered statistically significant.
3.3. RESULTS

3.3.1. Activated ERα is required for the survival and proliferation of MCF7

In order to determine whether estrogen signaling contributes to the survival and proliferation of breast cancer cells, I performed clonogenic assays and determined the effect of ERα-mediated transcription on the survival and proliferation of MCF7 breast cancer cells. Because MCF7 cells fail to continue growing for 14 days in phenol red-free medium supplemented with charcoal-stripped serum (data not shown), these cells are cultured in medium containing phenol-red, which is estrogenic. Possibly because of the abundance of estrogen in the medium, the addition of 17β-estradiol (10nM E₂, Figure 3-5A) does not show significant increase in colony formation. Therefore, I grow the cells in the presence of 17β-estradiol alone (E₂) or in addition to 4-hydroxy-tamoxifen (1μM 4-OHT + E₂, Figure 3-5A,), an antagonist of ERα [188]. The number of colonies formed from these treated cells after 14 days are compared to vehicle control (EtOH, Figure 3-5A). Colonies formed from 4-OHT treatment (Figure 3-5A) leads to significantly reduction in the number after 14 days, when compared to E₂-treated cells (Figure 3-5A, p-value<0.0001). In contrast, tamoxifen treatment does not affect colony formation in MDA-MB-231 cells (Figure 3-5B), which lacks endogenous ERα expression. These observations confirmed the inhibitory effect of tamoxifen in MCF7 cells and set up the growth condition for the following clonogenic experiments.
**Figure 3-5: 4-hydroxy-tamoxifen (4-OHT)-induced reduction of colony formation in MCF7 but not MDA-MB-231 cells.** (A) Parental MCF7 (wildtype) cells or (B) MDA-MB-231 cells were allowed to grow for 14 days in the presence of the indicated treatment: vehicle control (ethanol, EtOH), 10nM of 17β-estradiol (10nM E₂), or 1μM 4-OHT plus E₂. Colonies after 14 days were stained with crystal violet. Average numbers from six independent experiments. Error bars = SEM (Student t test, ***p-value<0.001, compared to EtOH control).
3.3.2. Depletion of TRIM24 affects survival and proliferation of MCF7

The addition of tamoxifen not only down-regulates estrogen-induced activation of ERα target genes GREB1 and PR (Figure 3-6). In TRIM24-depleted cells (Figure 3-6), the inhibitory effect by tamoxifen is additive to shTRIM24-mediated reduction on target gene induction. To test whether TRIM24 expression is critical for cell survival and proliferation, MCF7 cells engineered with Tetracycline (Tet)-inducible shRNA targeting TRIM24 (Tet-shTRIM24) or shRNA control (Tet-shControl) are utilized to perform clonogenic assays (representative images presented in Figure 3-7). These cells were generated by Chunlei Jin in our lab. In the absence of Tet induction, shRNA is not activated and there is no difference in colonies formed in shControl and shTRIM24 (10nM E2, Figure 3-8A). Addition of Also, 4-OHT treatment leads to significant decline in number of colonies in both shControl and shTRIM24 cells (1µM 4-OHT + E2, Figure 3-8A). These observations suggest that in the absence of Tet induction, both of these cell lines behave similarly to wild-type MCF7 parental cells (Fig. 3-5). Most importantly, the Tet-inducible construct is not leaky and does not cause any differences in shControl and shTRIM24 before Tet-induction.

When shRNA construct is activated by Tet treatment, depletion of TRIM24 significantly triggers the inhibition of cell proliferation and survival, when compared to Tet-shControl (Figure 3-8B, p-value<0.05), suggesting that depletion of TRIM24 leads to a significant reduction in the survival and proliferation of MCF7 cells. Notably, addition of ERα inhibitor 4-OHT reduced the numbers of colonies in both shControl and shTRIM24 MCF7 cells, to nearly
comparable levels (Figure 3-8B, p-value<0.001). Taken together, reduced survival and proliferation mediated by loss of TRIM24 may be compensated by ERα inhibition.

### 3.3.3. Inhibition of LSD1 by TCP affects survival and proliferation of MCF7

In the absence of Tet-induction, shControl and shTRIM24 cells respond similarly to increasing dosage of tranylcypromine (TCP). Significance reduction in colonies is observed in the presence of 30µM or 100µM TCP in both shControl and shTRIM24 cells (Figure 3-8A, p value<0.01). This observation again confirms that without Tet treatment, both cell lines respond similarly to TCP treatment.

When shRNA construct is activated by the addition of Tet, we saw limited effect of TCP on the formation of colonies by shControl MCF7 cells, which responded significantly only to high doses of TCP (100µM). In contrast, in combination with loss of TRIM24 in shTRIM24 MCF7 cells, colony formation was significantly affected by inhibition of LSD1 enzymatic activity by 10µM TCP (Figure 3-8B, p value<0.001). These observations suggest that treatment with TCP further reduces the survival and proliferation in MCF7 cells depleted of TRIM24.
Figure 3-6. Depletion of TRIM24 has additive effect in Tamoxifen-inhibited ERα target gene activation. Gene expression of estrogen-induced (A) GREB1 and (B) PR is inhibited by the addition of ERα inhibitor, 4-hydroxy-Tamoxifen, and the gene induction is progressively down-regulated in TRIM24 depleted MCF7 cells (shTRIM24). RNA levels are normalized to GAPDH; E2-only MCF7 is set as one. Averaged results from triplicates. Error bars = SEM.
Figure 3-7. Representative images of Tet-treated colonies in the presence of the indicated treatment. Tranylpromine (TCP): 0 µM, 2 µM, 10 µM, 30 µM, and 100 µM in shControl or shTRIM24 cells treated with or without 4-hydroxy-Tamoxifen (4-OHT)
**Figure 3-8A:** Depletion of TRIM24 expression leads to inhibit the survival of MCF7 breast cancer cells, and is highly additive to 4-hydroxy-tamoxifen (4-OHT)-induced survival inhibition. MCF7 shControl and shTRIM24 were used to perform clonogenic assays in the presence of 10nM E2 or 1µM 4-OHT + E2, (A) without or (B) with Tetracyclin (Tet). Average from triplicates. Error bars = SEM (Student t test: *p-value<0.05; **p-value<0.01; ***p-value<0.001).

A

**Clonogenic Assay**
MCF7 without TET

![Clonogenic Assay Graph](image)
Figure 3-8B: Depletion of TRIM24 expression leads inhibit the survival of MCF7 breast cancer cells, and is highly additive to 4-hydroxy-tamoxifen (4-OHT)-induced survival inhibition. MCF7 shControl and shTRIM24 were used to perform clonogenic assays in the presence of 10nM E$_2$ or 1µM 4-OHT + E$_2$, (A) without or (B) with Tetracyclin (Tet). Average from triplicates. Error bars = SEM (Student t test: *p-value<0.05; **p-value<0.01; ***p-value<0.001).
3.3.4. Knockdown of TRIM24 is highly additive to TCP- and 4-OHT-induced inhibition in colony formation

In the absence of Tet induction, treatment with 4-OHT lowered the baseline of colony formation by shControl MCF7 cells, regardless of TCP dosage (Figure 3-10A). On the other hand, addition of 4-OHT in Tet-induced shControl cells does not affect their response to TCP (Figure 3-10B). Similarly, treatment with 4-OHT lowers the baseline clonogenicity in shTRIM24 MCF7 cells, in which I saw even further loss in clonogenicity by 10µM TCP. Notably, in combination with 10µM TCP and 4-OHT, loss of TRIM24 in shTRIM24 MCF7 leads to further reduction in colony number. Taken together, LSD1 and TRIM24 function additively to regulate survival and proliferation of MCF7 cells, but the underlying mechanism is not solely dependent on ERα activation.
**Figure 3-9A**: Decreased TRIM24 expression sensitizes MCF7 cells to lower dosage of TCP-mediated reduction in colonies. Response of MCF7 shControl and shTRIM24 cells (before Tet-treatment) to increasing dosage of Tranylcypromine (TCP) in the (A) absence or (B) presence of TET. Average numbers of colonies formed after 14 days from triplicate experiments. Error bars = SEM (Student t test: *p-value<0.05; **p-value<0.01).

A Clonogenic Assay
MCF7 without TET

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- * p-value<0.05
- ** p-value<0.01
Figure 3-9B: Decreased TRIM24 expression sensitizes MCF7 cells to lower dosage of TCP-mediated reduction in colonies. Response of Tet-induced MCF7 shControl and shTRIM24 cells to increasing dosage of Tranylcypromine (TCP) in the (A) absence or (B) presence of TET. Average numbers of colonies formed after 14 days from triplicate experiments. Error bars = SEM (Student t test: *p-value<0.05; **p-value<0.01; ***p-value<0.001).
Figure 3-10A: Knockdown of TRIM24 is highly additive to TCP- and 4-OHT-induced inhibition in colony formation. Response of MCF7 shControl and shTRIM24 cells (before Tet-treatment) to increasing dosage of Tranylcypromine (TCP) with or without 4-OHT in the (A) absence or (B) presence of TET. Average numbers of colonies formed after 14 days from triplicate experiments. Error bars = SEM (Student t test: *p-value<0.05; **p-value<0.01).

A

Clonogenic Assay
MCF7 without TET

![Graph showing clonogenic assay results]
**Figure 3-10B: Knockdown of TRIM24 is highly additive to TCP- and 4-OHT-induced inhibition in colony formation.** Response of Tet-induced MCF7 shControl and shTRIM24 cells to increasing dosage of Tranylcypromine (TCP), with or without 4-OHT in the (A) absence or (B) presence of TET. Average numbers of colonies formed after 14 days from triplicate experiments. Error bars = SEM (Student t test: *p-value<0.05; **p-value<0.01; ***p-value<0.001).

B

Clonogenic Assay
3.4. DISCUSSIONS AND FUTURE DIRECTIONS

3.4.1. TRIM24 affects breast cancer cell survival and proliferation

In this thesis I demonstrated that TRIM24 expression is enriched and strongly binds to ERα target genes during G2/M phase of the cell cycle. I have also shown that depletion of TRIM24 reduces the survival and proliferation of MCF7 cells, as revealed by clonogenic assays. TRIM24 depletion also leads to additive effect of 4-OHT. Consistently, breast cancer patients with high level of TRIM24 are associated with poor prognosis and reduced survival [57,174], suggesting that TRIM24 plays essential roles in breast tumorigenesis.

3.4.2 Correlation of TCP effectiveness and TRIM24 expression?

I demonstrated in Chapter 2 how histone recognition by TRIM24 is affected by LSD1-mediated histone demethylation. Here, I demonstrated that MCF7 cells respond to TCP-induced inhibition of survival and proliferation in a dose-dependent manner when TRIM24 is depleted. Thus TRIM24 co-regulates specific genes that are critical for breast cancer-derived cells to respond to estrogen, and effects of LSD1 depletion or inhibition on estrogen response are primary mediated through TRIM24. Moreover, LSD1 and TRIM24 also interact with genes associated with breast cancer in an estrogen-independent manner [57,174,179], suggesting their involvement in estrogen-independent regulation of genes by mechanisms that remain to be determined.
Therefore, my results suggested that TRIM24 levels may be critical for the responsiveness to TCP in the cells. Future studies should investigate several breast cancer cell lines with various TRIM24 expression levels, and determine if TCP response is different in these cells. Knockdown or overexpression of TRIM24 will be an excellent tool to address whether differences in TCP responsiveness are directly related to TRIM24 expression. In addition, the potent LSD1 inhibitors (studied in Chapter 2) can also be tested in these experiments.

3.4.3. HDAC inhibitors and TRIM24 knockdown?

Hyperacetylated nucleosomes are known to prevent LSD1-mediated demethylation, indicating that deacetylation of nucleosomes by HDACs may cooperate with LSD1-mediated demethylation. Accordingly, the HDAC inhibitor Trichostatin A (TSA) leads to de-repression of LSD1 target genes. These observations suggest that combined treatment of HDAC and LSD1 inhibitors may serve as a potential therapeutics targeting breast cancer cells that do not respond to conventional therapeutics [189]. Notably, TRIM24-Bromo domain preferentially recognizes acetylated lysines, in addition to unmethylated H3K4 [57], leading to the questions of how TRIM24-depleted cells respond to HDAC inhibitors (when histones are hyperacetylated) and whether LSD1 inhibitors retain their additive effect to HDAC inhibitors when TRIM24 is knocked down.
3.4.5. Biological functions of LSD1 and TRIM24 in vivo?

Although the studies presented here are performed in human tumor-derived MCF cells, they provide important knowledge for further investigation in the mouse models. Based on the molecular studies of TRIM24 and its correlation in breast cancer patients, TRIM24 likely functions as an oncoprotein. Surprisingly, TRIM24 null mice develop hepatocellular carcinoma [68,69]. Note, however, that preliminary data from my lab suggested that absence of TRIM24 in the mouse model leads to aberrations in chromosomal segregation and many other cell cycle defects as well as metabolic dysfunctions in the liver, which may explain why these animals are more susceptible to tumorigenesis. TRIM24-transgenic and -knockout mice are now available in my lab and are under intensive investigation. Domain-specific TRIM24-transgenic will also be an excellent tool to dissect biological functions of each domain, especially PHD and bromodomains, which mediate chromatin interaction.

Similarly, despite the LSD1 overexpression in many cancers and its functions in driving cell proliferation, LSD1 inhibits breast cancer cell invasion and angiogenesis when ERα is not expressed [190,191]. These observations raise the question of whether LSD1 plays contradictory roles in tumorigenesis and metastasis. LSD1 knockout mice are also available in my lab through a collaborative project. These mouse models serve as a useful tool to determine how LSD1 contributes to breast cancer development and metastases, and determine how the collaborative roles of TRIM24 and LSD1 in chromatin regulation translate into biological functions in vivo.
Chapter 4: **CONCLUSION**

The histone code hypothesis proposes that single or combinatorial post-translation modifications of histones can be recognized by specific reader modules to amplify a cascade of downstream responses. Histone PTMs modulate chromatin structure and thus control DNA accessibility for estrogen-induced ERα binding. In fact, estrogen induces cyclical and sequential binding of ERα, the transcription machinery and co-regulators. H3K4-specific methyltransferases and demethylases have been shown to regulate ERα-mediated transcription activation, suggesting that this step-wise process likely involves histone methylation/demethylation to allow the recruitment of ERα and co-activator complexes. However, a comprehensive analysis and kinetic profile of H3K4 methylation is lacking. How demethylation of H3K4 by LSD1 and recognition by TRIM24 becomes the main focus of this thesis.

In first part, I demonstrated that TRIM24 and LSD1 expression allows for timely induction of ERα target gene activation induced by estrogen. TRIM24 is essential for mediating induced *GREB1* transcription at lower levels of estrogen. Importantly, re-induction of TRIM24-WT but not PHD-mutant is able to rescue TRIM24 functions in depleted cells. Importantly, estrogen triggers immediate gain of H3K23ac and H3K27ac on EREs, accompanied with demethylation of H3K4me3 to H3K4me2, and then to H3K4me1. I also showed that LSD1 and TRIM24 are cyclically recruited upon estrogen stimulation over a time course. LSD1 binding is inversely correlated with gain of H3K4me2 during each cycle. Importantly, at later cycle of LSD1, recruitment of LSD1 precedes that of TRIM24.
When LSD1 enzymatic activity is inhibited by TCP, estrogen-induced cells exhibit re-methylation of H3K4me2 as well as impaired TRIM24 and ERα binding, but no change in H3K4me1, H3K4me3, or H3K9me3. I also studied the effects of several LSD1 inhibitors in down-regulating ERα target gene activation and increasing total H3K4me2 levels. Notably, I have established a potential role of H3T6 phosphorylation in ERα regulation. Because H3T6ph inhibits demethylation of H3K4me1/2 by LSD1, as well as recognition of H3K4me0 by BHC80 and AIRE, I demonstrated here that this histone mark interferes TRIM24 and RBP2 from recognizing H3K4me0. I also showed that dephosphorylation of H3T6 is essential for estrogen-induced transcription activation of GREB1 and PR, which likely influences a number of readers and histone demethylases to mediate transcription regulation.

In the second part, I furthered my study of LSD1 and TRIM24 into the cooperative biological functions. I demonstrated that TRIM24 is highly enriched and binds to GREB1 and PR during G2/M transition of the cell cycle. Consistent with previous findings that poor survival of breast cancer patients associate with high TRIM24 level, I showed that MCF7 cells respond significantly affected by inhibition of LSD1 enzymatic activity by TCP when TRIM24 is depleted. Treatment with 4-OHT further reduces the survival and proliferation in shTRIM24 MCF7 cells treated with TCP. Taken together, LSD1 and TRIM24 function additively to regulate survival and proliferation of MCF7s, but the underlying mechanism is not solely dependent on ERα activation. My findings also suggested that TRIM24 level may determine responsiveness to LSD1 inhibitors.
Collectively, this dissertation demonstrated that function of TRIM24 is dependent on at least two histone modifications, unmethylated H3K4me2 and dephosphorylated H3T6. I also revealed the dynamic H3K4 methylation profile upon estrogen treatment, suggesting that several other H3K4-specific writers, readers, and erasers may be involved in mediating estrogen response. This work also emphasizes a highly time-dependent binding of LSD1 and TRIM24 to EREs upon estrogen. Importantly, inhibition of LSD1 and depletion of TRIM24 also have additive effects in inhibiting survival and proliferation in MCF7 cells. Taken together, this work supported the “histone code” hypothesis and provided important knowledge in how histone PTMs translate into gene transcription through a reader (TRIM24) and an eraser (LSD1). This project established the fundamental framework for future investigation of the roles of LSD1 and TRIM24 in the mouse models.
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