Components of the histone modifying CoREST/LSD1 complex regulate breast tumorigenesis and breast cell biology

A thesis

submitted by

Sohini Mazumdar

In partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

in

Genetics

TUFTS UNIVERSITY

Sackler School of Graduate Biomedical Sciences

August, 2013

ADVISER: Dr. Grace Gill

List of abbreviations

ACTA2: Aortic smooth muscle actin (see also SMA)

AF4: AF4/FMR2 family member 1

AFF4: AF4/FMR2 family, member 4

ANG: Angiopoietin

AR: Andogen receptor

Bmi1: B lymphoma Mo-MLV insertion region 1 homolog

BRCA1: Breast cancer type 1 susceptibility protein

BRCA2: Breast cancer type 2 susceptibility protein

BSA: Bovine serum albumin

CD24: Cluster of differentiation 24

CD31: Cluster of differentiation 31

CD49f: Cluster of differentiation 49f

cDNA: Complementary DNA

ChIP: Chromatin immunoprecipitation

CK5: Cytokeratin 5

CK17: Cytokeratin 17

CK18: Cytokeratin 18

CK19: Cytokeratin 19

CM: Conditioned media

CoREST: Corepressor of REST

CoREST1/RCOR1: Corepressor of REST-1

CoREST2/RCOR2: Corepressor of REST-2

CoREST3/RCOR3: Corepressor of REST-3

CtBP: C terminal binding protein 1

CXCL16: Chemokine (C-X-C motif) ligand 16

CXCR4: C-X-C chemokine receptor type 4

DNA: Deoxy ribonucleic acid

Dnmt1: DNA methyltransferase 1

E cadherin: Epithelial cadherin

EBM: Endothelial cell culture media

EDTA: Ethylenediaminetetraacetic acid

ELL: RNA polymerase II elongation factor ELL

ELM2: EGL-27 and MTA1 homology domain

EMT: Epithelial mesenchymal transition

EPCAM: Epithelial cell adhesion molecule

ER: Estrogen receptor

ERBB2: V-Erb-B2 Erythroblastic Leukemia Viral Oncogene Homolog 2

ESC: Embryonic stem cell

Ezh2: Enhancer Of Zeste Homolog 2

F4/80: mouse homolog of human EGF-like module-containing mucin-like hormone receptor-like 1 (EMR1)

FACS: Fluorescence activated cell sorting

FDA: Food and Drug Administration

FGF: Fibroblast growth factor

G9a/EHMT2: Histone-lysine N-methyltransferase EHMT2

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

Gata3: Trans-acting T-cell-specific transcription factor GATA-3

GM-CSF: Granulocyte-macrophage colony-stimulating factor

H2A: Histone 2A

H2B: Histone 2B

H3K4me2: dimethylated lysine residue 4 on histone 3

H3: Histone 3

H3K4: Histone 3 Lysine 4

H3K4me1: Histone 3 Lysine 4 monomethyl

H3K4me2: Histone 3 Lysine 4 dimethyl

H3K4me3: Histone 3 Lysine 4 trimethyl

H3K9: Histone 3 Lysine 9

H3K9ac: Histone 3 Lysine 9 acetyl

H3K9me2: Histone 3 Lysine 9 dimethyl

H3K9me3: Histone 3 Lysine 9 trimethyl

H3K27: Histone 3 Lysine 27

H3K27me3: Histone 3 Lysine 27 trimethyl

H3K36: Histone 3 Lysine 36

H3K36me3: Histone 3 Lysine 36 trimethyl

H4: Histone 4

H4K16: Histone 4 Lysine 16

H4K16ac: Histone 4 Lysine 16 acetyl

H4K20me3: Histone 4 Lysine 29 trimethyl

H&E staining: Hematoxylin and eosin staining

HAT: Histone acetyltranferase

HCl: Hydrochloric acid

HDAC1/2: Histone deacetylase 1/2

HDAC6: Histone deacetylase 6

HIF/HIF1/HIF1a: Hypoxia inducible factor-1 alpha

HME621: Human mammary epithelial cell line- patient 621

HME630: Human mammary epithelial cell line- patient 630

HME636: Human mammary epithelial cell line- patient 636

HMEC: Human mammary epithelial cells

HMT: Histone methyltransferase

HOTAIR: HOX antisense intergenic RNA

HOX: Homeobox

HP1: Heterochromatic protein-1

HSPC: Hematopoeitic stem progenitor cell

HUVEC: Human umbilical vein endothelial cells

IGF-1: Insulin derived growth factor-1

IL8: Interleukin 8

lncRNA: long non coding RNA

IP: immunoprecipitation

JMJD1A: Jumonji Domain Containing 1A

K-ac: Lysine (K) acetylation

K-me1: Lysine (K) monomethyl

K-me2: Lysine (K) dimethyl

K-me3: Lysine (K) trimethyl

K-su: Lysine (K) SUMO

K-ub: Lysine (K) Ubiquitin

LOCK: Large organized chromatin K9-modifications

LSD1/KDM1A/AOF2: Lysine demethylase-1

LSD2/AOF1: Lysine demethylase 2

MCP1: Monocyte chemoattractant protein-1

MET: Mesenchymal-epithelial trasnition

miR-210: MicroRNA-210

miRNA: MicroRNA

mRNA: Messenger RNA

ncRNA: Non coding RNA

NOD/SCID: Non-obese diabetic severe combined immunodeficient

OxLDL: oxidized low density lipoprotein

p53: Tumor protein 53

PARP: Poly (ADP ribose) polymerase

PBS: Phosphate buffered saline

PcG: Polycomb group protein

PDGF: Platelet derived growth factor

PEDF: Pigment epithelium derived factor

PMSF: Phenylmethylsulfonyl fluoride

pTEFb: Positive transcription elongation factor

PTM: Posttranslational modification

PRC2: Polycomb Repressive Complex 2

qPCR: Quantitative polymerase chain reaction

R-me1: Residue-monomethyl

R-me2a: Residue- asymmetrically dimethylated arginine

R-me2s: Residue- symmetrically dimethylated arginine

Rb: Retinoblastoma protein

RNA: Ribonucleic acid

REST/NSRF: Re-1 silencing transcription factor

RNAse: Ribonuclease

RNH1: Ribonuclease/Angiogenin Inhibitor 1

RT-qPCR: Reverse transcribed quantititative PCR

S-ph: Serine phosphorylation

SANT: Swi3, Ada2, N-Cor, and TFIIIB domain

SDF1a: Stromal derived growth factor-1a

SMA: Smooth muscle actin

Snai1: Snail 1 (Snail)

Snai2: Snail 2 (Slug)

shRNA: Small hairpin RNA

siRNA: Short interfering RNA

SIRT1: Sirtuin-1

Sox2/SRY-2: SRY (sex determining region Y)-box 2

SUMO: Small ubiquitin like modifer

Suv39H1: Suppressor Of Variegation 3-9 Homolog 1

T-ph: Threonin phosphorylation

TAM: Tumor associated macrophage

TATA-TBP: TATA binding protein

TCA: Trichloroacetate

TE: Tris-EDTA

TGF-β: Transforming growth factor- β

TNBC: Triple negative breast cancer

TSP-1: Thrombospondin-1

TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling

VEGF/VEGF-A: Vascular endothelial growth factor

VEGFR-1/Flt-1: Vascular endothelial growth factor receptor-1

VEGFR-2/ Flk-1: Vascular endothelial growth factor receptor-2

WB: Western blot

WHO: World Health Organization

ZNF198: Zinc finger protein 198

ZNF217: Zinc finger protein 217

Abstract

Multiprotein complexes that regulate histone modifications and chromatin structure play critical roles in determining gene expression patterns important for cellular differentiation and neoplasia. This work explores the role of CoREST1 (corepressor of REST-1) and LSD1 (Lysine specific demethylase 1) in breast cancer tumorigenesis and epithelial breast cell differentiation, respectively. CoREST1 and LSD1 are associated in multiprotein complexes, and CoREST1 is important for LSD1 enzymatic activity as a histone lysine demethylase (Lee et al., 2005; Ouyang et al., 2009; Shi et al., 2005). While high levels of LSD1 have been observed in aggressive, basal-type breast cancers (Lim et al., 2010), nothing is known about the role of CoREST1 in breast cancer initiation and/or progression. We investigated the role of CoREST1 in tumorigenesis by knocking down CoREST1 in the invasive, metastatic cell line, MDA MB 231 that lacks expression of hormone receptors, estrogen receptor (ER), progesterone receptor (PR) and HER2. In xenograft studies, we observed that CoREST1 knockdown cells led to significantly smaller tumors with a marked decrease in angiogenesis. Levels of several secreted pro-angiogenic and pro-inflammatory factors were reduced in CoREST1 depleted cells. Many of these changes occurred at the transcriptional level, indicating that CoREST1 acts to promote expression of pro-angiogenic and pro-inflammatory genes. We further showed that, upon CoREST1 depletion, the MDA MB 231 secretome had reduced ability to stimulate endothelial cell tube formation and

migration. These findings underscore an unanticipated function for a chromatin modifier acting in tumor cells to exert profound non-cell autonomous effects on the tumor microenvironment.

In a collaborative study with the Kuperwasser lab, we also investigated the role of LSD1 in breast cell differentiation. We observed an interaction between LSD1 and the EMT transcription factor Slug, which has an important role in mammary epithelial cell differentiation (Come et al., 2004; Nassour et al., 2012). Based on this observation, we hypothesized that elevated levels of the LSD1/Slug complex contribute to breast cell fate determination by repressing transcription of luminal differentiation genes favoring maintenance of a basal cell state. To investigate this, we depleted LSD1 and Slug in human mammary epithelial cells (HMECs). Gene expression profiling identified 30% overlap between LSD1 and Slug target genes. Endogenous coimmunoprecipitations confirmed that LSD1 and Slug formed a complex in HMECs, and additional binding partners, including CoREST3, were identified. FACS analyses revealed a more pronounced luminal phenotype upon LSD1 or Slug knockdown and chromatin immunoprecipitation studies demonstrated that LSD1 and Slug were recruited to luminal gene promoters such as CK18. Taken together, these studies advance understanding of the molecular changes in breast cell fate determination and lend a better understanding of the factors that promote basal cell fate, which will inform us about better treatment of treatment refractory basal-like breast cancers.

Taken together, the studies presented here reveal novel and important roles of CoREST1 and LSD1 in tumorigenesis and epithelial cell specification, respectively. Further, our studies suggest that there are multiple LSD1 and/or CoREST1 complexes with distinct functions. Our findings illustrate the complexity of regulation that multiprotein complexes with epigenetic modifier activity have in development. Further understanding of those processes will potentially aid in the development of sophisticated cancer therapies like targeting anti-angiogenic therapies specifically to tumors, and reprogramming the molecular phenotype of a tumor from a refractory to a more tractable one.

Acknowledgements

I would like to start by thanking my mentor, Grace Gill, for making me a part of the Gill lab team; they have been a fantastic support system throughout my graduate life. Thank you Grace, for all your support- science related and otherwise and for having faith in me when it didn't seem like there was a reason you should have any. I appreciate everything you have taught me, for knowing when to take the training wheels off, but never being too far away, in case I needed you. A shout out to my wonderful thesis committee, particularly Charlotte Kuperwasser. You have been a second mentor to me, guiding me every step of the way and helping me learn about the exciting field of breast cancer. My thesis project stands on the pillars of two wonderful collaborations with the Kuperwasser Lab. Thank you Lisa and Sarah, for being part of my graduate school journey, and to all the other K-Lab members (past and present) for your help and advice.

Although this is a single author document, it would not exist today without help from my "personal pep squad". There are so many of you, it would really add pages to my thesis, if I started to name you all. But please know, I am truly thankful to you for your guidance, pep talks, proof reading skills, and for helping me get through this exciting, as well as overwhelming year. I want to thank my husband Joy, for coming a full circle with me, from graduate school applications to thesis writing; you have supported me through it all. And finally, my wonderful parents! You let me come half way across the world to follow my dream of a

better education, even though I am all you have. Thank you for everything, and I hope that I can make you proud. I am truly fortunate to have your love and unconditional support.

Table of Contents

List of Figures

List of tables

List of Supplementary Figures

CHAPTER I

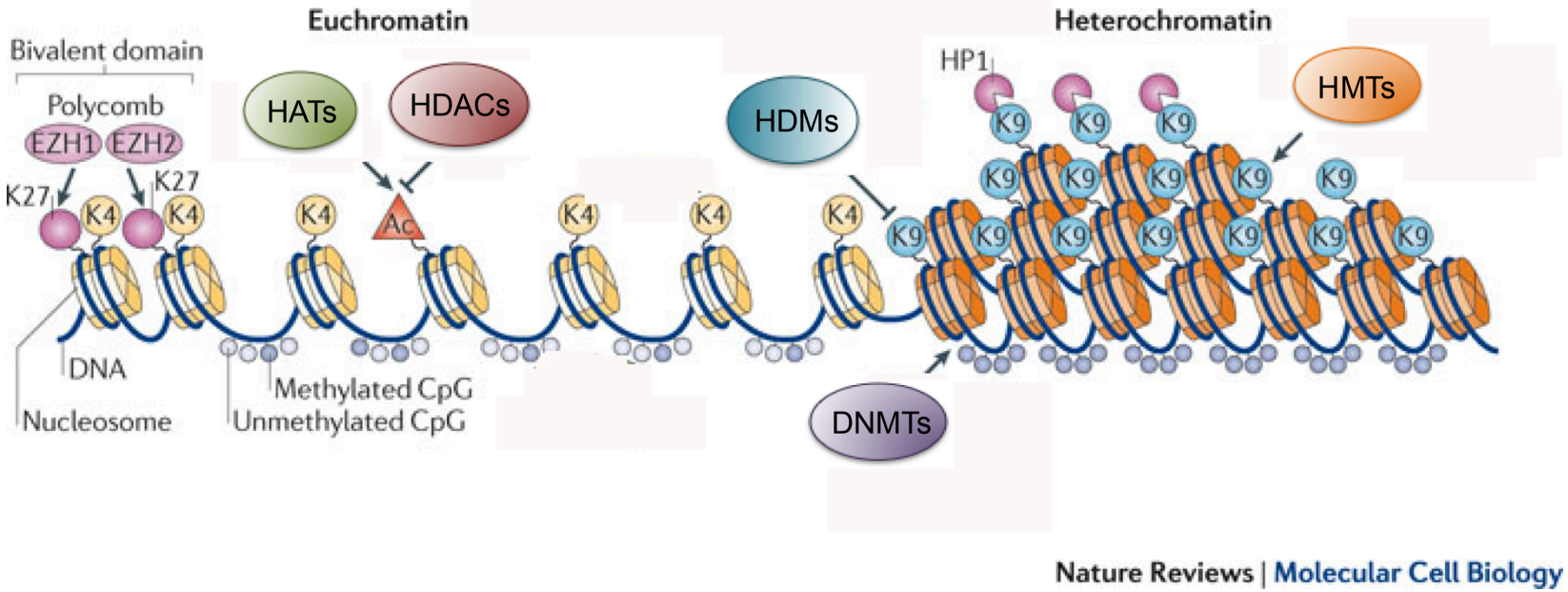
Introduction

1.1 Chromatin and transcription

Eukaryotic DNA is packaged in an orderly fashion into units of DNA wrapped in protein called nucleosomes, which condense further to form chromatin fibers that can be further compacted. The nucleosome is an octamer of four core histones (H3, H4, H2A, H2B), which allows for approximately 147 base pairs of DNA to be wrapped around it. The core histones form a structured unit with flexible N terminal “tails” that contain residues that can be modified by posttranslational modifications (PTMs). PTMs including acetylation, methylation, sumoylation and phosphorylation are dynamic responses to cellular stimuli and determine chromatin conformation, which can regulate signal dependent, tissue specific gene expression, by restricting or allowing access to transcriptional machinery based on developmental and contextual cues (Kouzarides, 2007; Serrano et al., 2013). Chromatin modifiers play an important role in transcription programs that dictate cell fate and when they go awry they can contribute to disease. Cell fate can be defined as the functional state of the cell that confers it with distinct functions and properties and is dictated by differentially expressed genes.

Chromatin modifiers can determine cell fate as well as disease states because they dictate if chromatin exists in open (euchromatin) or closed (heterochromatin) conformation (Figure 1.1). Open chromatin facilitates the binding of transcriptional machinery to the promoter and allows productive elongation resulting in gene transcription. Heterochromatin not only prevents the

important transcriptional machinery from binding to the promoter; heterchromatic regions do not get transcribed.



Nature Reviews | Molecular Cell Biology

Figure 1.1: Epigenetic modifications regulate the chromatin landscape.

Histone modifications recruit readers; example shown here heterochromaic protein 1(HP1) binds to H3K9me, writers—histone methyltrasnferases and histone acetyltransferases (HMTs and HATs) and erasers—histone demethylases and histone deacetylases (HDMs and HDACs) to regulate the chromatin landscape. Also shown are DNA methyltransferases (DNMTs) that add methyl groups to DNA at CpG islands. Adapted from Alexandre Gaspar-Maia, Adi Alajem, Eran Meshorer & Miguel Ramalho-Santos Nature Reviews MCB 12, 36-47.

Certain posttranslational modifications like histone acetylation, methylation, phosphorylation, and ubiquitination are associated with gene activation, while histone methylation, ubiquitination, sumoylation, deimination, and proline isomerization are associated with gene repression. However, any modification can be activating or repressing dependent on context. For example, H3K9 methylation is an activating modification when present in the body of a gene and has a repressive effect when found in the promoter region. (Vakoc et al., 2005)

1.2 Epigenetics

The epigenetic modification repertoire can be broadly classified into three main categories: DNA methylation changes, histone modifications and others, including microRNAs, siRNAs and lncRNAs (Figure 1.2).

1.2.1 DNA methylation

DNA methylation was one of the epigenetic modifications shown to be important in cancer progression (Feinberg and Vogelstein, 1983), and occurs at CpG dinucleotides that are common in the promoter regions of the genes. The human genome is extensively methylated throughout most developmental stages and in different tissues, and DNA methylation is and involved in numerous developmental and regulatory processes. DNA methylation can suppress proliferation of transposable elements, regulate RNA polymerase II promoter as well as occupancy of histone modifiers on chromatin (Bird, 2002).

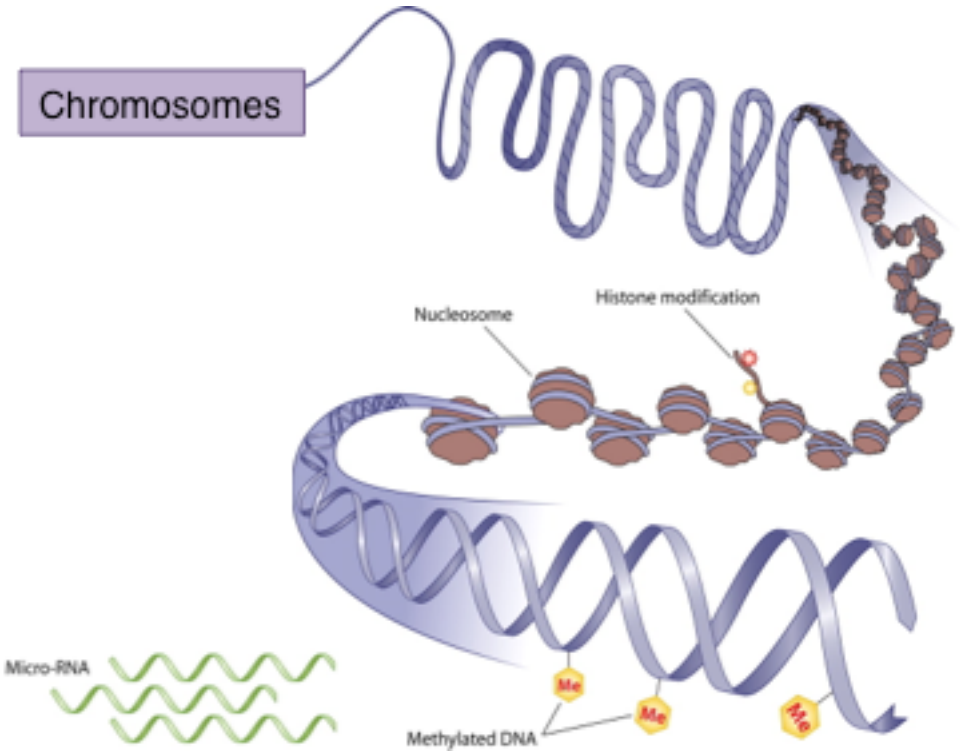

Chromosomes
Nucleosome
Histone modification
Micro-RNA
Me
Me
Me
Methylated DNA

Figure 1.2 Classes of epigenetic modifications.

The three main categories of epigenetic modifications: DNA methylation (yellow), histone modifications (orange and yellow stars) and micro RNAs (green). From Zaidi et al, Mol Cell Biol. 2010 Oct; 30(20): 4758-66.

DNA methyltransferases are responsible for establishing and maintaining patterns of DNA methylation that can result in gene repression. Atypical changes in DNA methylation (global hypomethylation and CpG island hypermethylation) occur in cancer due to deregulation of DNA methyltransferases (Heichman and Warren, 2012). Recent studies suggest that there is crosstalk between DNA methylation and histone modification machinery and DNA methylation can even be guided by histone modifications (Cedar and Bergman, 2009). We have only begun to understand the different layers of complexity and dynamic interplay between enzymes that are responsible for DNA and histone modifications, which in turn control transcription and potentially promote deregulation of gene expression in tumors (Vaissiere et al., 2008).

1.2.2 Histone modification

Post-translational modification of histones is most prevalent at specific lysine and arginine residues in histone N-terminal tails and has a variety of functional consequences. Strahl and Allis proposed the 'histone code' hypothesis based on studies of histone modifications and transcriptional regulation (Strahl and Allis, 2000; Turner, 2000). The histone code hypothesis postulates that unique combinations of covalent histone PTMs regulate chromatin structure and transcription. Histone modifying enzymes can be broadly classified as readers, writers and erasers. Enzymes such as histone methyltransferases (HMTs) and histone acetyltransferases (HATs) are referred to as "writers" because they add histone marks; enzymes such as histone demethylases and

histone deacetylases remove histone marks from residues and are called “erasers” (Strahl and Allis, 2000). Readers recognize histone modifications and translate the “histone code” into a transcriptional outcome, activation or repression of genes (Ruthenburg et al., 2007). For example, HP1 binds to H3K9me3 and recruits transcriptional machinery to chromatin promoting gene silencing (Munari et al., 2012). In concert, these enzymes regulate the epigenetic landscape that can determine cell fate decisions during development and maintenance of stem cell states as well as differentiated cellular states.

Histone acetylation and methylation:

Histone acetylation regulates the degree of chromatin folding and promotes an open chromatin configuration that allows transcriptional machinery to be recruited to access the promoter region. Histone acetylation strongly correlates with gene transcription, and moreover, histone acetylation sites and histone acetyltransferases (HATs) are required for gene activity.

Histones are acetylated and deacetylated in two ways: first, activator and repressor complexes can directly recruit HATs and histone deacetylases (HDACs) to the regulatory sequences; second, both HATs and HDACs can modify histone acetylation in a global, genome wide manner to alter levels of gene transcription. Broadly speaking, histone acetylation is linked to active gene transcription, and deacetylation to promoting gene repression. However, transcriptional regulation is a complex process and histone deacetylation is also

critical in active gene transcription, depending on the context of other histone marks present on the chromatin. Both HATs and HDACs function in multiprotein complexes with a variety of factors that dictate their substrate specificity, and ultimately their activity (Shahbazian and Grunstein, 2007).

Histones can be methylated at several key residues including histone 3 lysine 4 (H3K4), histone 3 lysine 9 (H3K9) and histone 3 lysine 27 (H3K27). The number of methyl groups on the lysine residue (mono-, di- or tri-methylation) has diverse functions including transcriptional activation or repression and response to DNA damage. Methylation at different lysine residues can result in different outcomes of transcriptional regulation, for e.g., histone 3 lysine 4 mono and tri methyl (H3K4me1 and me3) are typically correlated with active gene expression, histone 3 lysine 36 trimethyl (H3K36me3) with transcriptional elongation, and histone 3 lysine 9 trimethyl (H3K9me3) and histone 3 lysine 27 trimethyl (H3K27me3) are typically markers of silenced genes (Serrano et al., 2013). LSD1 can demethylate H3K4me1/2 to silence genes in several cell types and H3K9me1/2 to promote androgen dependent gene transcription (Metzger et al., 2005). In addition, genes can also have bivalent marks such as the H3K27me3 repressive mark and the H3K4me3 active mark. These are considered to be in a poised state in which the transcriptional machinery is present on the promoter but not active (Serrano et al., 2013).

1.2.3 Noncoding RNAs (ncRNAs)

Non-coding genomic DNA comprises nearly 50% of the human genome and was considered untranscribed “junk” DNA until recently. Over the last decade, scientists have discovered that large parts of noncoding genomic DNA are transcribed into ncRNAs, and are important in epigenetic regulation and transcriptional programs. ncRNAs have been implicated in mechanisms of chromatin reorganization and silencing. For example, the long anti-sense ncRNA, HOTAIR mediates epigenetic silencing of the HOX gene cluster in trans, by guiding the H3K27me3 histone modifier, Polycomb repressive complex 2 (PRC2) (Costa, 2008; Rinn et al., 2007). Interestingly, HOTAIR serves as a scaffold for another epigenetic complex, the LSD1/CoREST1/REST complex (discussed in greater detail below). HOTAIR thus acts to co-ordinate enzymatic activities of PRC2 and LSD1, coupling H3K27 methylation and H3K4 demethylation, thereby promoting specific combinations of histone modifications on different target genes (Tsai et al., 2010).

miRNAs

MicroRNAs (miRNAs) are a class of regulatory, small non-coding RNAs involved in developmental processes and in pathologies like breast cancers when they are dysregulated (Liu, 2012). They are tightly regulated, posttranslational modifiers that regulate expression of genes by translation repression or degradation of transcripts. Experimental data suggests that dysregulated expression of miRNAs play roles in carcinogenesis, in tumor as well as stromal cells. There is evidence to suggest that epigenetic modulators are responsible for

these altered levels of miRNAs found in cancers compared to normal tissue (Baer et al., 2013). A subset of miRNAs dubbed “epi-miRNAs” can directly influence the activity of epigenetic regulators like methyltransferases and histone deacetylases, which in turn regulate the expression of tumor suppressor genes (Fabbri and Calin, 2010).

Of note, miRNAs can also be regulated by hypoxic conditions. For example, miR-210 has been the single miRNA consistently induced by hypoxia via HIF-1α, and correlated with poor prognosis of cancers (Soon and Kiaris, 2013).

Aberrant CpG methylation can result in epigenetic repression of miRNAs. Histone profiling studies in mammary fibroblasts and epithelial cells indicate that some promoters were silenced in a tissue-specific manner by DNA methylation, others were silenced by H3K27 trimethylation, and a subset of miRNA promoters displayed both modifications, suggesting that DNA methylation and histone modifications can co exist to form the chromatin landscape (Baer et al., 2013).

1.3 Epigenetic modifiers: CoREST1 and LSD1

CoREST1 was originally discovered as a corepressor for the transcription factor, REST (also known as NRSF); DNA-bound REST functions as a scaffold to recruit the CoREST1/LSD1/HDAC complex and repress neuronal genes in non neuronal cells (Ballas et al., 2001; Ballas et al., 2005; Hakimi et al., 2002; Nishizawa et al., 1992; Shi et al., 2005). The CoREST family has three known members-- CoREST1 (RCOR1), CoREST2 (RCOR2) and CoREST3 (RCOR3)-- that have similar protein domains (Figure 1.3). CoREST1 (RCOR1) is

ubiquitously expressed in all tissues, and is currently the most well studied CoREST family member. CoREST family members contain different protein domains that allow for it to bind to other proteins in multiprotein transcriptional complexes. More specifically, CoREST1 contains ELM2 and SANT domains that allow for interaction with HDAC1, and a second SANT domain that binds LSD1.

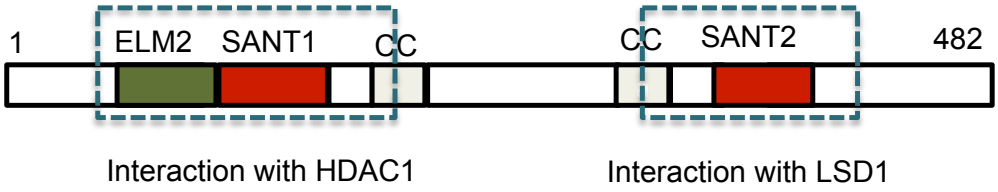


Figure 1.3 Protein structure of the CoREST1 co repressor.

CoREST1 interacts with HDAC1 via the ELM2 and SANT1 domains (You et. Al, 2000); CoREST1 also interacts LSD1 via the SANT2 domain (Shi et. al, 2005)

Very little is known about CoREST2 and CoREST3, although both have been shown to bind LSD1 (Ouyang et al., 2009) Recently, CoREST2 has been identified in a complex with LSD1 in embryonic stem cells (ESCs) (Yang et al., 2011). Interestingly, the study also showed that exogenous expression of CoREST2 in mouse and human somatic cells can replace Sox2 expression in somatic reprogramming (Yang et al., 2011). Serum CoREST3 levels can be a potential prognostic marker in Hepatitis B (Xue et al., 2011).

LSD1 (AOF2 or KDM1A) is a histone demethylase capable of demethylating mono- and dimethylated lysine 4 of histone H3 (H3K4me1 and H3K4me2) (Lee et al., 2005; Shi et al., 2004) and is a part of the flavin monamine oxidase family. Other members include LSD2 (AOF1) that has a distinct function to demethylate mono- and dimethylated H3K4 in the highly transcribed coding region enriched with trimethylated histone H3 at lysine 36 (H3K36) (Fang et al., 2010).

LSD1 and CoREST1 complexes:

Affinity purification studies demonstrate that LSD1 is a part of several large protein complexes and intimately interacts with CoREST1 and histone deacetylases 1 and 2 (HDAC1/2) (Hakimi et al., 2003; Humphrey et al., 2001; Shi et al., 2003; Shi et al., 2005; You et al., 2001). CoREST1 and LSD1 directly bind each other and this interaction aids LSD1-mediated demethylation of nucleosomal substrates (Foster et al., 2010; Lee et al., 2005; Shi et al., 2005;

Yang et al., 2006). The presence of LSD1 and HDACs supports the idea of multifunctional enzymatic complexes that coordinate histone deacetylation and demethylation activities to repress gene expression (Lan et al., 2008; Shi et al., 2003). LSD1 is also found in a complex with SIRT1, and together they co ordinate H4K16 deacetylation and H3K4 demethylation to repress target genes in the Notch signaling pathway (Mulligan et al., 2011).

Crystal structure studies of CoREST1-LSD1 complex demonstrate a high degree of substrate specificity of LSD1 for histone tails. The catalytic domain of LSD1 and the SANT2 domain of CoREST1 (DNA binding domain) interact and LSD1 recognizes a large portion of the H3 tail through a deep, negatively charged pocket at the active site and potentially a shallow groove on its surface (Yang et al., 2006). CoREST1-bound LSD1 molecules also showed increased catalytic activity in enzyme kinetic assays. These studies indicate that CoREST1 and other members of the LSD1 complex are key regulators of LSD1 recruitment to specific genes (Figure 1.4). The LSD1/CoREST1/HDAC complex has been known to interact with corepressor CtBP, which can directly bind many DNA-binding transcription factors (Chinnadurai, 2002), and direct binding of CoREST1 with CtBP contributes to repression of a subset of CtBP target genes (Cowger et al., 2007; Kuppuswamy et al., 2008). Biochemical studies show that CoREST1 can also interact with post translational modifications such as SUMO creating an interface for other proteins to bind that does not exist in the absence of SUMO (Ouyang et al., 2009). Crystal structure studies of CoREST1-LSD1 complex

demonstrate a high degree of substrate specificity of LSD1 for histone tails. The catalytic domain of LSD1 and the SANT2 domain of CoREST1 (DNA binding domain) interact and LSD1 recognizes a large portion of the H3 tail through a deep, negatively charged pocket at the active site and potentially a shallow groove on its surface (Yang et al., 2006). CoREST1-bound LSD1 molecules also showed increased catalytic activity in enzyme kinetic assays. These studies indicate that CoREST1 can play a dual role in enhancing LSD1 activity; by maintaining the structure of the peptide-binding site, and by interacting with the nucleosomes, facilitating their recognition by LSD1 (Forneris et al., 2007). Additional crystallographic studies show that an N terminal peptide of the transcription factor, Snail (Snai1) mimics the H3 tail when bound to LSD1-CoREST1 (Baron et al., 2011). This mode of binding is likely to occur for all transcription factors with the related SNAG domain, including Slug (see chapter III).

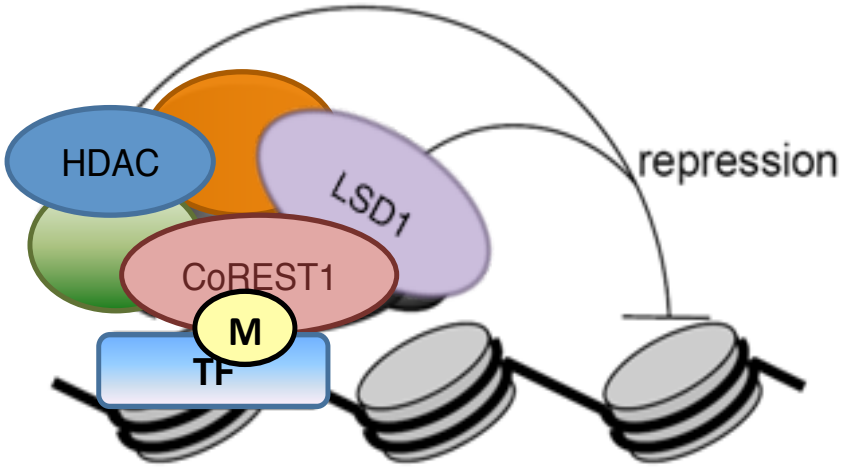


Figure 1.4 Schematic of the LSD1-CoREST1- HDAC complex.

The canonical CoREST1 repressor complex can be recruited to gene promoters by transcription factors that can potentially be posttranslationally modified. Abbreviations: LSD1- histone demethylase; HDAC- histone deacetylase; TF- transcription factor; M-modification, post translational modification SUMO is an example of one such modification.

1.4 Epigenetics in development

"An epigenetic trait is a stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence" (Berger et al., 2009). These heritable changes play key roles in cellular reprogramming and cell fate decisions. Studies in cellular and animal models have established the role of epigenetic regulators in cellular development and differentiation. They have also helped lend credence to the hypothesis that epigenetic regulators are key in determining cell fate and contribute to the transcriptional program of the cell. Transcription factors form different flavors of complexes with epigenetic regulators to alter the chromatin landscape of key developmental regulatory genes. This results in gene expression changes that allow progenitor cells to form differentiated cells in different organ systems. Epigenetics can influence not only the local chromatin landscape, but also higher order chromatin structure and nuclear organization (Serrano et al., 2013).

Experimental data suggest that pluripotent cells are in a state of widespread transcriptional hyperactivity (Efroni et al., 2008). For example, repetitive DNA that is normally silenced is active in pluripotent cells; in contrast, tissue specific genes display very little activity in these cells. As cells go from stem cell state to differentiated state there is extensive heterochromatinization. Differentiated cells accumulate large regions of H3K9me2 or large organized chromatin K9-modifications (LOCKs) (Wen et al., 2009).

Bivalent domains are domains that have more than one type of histone mark on them (for e.g. an repressive H3K27me3 with a active H3K4me3). Bivalent domains correlate with low gene expression and are thought to be a process by which important developmental genes are kept silenced, poised for future activation or repression. While this was initially thought to be an exclusive property of stem cells, some bivalent domains have been observed in differentiated cells as well (Serrano et al., 2013).

1.4.1 CoREST1 and LSD1 in development and differentiation

There is a considerable body of literature highlighting the importance of CoREST1 in development and in determination of cell fate in diverse cell types including neuronal cells where CoREST1 was originally discovered (Abrajano et al., 2009a, b; Andres et al., 1999; Ballas et al., 2001; Ballas et al., 2005; Hakimi et al., 2002; Lakowski et al., 2006; Qureshi et al., 2010; Saijo et al., 2009). CoREST1 also has an established role in determining cell fate in hematopoietic lineages (Chowdhury et al., 2013; Hu et al., 2009; Saleque et al., 2007). CoREST1 expression is more restricted in comparison to REST and is downregulated at the time of birth (Tontsch et al., 2001). Further, CoREST1 is more evolutionarily conserved than REST indicating that CoREST1 may have functions beyond REST (Lakowski et al., 2006). Studies show that REST and CoREST1 have the ability to interact with a diverse array of regulatory factors including DNA methyltransferases (Esteve et al., 2009), chromatin remodelers (Kuppuswamy et al., 2008) and transcription factors (Kim et al., 2006)

highlighting the importance CoREST1 beyond its role as a repressor of REST. REST and CoREST1 help determine cell fate and maintain differentiated cellular states by directly or indirectly recruiting multiprotein complexes to regulate developmental genes. REST and CoREST1 have also been known to interact with Polycomb group (PcG) proteins, Bmi1 and Ezh2, both of which are implicated in regulating neural stem cell renewal and maintenance (Pereira et al., 2010; Wang et al., 2010). Previously performed ChIP-on-chip studies examining REST and CoREST1 target genes show that a significant number of CoREST1 target genes did not have REST binding motifs indicating REST independent functions for CoREST1 at these promoters (Abrajano et al., 2010). Additionally, our own affinity purification studies using CoREST1 as bait did not pull down any REST peptides in our mass spectrometry results (see Appendix 1). These aforementioned studies establish an important role for both REST dependent and independent CoREST1 mediated gene regulation. This also suggests that other REST/CoREST1 interactors may have currently unrecognized REST independent roles in gene regulation. All the in vivo studies with CoREST1 are currently in non-mammalian systems like Drosophila and no published mouse knockouts are available (Curtis et al., 2013; Dallman et al., 2004; Domanitskaya and Schupbach, 2012; Zhang et al., 2013).

LSD1 has been most well documented as a repressor and is known to associate with factors like HDAC1/2, CoREST1 and BHC80 that have repressive or silencing enzymatic activity (Banck et al., 2009; Ceballos-Chavez et al., 2012;

Kim et al., 2013; Laurent et al., 2012; Mulligan et al., 2011; Ouyang et al., 2009; Tsai et al., 2010; Zhang et al., 2013). LSD1-null mice are embryonic lethal highlighting the importance of LSD1 in developmental programs (Wang et al., 2007). Further, LSD1 is a component of the CoREST1-CtBP complex that is essential for cell fate determination during pituitary organogenesis and can have varied functions depending on whether LSD1-containing repressor or activator complexes are recruited to promoters. In this system, temporal recruitment of different LSD1 complexes can modulate multiple gene expression programs (Wang et al., 2007). Additionally, LSD1 is also necessary for hematopoietic differentiation and represses hematopoietic stem and progenitor cells (HSPCs). Loss of LSD1 results in failure to repress HSPC genes and results in ineffective differentiation of hematopoietic stem cells and maturation of blood cell lineages (Kerenyi et al., 2013).

As described above, LSD1 can function as an activator in certain contexts. For example, LSD1 interacts with androgen receptor (AR) and estrogen receptor (ER) and is essential for activation of some AR and ER target genes, by demethylation of the repressive H3K9me2 (Metzger et al., 2005). LSD1 is also found in a transcription elongation complex that contains factors like ELL (elongation factor RNA polymerase II), pTEFb, AF4, and AFF4 (Biswas et al., 2011). Studies also suggest that LSD1 is a component of an MLL supercomplex associated with active transcription (Nakamura et al., 2002). Furthermore, although LSD1 is a histone demethylase, it also has non histone targets such as

p53 and Dnmt1. LSD1 represses p53-mediated gene activation by preventing interaction of p53 with 53BP1(Huang et al., 2007a). LSD1 can demethylate Dnmt1 thereby stabilizing its levels (Esteve et al., 2009).

1.5. The biology of cancer:

1.5.1 Hallmarks of cancer:

Hanahan and Weinberg have described the characteristics and properties acquired by normal cells as they become tumorigenic, often referred to as the hallmarks of cancer. There are six main properties that all tumorigenic cells share: sustained proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis and activating invasion and metastasis (Figure 1.5). In addition to these hallmarks, cancer cells also acquire enabling hallmarks such as avoiding immune destruction, deregulating cellular metabolism, upregulating tumor promoting inflammatory processes and genomic instability (Hanahan and Weinberg, 2011).

Untransformed cells tightly regulate the balance between pro and anti proliferative signals thereby ensuring balance in cell number and maintenance of normal tissue structure and function. Cancer cells can hijack these signaling pathways to sustain proliferation. They do this by altering levels of ligands, receptors and other downstream signaling in both autocrine and paracrine fashion. Alternatively, they can also become independent of growth signals by altering regulatory signals in growth pathways and disrupting feedback mechanisms.

Figure 1.5. Hallmarks of cancer.

Tumors progress by sustaining proliferation, evading growth inhibitory signals and avoiding immune detection, deregulating their metabolism and resisting cell death. They accumulate mutations that allow them to induce angiogenesis and activate the invasion and metastasis cascade and recruit tumor promoting inflammatory cells. From Hanahan and Weinberg, 2011.

Cancer cells also need to overcome strong negative regulators of proliferation, many of which are dependent on tumor suppressors like Rb and p53. Additionally, tumor cells lose contact inhibition and develop ways to resist cell death. There is substantial evidence to support the idea that the apoptotic response is a barrier to tumor pathogenesis (Attardi, 2005). Both hyperproliferation and DNA damage are also apoptotic triggers and are common in cancer cells. Cancer cells that can circumvent the apoptotic machinery become resistant to therapy (Lowe and Lin, 2000). Most common occurrences are loss of p53 function (which prevents cells from sensing apoptotic triggers) and upregulation of anti-apoptotic regulators.

Like apoptosis, cancer cells can also hijack the autophagic stress response to survive in stressed, nutrient limited environments that cancer cells often experience. Unlike apoptosis and autophagy, necrosis releases proinflammatory signals in the tissue microenvironment. These signals recruit tumor promoting inflammatory cells that can induce angiogenesis, proliferation and invasiveness (Grivennikov et al., 2010).

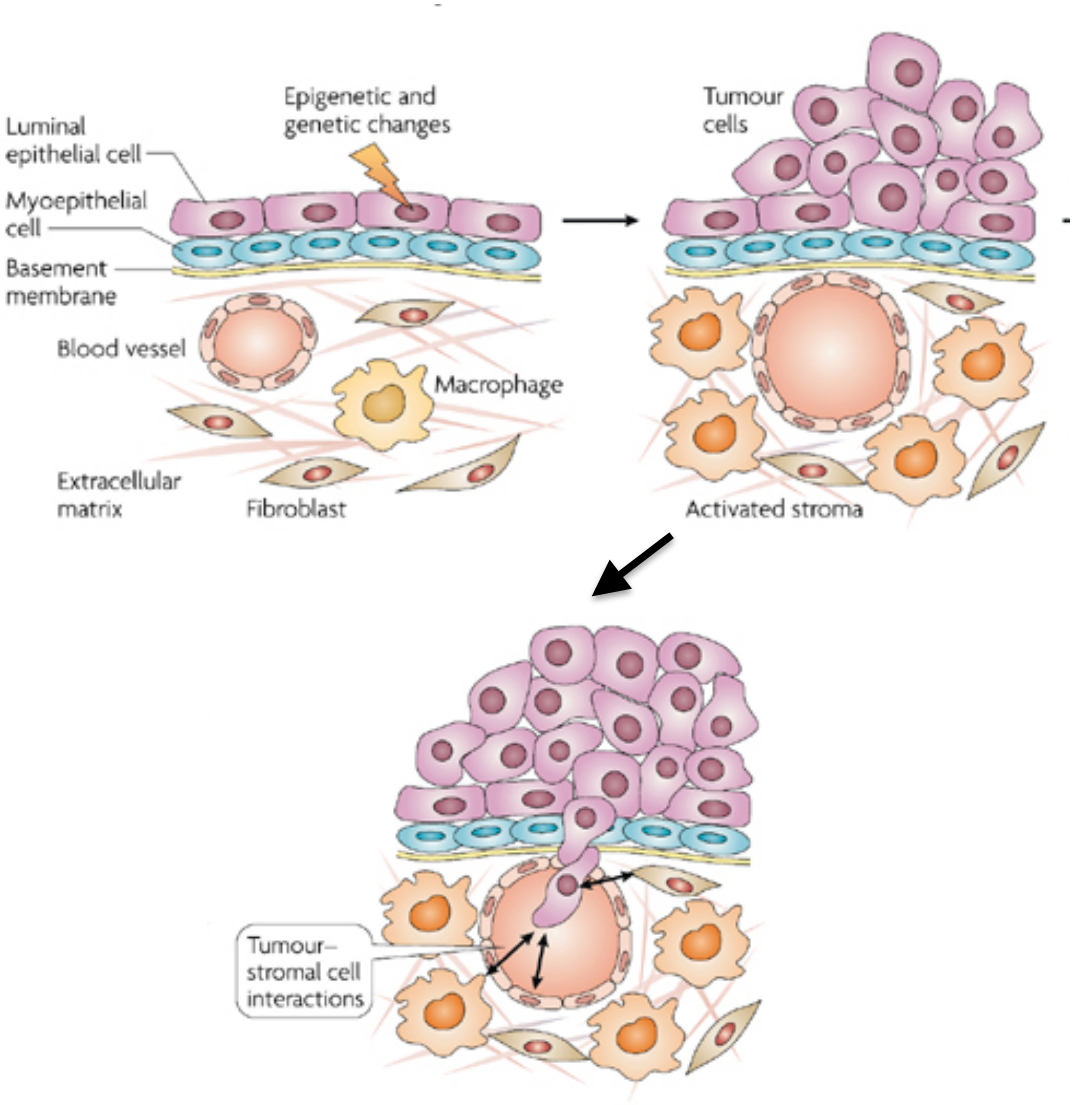


Figure 1.6. Tumor development is a complex process that relies on dynamic interactions between the tumor and surrounding stroma.

Breast epithelial cells undergo neoplastic transformation to give rise to metastatic cancer as a result of genetic and epigenetic changes within the tumor and surrounding microenvironment. Aberrant tumor stroma interactions facilitate dysregulation of proliferation; survival and migration are impaired resulting in tumor growth and maintenance. Tumor cells also produce a number of cytokines

that act as chemoattractants for different inflammatory cells, including macrophages and endothelial cells. Additionally, activated fibroblasts and infiltrating inflammatory cells secrete proteases and cytokines involved in neovascularization. These factors stimulate tumor growth and stimulate angiogenesis. Adapted from Tracy Vargo-Gogola & Jeffrey M. Rosen, Nature Reviews Cancer 7, 659-672.

Cells that progress to neoplasia are said to have flipped the “angiogenic switch”, causing the otherwise dormant vasculature to become active and sustain the tumor cells by providing nutrients and oxygen and expelling metabolic waste. The balance between pro- and anti-angiogenic factors like vascular endothelial growth factor A (VEGF-A) and thrombospondin 1 (TSP-1) regulates the angiogenic switch. Angiogenesis is one of the earlier hallmarks of cancer. Histological analyses of premalignant, non-invasive lesions reveal signs of neovasculature (Hanahan and Folkman, 1996). Until recently, angiogenesis was considered an important phenomenon after tumors had formed, but studies suggest that angiogenesis is responsible for microscopic tumor progression and premalignant phases of tumorigenesis (Raica et al., 2009).

The final hallmark of cancer is the ability to invade and metastasize, which is a multi-step process beginning with invasion, intravasation of cancer cells, circulation within the blood and lymphatic systems followed by extravasation, formation of micrometastases which colonize to form tumors. This process (except for colonization) is regulated in part by transcription factors that control epithelial to mesenchymal transition like Snail, Slug, Twist, and Zeb1/2 (Micalizzi et al., 2010).

1.5.2 **Breast cancer**:

Breast cancer is the leading cause of death in women worldwide and accounts for 13% of all mortalities (WHO) underscoring the need for targeted

therapies. It is not a homogenous disease; rather it is a combination of breast pathologies, genetic and epigenetic variations and clinical outcomes (Figure 1.7). Clinicians categorize breast cancer patients into different molecular subtypes, which allows them tailor therapies predict prognoses based on molecular subtype. Broadly, breast cancer can be classified into luminal A, luminal B, basal, ERBB2+ and triple negative subtypes (Figure 1.7). Cell lines have been derived from these different types of primary tumors and are used in the laboratory to study the molecular profiles of these different subtypes. Basal and ERBB2+ types show the worst disease prognosis, while luminal A cancers show the best prognosis (Sorlie, 2004). Luminal subtype A tumors have the highest expression of estrogen receptor (ER), and luminal subtype B show low to moderate expression of luminal-specific genes like ER cluster but are different from luminal subtype A. The basal epithelial-like subtype is characterized by high expression of keratins like CK5 and CK17, and is negative for the estrogen receptor (ER), and the ERBB2+ subtype has an overexpression of genes in the ERBB2 cluster (Sorlie, 2004). Since basal tumors have the poorest disease prognosis, understanding more about how these tumors form and sustain growth will be a significant contribution to the field of breast cancer.

Breast epithelial cells can undergo aberrant genetic and epigenetic changes to transform within the microenvironment. The gene expression profile of the cells prior to their transformation into a cancer cell will dictate the nature of the tumor the cell gives rise to (Gorski et al., 2010; Proia et al., 2011). During this

process of neoplastic transformation, cellular processes like proliferation, survival, apoptosis, differentiation and migration are dysregulated. One of the biggest hurdles in advancing the field of cancer biology is the lack of model systems that can recapitulate the different pathologies and aspects of the disease (Vargo-Gogola and Rosen, 2007).

Breast cancer derived cell lines serve as tractable systems in which scientists can model the progression of cancer. A comprehensive study showed that a panel of 51 breast cancer lines show the heterogeneity and recurrent aberrations found in many primary tumors indicating that while no single cell line can recapitulate all the facets of breast cancer, they represent a system that is easy to study and that resembles a genetic environment found in tumors (Neve et al., 2006).

Studies in breast cancer lines have helped expand our knowledge of genetic variations in cancer as well as different signaling pathways. Cell lines are derived from human tumors and model aspects of the human disease like hormone-dependent signaling pathways. Cell lines can also be used in xenograft studies that are particularly useful in studying tumor-stroma interactions (Clarke, 1996).

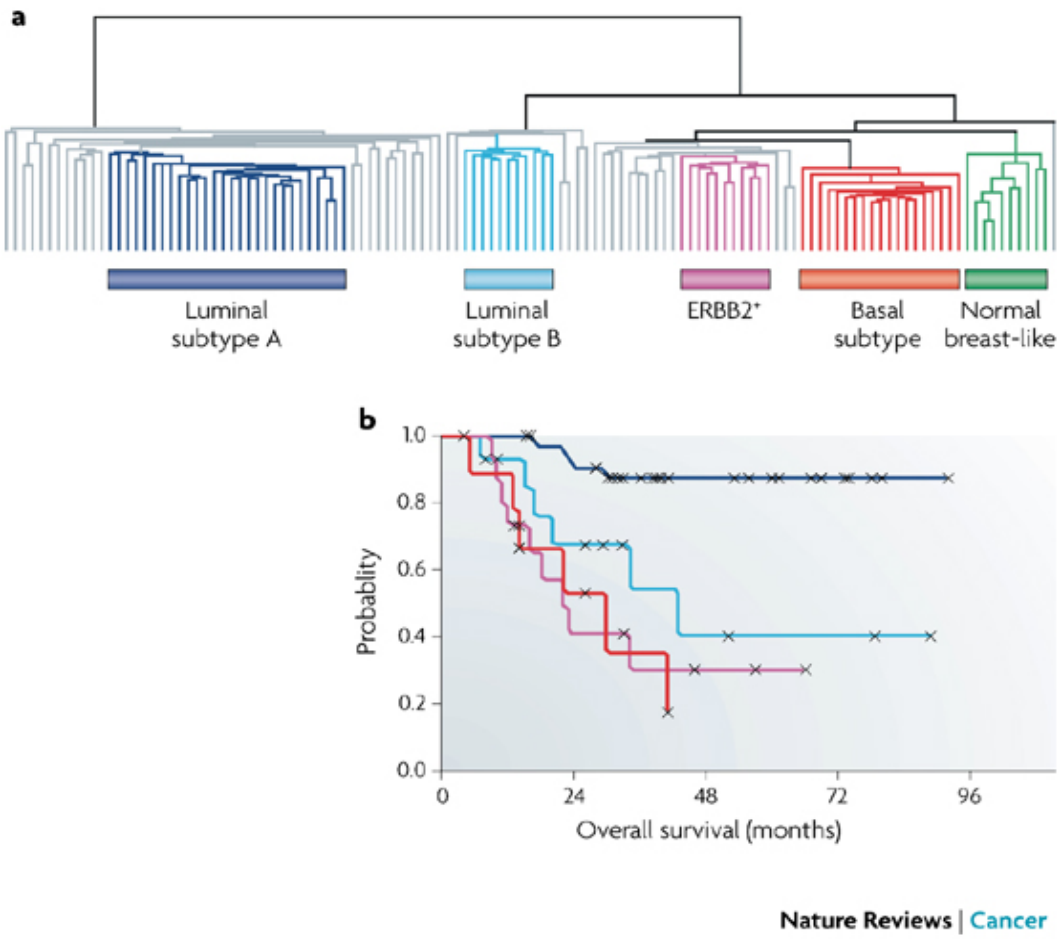


Figure 1.7. Molecular subtypes of cancer.

(a) Breast cancer can be broadly classified into 5 molecular subtypes based on gene expression profiling. Hierarchical clustering of 115 tumor tissues and seven non-malignant tissues using the intrinsic gene set. Experimental dendogram shows the clustering of the tumors into 5 subgroups. Branches corresponding to tumors with low correlation to any subtype are shown in grey. (b) Probability of overall survival for the different molecular subtypes. Luminal A subtype (dark blue) has the best prognosis while ERRB2+ (pink) and basal (light blue) subtypes have the worst prognosis. From Sorlie *et al.,* 2004.

1.5.3 The tumor microenvironment

The tumor is no longer considered an isolated foreign entity in the body and researchers now recognize that the complexity of tumor/stroma interactions parallels and can even surpass that of normal organ systems (Swartz et al., 2012). To truly understand carcinogenesis, it is important to study tumors and their interaction with their microenvironment, rather than just their cell autonomous properties. Since the tumor heavily relies on its surrounding stromal compartment to progress and metastasize, targeting tumor stromal compartments with therapies like bevacizumab and sunitinib has become a popular approach (Bisagni et al., 2013; Clavarezza et al., 2013; Curigliano et al., 2013; Gianni et al., 2013; Hanahan and Weinberg, 2011).

1.5.4 Angiogenesis and inflammation in cancer:

Solid tumors are often infiltrated with inflammatory and immune cells. Cancer cells can take advantage of this normal immune response to the tumor (also called tumor immunosurveillance) (Dunn et al., 2004; Grivennikov et al., 2010). The immune system can recognize premalignant cells based on the tumor antigens they express, and other signals that help the immune system recognize tumors as “non-self”. The cancer cells avoid detection by downregulating the expression of tumor antigens, or hijacking the immune response for neoplastic progression (often referred to as tumor escape) (Poggi and Zocchi, 2006). Immune cells including lymphocytes, macrophages and mast cells are found in large numbers in the tumor stroma, suggesting that they are actively recruited in

response to signals from the tumor cells (de Visser et al., 2006). It is also possible that they are initially recruited to the tumor site as part of tumor surveillance but later commandeered for tumor progression. Inflammatory mediators are pro survival signals for premalignant cells and can stimulate tumor angiogenesis (Grivennikov and Karin, 2010).

The growth of solid tumors depends on their ability to acquire nutrients and oxygen and get rid of metabolic waste through neovascularization, one of the essential hallmarks of cancer progression. The “angiogenic switch” occurs when there is an imbalance between pro- and anti-angiogenic signals, allowing for proliferation, migration and increased survival of endothelial cells in an otherwise dormant vascular system. Tumor associated endothelial cells can attract bone marrow derived progenitors to differentiate them into endothelial cells or pericytes and form new vessels (Butler et al., 2010). There is evidence to support the idea that tumor associated angiogenesis is a complex relationship between the tumor microenvironment, cancer cells, stimulated endothelial cells and activated fibroblasts (Weis and Cheresh, 2011).

Tumor vasculature is characterized by excessive vessel branching and capillary sprouting, leakiness and increased endothelial cell proliferation and migration (Nagy et al., 2010). Leaky and defective blood vessels result in a hypoxic tumor microenvironment, which further perpetuates the angiogenic response. This constant feedback loop can be one of the reasons why using angiogenic inhibitors can promote tumor progression instead of preventing

neovascularization, and actually resulting in an enhanced tumor angiogenic response and ultimately fail as therapeutics.

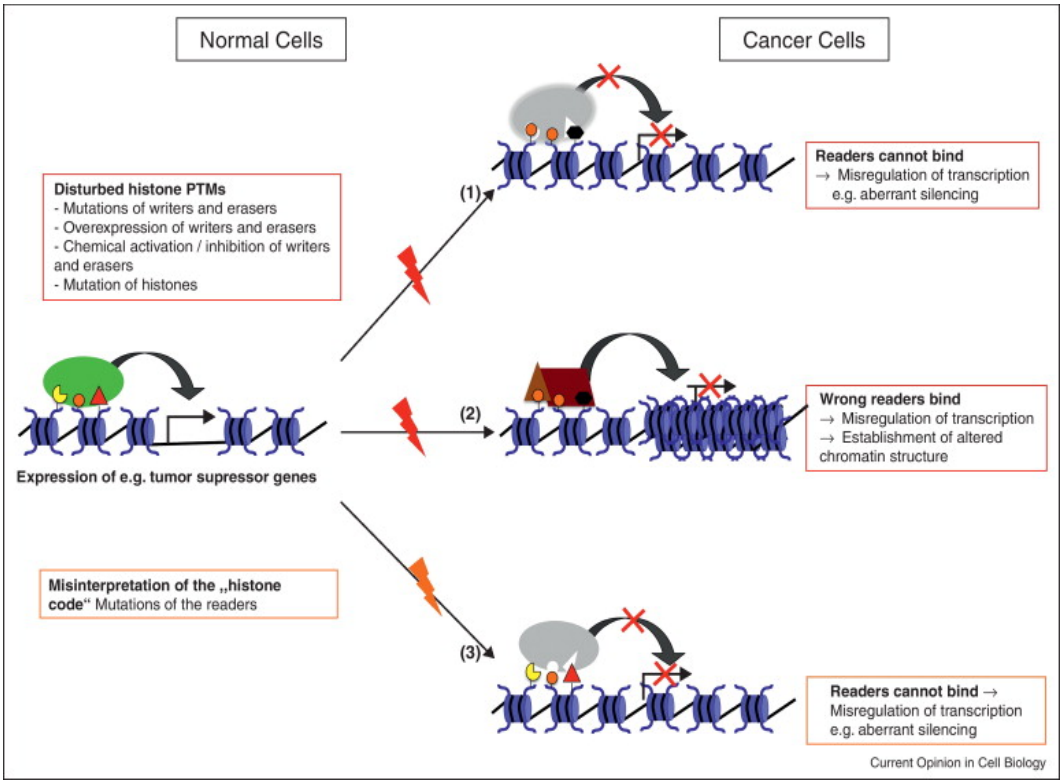
Tumors secrete an abundance of inflammatory regulators like growth factors and cytokines, which can activate immune cells (mainly tumor associated macrophages), these, in turn secrete growth signals for endothelial cells and fibroblasts. The immune cells also produce proteases that allow for capillary sprouting and stimulate angiogenesis and metastasis (Coussens and Werb, 2002). The tumor-associated macrophages (TAMs) accumulate in hypoxic areas of tumors (Pollard, 2004) and are the main source for cytokines and proteases that affect endothelial, epithelial and mesenchymal cells in the tumor microenvironment (Porta et al., 2007). The fine balance between pro and anti-angiogenic signals during inflammation and neoplastic progression is complex and depends on cellular context (Nyberg et al., 2008).

1.6 Epigenetics in cancer

The majority of initial studies of the role of histone modifications in cancer focused on global changes in different tumor types. For example, a global loss of H4K16ac and H4K20me3 appears to be a signature of many human cancers (Fraga et al., 2005). Recent studies have shown that many changes in histone posttranslational modifications are tumor type specific. An example of this specificity is seen in breast cancer cells, which show low levels of H3K4me2 and H3K9ac (Elsheikh et al., 2009), and in lung cancers that show low levels of

H3K4me2 but high levels of H3K9ac (Barlesi et al., 2007) (Figure 1.8). There has been considerable interest in utilizing histone modifications as prognostic markers for different cancers. Global loss of particular modifications is suggestive of poor prognosis and high risk of recurrence (Ellinger et al., 2010; Kurdistani, 2011; Schneider et al., 2011; Seligson et al., 2009; Seligson et al., 2005). Another study shows that prostate cancers can be classified into prognostic groups based on their patterns of H4K20 methylation (mono-, di- or tri-methylation) (Behbahani et al., 2012). Taken together these studies suggest that altered levels of histone modifications is a hallmark of most cancer types (Waldmann and Schneider, 2013).

A.



B.

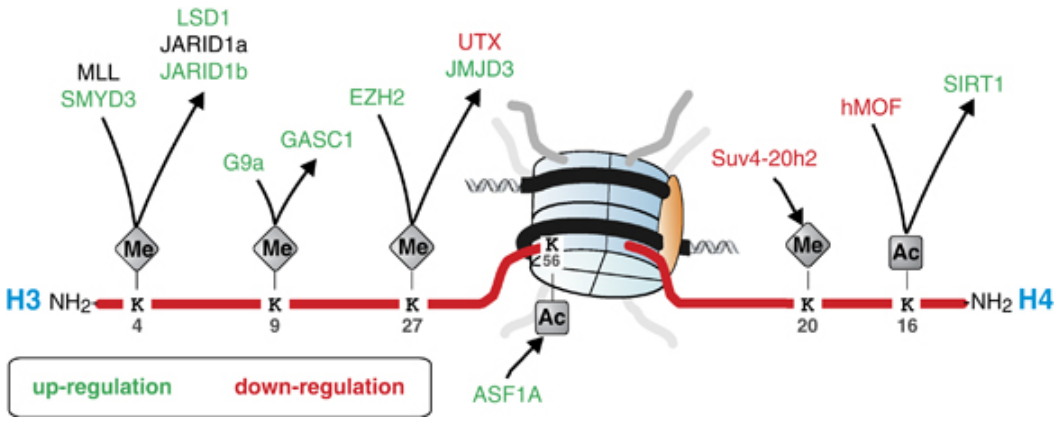


Figure 1.8. Aberrant histone modifications in cancer

There are several ways in which aberrations in histone PTMs and misinterpretation of the histone code in normal cells leads to tumorigenesis. These include mutations of writers and erasers, overexpression of writers and readers and mutations of the histones themselves. Mutations in reader proteins

can also result in misinterpretation of the histone code (A.1) Mutations prevent enzymes from binding, resulting in inappropriate silencing or activation. (A.2) Mutations cause different enzymes to bind resulting in deregulated transcription or altered chromatin structure. (A.3) Misinterpretation of the histone code results in inappropriate silencing of genes. Adapted from Waldmann T and Schneider R, Curr Opin Cell Biol. 2013 Apr; 25(2): 184-9. (B) Factors in green including LSD1 are upregulated in cancer; factors in red are downregulated in cancer. From J Füllgrabe, E Kavanagh and B Joseph; Oncogene (2011) 30, 3391–3403.

1.6.1 Epigenetic modulators as cancer therapeutics:

Aberrant levels of several epigenetic modulators are prevalent in breast cancers, making them clinically relevant targets. For example, higher levels of HDAC6 mRNA are frequently expressed in breast cancer patients with low grade, hormone receptor positive tumors compared to higher-grade tumors (Zhang et al., 2004). Global DNA hypomethylation is more common in breast cancer and correlates with poor prognosis (Soares et al., 1999), however, specific breast cancer-related genes like ER, Twist and E-cadherin are hypermethylated and thus silenced relative to normal tissue (Widschwendter and Jones, 2002). For instance, the ER promoter contains CpG islands, which are unmethylated in normal tissues and methylated in triple negative breast cancers (Lapidus et al., 1998).

The question of whether methylation of specific breast cancer genes can predict response to cancer therapeutics has been an area of considerable interest. For example, DNA methylation can inactivate an otherwise wild type BRCA1 gene in sporadic breast and ovarian cancer and poly(adenosine diphosphate)-ribose polymerase (PARP) inhibitors are being tested in clinical trials for BRCA1/BRCA2-associated cancers (Fong et al., 2009). Recent studies show that both BRCA1 mutations and hypermethylation of the BRCA1 promoter correlated with sensitivity to PARP inhibitors. Since a subset of “triple-negative” breast cancer patients exhibits BRCA1 promoter methylation, these patients might be ideal candidates for PARP inhibitors in the clinic.

Based on the success of HDAC inhibitors like Vorinostat and DNMT inhibitors like AZA in hematological malignancies, these molecules are being evaluated in the clinic as potential therapeutics for solid tumors including breast cancer (2003; Munster et al., 2011). While they have shown great promise in the laboratory, partially successful clinical trials in patients indicate that the dosage and the ideal combination of epigenetic inhibitor therapies with other anti cancer agents is still to be elucidated (Connolly and Stearns, 2012).

1.6.2 LSD1 and LSD1 inhibitors in breast cancer

LSD1 activity (in HDAC and other complexes) can promote carcinogenesis (Althoff et al., 2013; Amente et al., 2013; Bennani-Baiti, 2012; Harris et al., 2012; Jie et al., 2013; Lim et al., 2010; Lv et al., 2012; Metzger et al., 2005; Rudolph et al., 2013; Serce et al., 2012; Zhao et al., 2012). Additionally, high levels of LSD1 expression in tumors correlate with poor disease prognosis, further highlighting the clinical importance of LSD1 in cancer pathologies (Lim et al., 2010).

LSD1 expression levels are elevated in pluripotent cancer cells and LSD1 depletion selectively inhibits the growth of pluripotent cancer cells indicating that therapeutic inhibitors of LSD1 maybe promising when both, used alone and in combination with other agents that target epigenetic regulation (Huang et al., 2007b; Huang et al., 2009a; Huang et al., 2009b; Huang et al., 2012; Wang et al., 2011).

LSD1 demethylates mono- and di-methylated H3K4 (Agger et al., 2008) and depletion of LSD1 causes the accumulation of these methylated forms of

H3K4. This resultant accumulation of H3K4 methylated substrates can lead to reactivation of repressed target genes in vitro (Shi et al., 2004). In some contexts, LSD1 can also be an activator; for example, LSD1 participates in estrogen receptor (ER) mediated activation of ER target genes. Estrogen stimulation can result in LSD1 mediated H3K9me2 demethylation and activated of estrogen responsive genes (Ombra et al., 2013).

LSD1 inhibitors can reduce expression of genes involved in cellular differentiation indicating that LSD1 regulates cellular differentiation processes (Wang et al., 2007). High levels of LSD1 correlate with expression of stem cell markers like Oct4 and Sox2, which are significantly downregulated upon LSD1 inactivation. Immunostaining data from human tumor tissue microarrays indicate that LSD1 levels are high in tumor tissues (with stem cell properties) compared to normal tissue counterparts (Wang et al., 2011). These data suggest that LSD1 regulates proliferation of stem cell-like cancer cells by influencing expression levels of essential stem cell genes like Sox2 and Oct4 (Wang et al., 2011).

Knockdown of LSD1 resulted in downregulation of most classes of HDAC mRNAs (HDAC1, 2, 3, 6, 8,10) in triple-negative breast cancer (TNBC) cells (Vasilatos et al., 2013). Combination treatment of pargyline and the HDAC inhibitor, Vorinostat, led to inhibition of proliferation and apoptosis in TNBC cells. Knockdown of LSD1 with Vorinostat treatment results in derepression of tumor suppressive genes like E-cadherin. These data highlight the role of LSD1

dependent HDAC activity in TNBC cells, with implications for therapeutic efficacy of HDAC inhibitors in the clinic.

HDAC inhibitors, vorinostat and romidepsin are FDA approved therapeutics for cutaneous T-cell lymphoma. Although they have shown promising results in treating hematological cancers, they have not been effective single agents against solid tumors including breast cancer in early stage clinical trials. One of the reasons for this lack of efficacy is our incomplete knowledge of HDAC biology in breast cancer. Current research suggests that HDAC inhibitors are more effective at inhibiting tumor growth in combination with other anti cancer therapies (Vasilatos et al., 2013). LSD1 inhibitors are in preclinical development for a variety of solid tumors and blood cancers (Figure 1.9) (Dawson and Kouzarides, 2012).

1.6.3 CoREST1 in cancer

CoREST1 interacts with several tumor supressors and oncogenes, however, the role of CoREST1 in cancer has not been established. CoREST1 was reported not to be essential for the tumor suppressor function of REST (Mulligan et al., 2008). CoREST1 interacts with ZNF217, an important oncogene amplified in several types of cancer including breast cancer (Banck et al., 2009) and prognostic marker (Littlepage et al., 2012). CoREST1 and ZNF217 have been shown to bind tumor suppressor gene p15ink4b (Thillainadesan et al., 2008). CoREST1 is found in complexes with zinc finger proteins like ZNF217 and ZNF198 that have DNA binding modules called zinc finger binding domains. ZNF198 is a MYM type

zinc finger protein that can bind the CoREST1-LSD1-HDAC1 repressor complex and be recruited to gene promoters. ZNF198 like proteins are known to repress E-cadherin, which is also an LSD1 and CoREST1 target gene (Gocke and Yu, 2008).

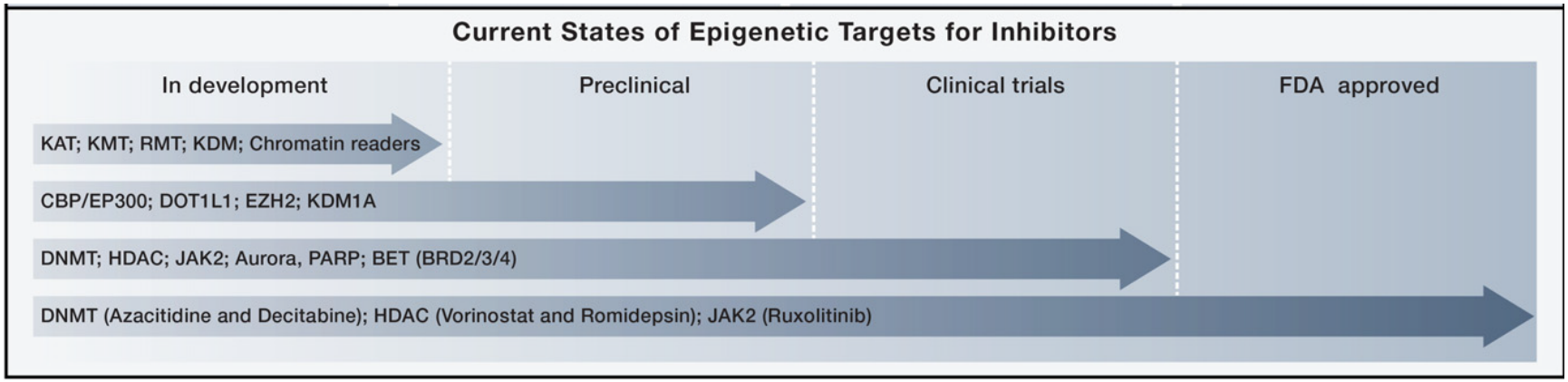

Current States of Epigenetic Targets for Inhibitors
In development
Preclinical
Clinical trials
FDA approved
KAT; KMT; RMT; KDM; Chromatin readers
CBP/EP300; DOT1L1; EZH2; KDM1A
DNMT; HDAC; JAK2; Aurora, PARP; BET (BRD2/3/4)
DNMT (Azacitidine and Decitabine); HDAC (Vorinostat and Romidepsin); JAK2 (Ruxolitinib)

Figure 1.9. Epigenetic modifiers are targets of several promising clinical trials. Candidate inhibitors are first tested in vitro for specificity and phenotypic responses including assessment of inhibition of proliferation, induction of apoptosis, or cell-cycle arrest. These assays are used in conjunction with genomic and proteomic methods to identify potential molecular mechanisms. Inhibitors that demonstrate potential in vitro are then tested in vivo in animal models of cancer to ascertain toxicity and pharmacokinetic properties of the drug and whether they may provide therapeutic benefit in terms of survival. Based on these preclinical studies, molecules move to testing in clinical trials, following which, they are approved for routine clinical use by the FDA (in the US). KAT, histone lysine acetyltransferase; KMT, histone lysine methyltransferase; RMT, histone arginine methyltransferase; and PARP, poly ADP ribose polymerase.Of note, LSD1 (KDM1A) inhibitors are in preclinical development. Dawson MA, Kouzarides T Cell. 2012 Jul 6;150(1):12-27.

1.7 Significance of the project:

As described earlier, there is now considerable evidence that epigenetic modifiers play critical roles in tumorigenesis. This study presents a pro-tumorigenic role for CoREST1 in tumor cells where it exerts profound, non-cell autonomous influences on the surrounding microenvironment. Our data demonstrate a role for CoREST1 in tumor angiogenesis and tumor stroma interactions. Surprisingly, we found that depletion of CoREST1 in tumor cells resulted in downregulation of genes associated with the angiogenic program, suggesting an oncogenic co-activator function for CoREST1.

We also hypothesize that high levels of a complex between LSD1 and the transcription factor Slug contributes to the breast epithelial cell differentiation by repressing transcription of specific genes associated with the luminal differentiation program. Our studies suggest a role for epigenetic modifier, LSD1 and transcriptional factor, Slug in determining cell fate decisions, and maintainence of different cellular differentiation states.

Taken together, the studies presented here underscore the importance of epigenetic modifiers in breast epithelial cell differentiation as well as tumorigenesis. Our data with CoREST1 illustrates the importance of understanding tumor/stroma interactions in order to identify better and more effective therapeutics against cancer. Our findings on LSD1 and Slug highlight the role of epigenetic modifiers in determining and/or maintaining cell fate. Cell

fate choices are important not only from a developmental perspective, but also in the context of transformation. The nature of the tumors that arise from a cell is dictated by its cell fate at the time of transformation.

1.8 Specific aims:

Specific aim 1:

a. To determine the role of CoREST1 in modulating the tumor promoting ability of MDA MB 231s
b. To determine the role of CoREST1 in tumor angiogenesis

Specific aim 2:

a. To determine the changes in gene expression profiles upon ablating LSD1 and Slug in patient derived human mammary epithelial cell lines and the cellular consequences of these changes in gene expression
b. To determine the formation of LSD1 Slug complexes in human mammary epithelial cells and their role in altering the luminal differentiation program.

CHAPTER II

Biological role for CoREST1 in tumor formation and tumor stroma interactions

2.1 Abstract

There is increasing evidence that regulators of chromatin structure and gene expression play key roles in contributing to tumor formation and maintenance. Recently, high levels of the histone demethylase LSD1 (lysine specific demethylase 1) have been observed in aggressive, ER negative, basal-type breast cancers (Lim et al., 2010). CoREST1 (co-repressor of REST) and LSD1 are often found in the same complex and CoREST1 is important for LSD1 stability and enzymatic activity on nucleosomes (Ouyang et al., 2009). However, the role of CoREST1 in initiation and/or progression of breast cancer is currently unclear. We investigated the role of CoREST1 in an invasive metastatic cell line, MDA-MB-231 and found that knockdown of CoREST1 led to significantly smaller tumors than controls in xenograft studies. Notably, tumors formed from CoREST1 knockdown cells revealed a marked decrease in angiogenesis. Depletion of CoREST1 led to a decrease in secreted angiogenic and inflammatory factors, both of which influence the tumor microenvironment. Some of these factors were decreased at the transcriptional level, indicating that CoREST1 promotes expression of genes related to the angiogenesis program. Tube formation and wound healing assays with HUVECs reflected these changes in the MDA MB 231 secretome upon CoREST1 depletion. This is an exciting finding where a putative epigenetic modifier in tumor cells has profound non-cell autonomous effects on surrounding cells and the tumor microenvironment.

2.2 Introduction

Tumor angiogenesis is critical for tumor progression and occurs when the pro angiogenic signals from the tumor cells are greater than the antiangiogenic response. This could be direct modulation of pro- and anti-angiogenic factors or modulation of changes in tumor secretion of factors such as inflammatory cytokines, which in turn affects the angiogenic response. Hypoxia, tumor angiogenesis and tumor progression are interlinked phenomena; as solid tumors like breast cancer increase in size, the tumors cells in the interior of the tumor experience hypoxia and signal to their surrounding stroma to recruit more oxygen and nutrient supplying blood vessels (Folkman, 1971). This allows the tumor to grow further, creating positive feedback loop between the hypoxic signals that can regulate angiogenic and inflammatory factors including VEGF, which can have dramatic influence on tumor growth and invasion (Semenza, 2000).

CoREST1 and LSD1 are part of multiprotein complexes that regulate histone modifications and transcription. CoREST1 plays a vital role in LSD1 activity and stability (Ouyang et al., 2009; Shi et al., 2005). High levels of the histone demethylase LSD1 have been correlated with aggressive, ER negative, basal-type breast cancers (Lim et al., 2010); however the role of CoREST1 in cancer progression is still unclear. CoREST1 is found in complexes with different tumor promoting factors including REST, ZNF217, ZNF198, HDAC1/2 and factors such as SIRT1 that have dual roles in tumorigenesis and tumor suppression

(Banck et al., 2009; Cowger et al., 2007; Gocke and Yu, 2008; Kuppuswamy et al., 2008; Mulligan et al., 2011; Thillainadesan et al., 2008; You et al., 2001)

Here we investigated the role of CoREST1 in tumor formation by breast cancer cells. We report that knockdown of CoREST1 reduced tumorigenesis and led to decreased secretion of pro-angiogenic and pro-inflammatory factors. Our data demonstrate that CoREST1 regulates angiogenic and inflammatory genes; expression of which is vital in the processes of neoangiogenesis and tumor induced inflammatory responses. Our findings implicate a necessary role for CoREST1 in tumor angiogenesis and allude to its importance in tumor/stroma interactions including recruitment of inflammatory cells like macrophages.

2.3 Results

Data in human patients as well as cell and animal models support a role for LSD1 in promoting breast tumor growth (Huang et al., 2012; Lim et al., 2010; Schulte et al., 2009; Zhu et al., 2012). LSD1 and CoREST1 are intimately associated and, in some contexts, CoREST1 is required for LSD1 activity (Lee et al., 2005; Ouyang et al., 2009). We therefore investigated whether CoREST1 contributes to tumorigenesis in a breast cancer model. We used two different shRNAs to stably deplete CoREST1 in invasive, metastatic, hormone unresponsive, triple negative and basal breast cancer cell line, MDA-MB-231. As shown in Figure 2.1A, the levels of CoREST1 mRNA and protein were significantly reduced in the shCoREST1 cells.

In order to determine if downregulation of CoREST1 has an effect on the tumor forming ability of the MDA MB 231 cells, we performed xenograft assays using mammary fat pad injections in NOD/SCID mice. Breast tumor xenografts are ideal for studying tumor stroma interactions and immunosurveillance of tumor cells like macrophage infiltration (Clarke, 1996). Cells were allowed to grow into tumors for 6-7 weeks and tumors were harvested for histological analyses.

As expected, control MDA MB 231 cells gave rise to tumors in 100% of injected animals. However, knockdown of CoREST1 in MDA MB 231 cells resulted in a 50% reduction in tumor incidence (growths greater than 3mm were considered tumors). The tumors that formed were significantly smaller both by

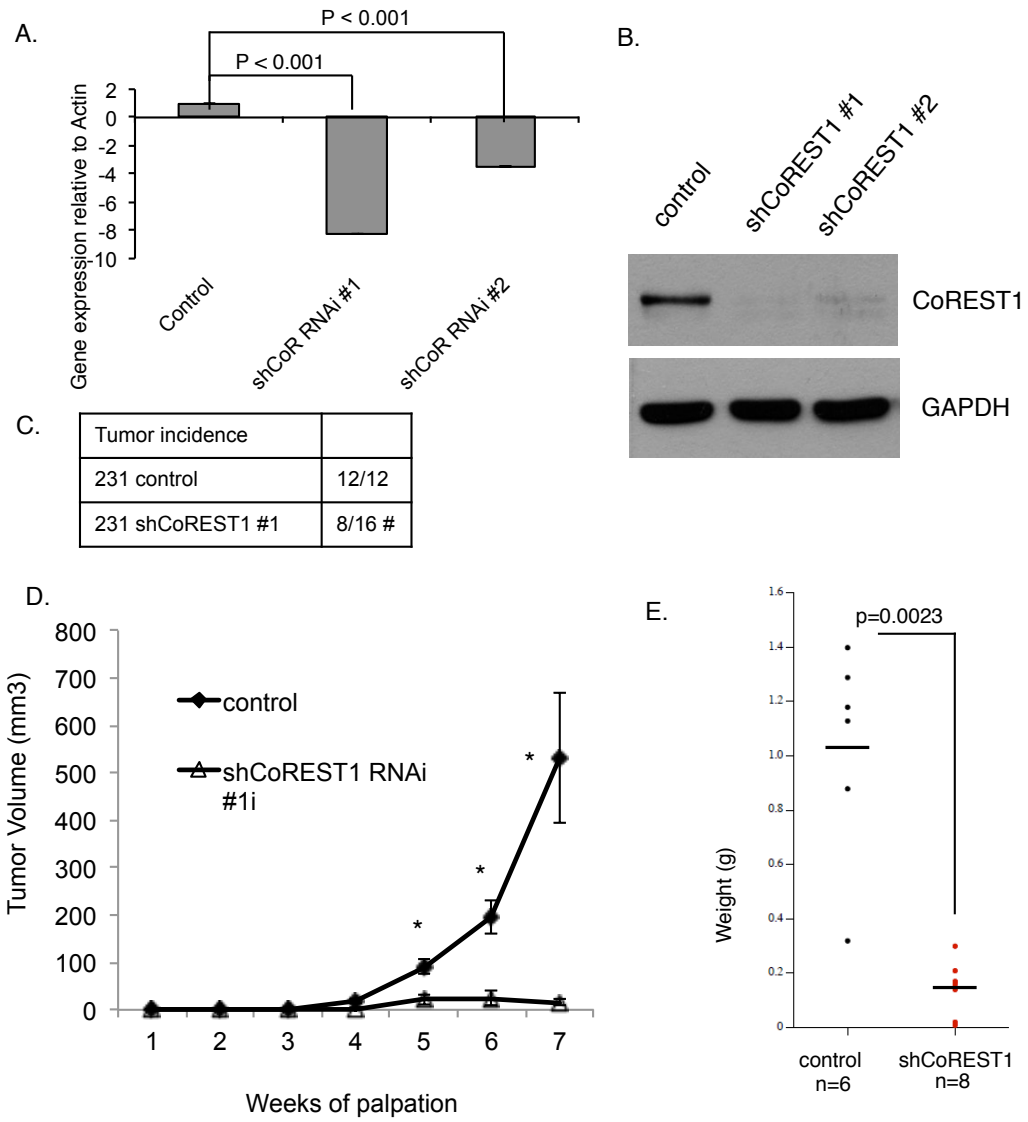


Figure 2.1. Depletion of CoREST1 in MDA MB 231s decreases the ability of MDA MB 231s to form tumors in vivo.

(A) Levels of CoREST1 knockdown in MDA MB 231 cells by RNA (RT-qPCR) and (B) protein in RIPA extracted whole cell lysates (Western Blot analysis) (C) Tumor burden in mice injected with shCoREST1- MDA MB 231s or control cells. # any mass greater than a diameter of 3mm was considered a tumor, $p < 0.001$ by Fischer's exact test (D) Tumor incidence in mice injected with shCoREST1-

MDA MB 231s or control cells. (* is p value< 0.005, Mann Whitney test) (E) Differences in tumor weights between control and shCoREST1-231 injected cells. All tumor injections and harvesting performed in collaboration by Dr. Lisa Arendt, Kuperwasser lab.

volume and weight (Figure 2.1 A and B). Thus reduced CoREST1 in the tumor cells failed to promote and sustain tumor growth in this breast cancer model.

To identify the mechanism by which CoREST1 promotes tumorigenesis we performed several analyses to identify differences upon CoREST1 depletion. No changes in proliferation rates were observed upon CoREST1 knockdown (Figure 2.2 A). This is noteworthy, because pharmacological inhibition or knockdown of LSD1 (commonly found in complexes with CoREST1) inhibits cell growth in MDA MB 231 cells (Pollock et al.; Zhu et al.). This inhibition of cell growth could be attributed to changes in cell cycle regulators or mechanisms such as increased cell death or apoptosis. Knockdown of CoREST1 in MDA-MB-231s did not lead to obvious changes in cell morphology indicating no obvious signs of epithelial to mesenchymal transition (EMT) (Figure 2.2 B). Visual signs of EMT include change in cobblestone shaped appearance of epithelial cells into dissociated spindly or elongated cells. MDA MB 231 cells are a heterogenous population of epithelial and basal cells and no obvious changes were observed in cell morphology upon CoREST1 depletion (Figure 2.2B).

Additionally, no significant changes were observed in global H3K4me2 levels, indicating that depletion of CoREST1 is not compromising the activity of LSD1 in MDA MB 231s (Figure 4. 1). H&E staining revealed that, although they were smaller, tumors formed upon CoREST1 knockdown had larger necrotic areas (Figure 2. 3 A). Ki 67 staining revealed no differences in cellular

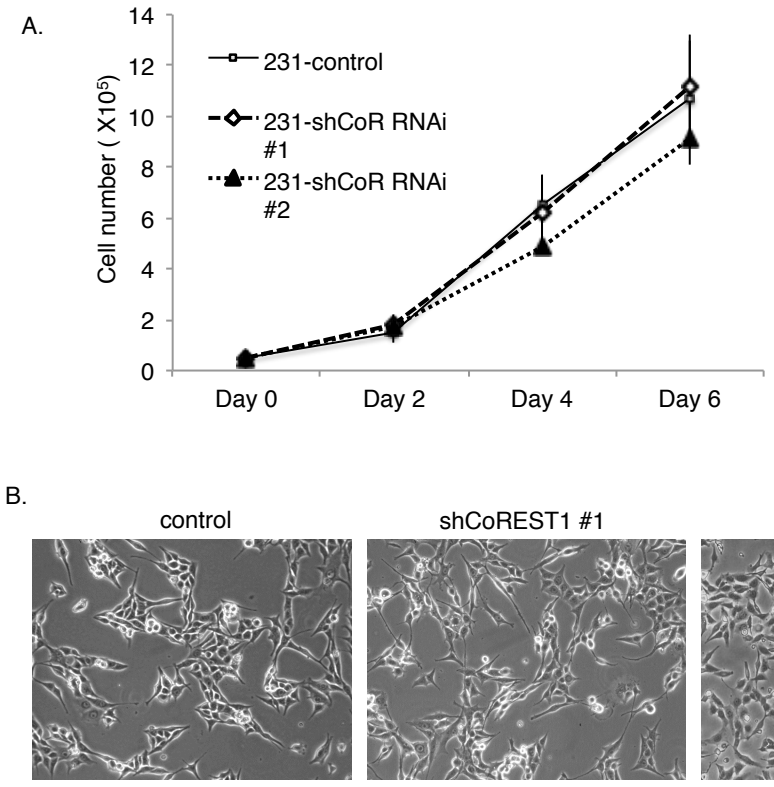


Figure 2.2. Depletion of CoREST1 in MDA MB 231 cells does not show changes in proliferations or visible morphological changes like epithelial to mesenchymal transition.

(A) Proliferation rates of control and shCoREST1 MDA MB 231 cells. 50,000 cells were plated and counted at intervals over a 6-day period using an automated cell counter (B) Phase contrast images analysis of the MDA MB 231 cells with and without CoREST1 depletion display no gross, visible morphological changes.

in the tumors (Figure 2.3 B), consistent with our finding that CoREST1 depletion did not alter growth rates in vitro (Figure 2.2 A).

Since obvious cell autonomous mechanisms of tumor formation other than necrosis were not affected, we examined non-cell autonomous mechanisms. Gross visual examination of the control and CoREST1 depleted tumors while harvesting tumors from animals, revealed that control tumors had a more reddish appearance compared to CoREST11 depleted tumors (data not shown). The H&E staining with the necrotic indices suggest that lack of CoREST1 in the tumor cells could be influencing the blood supply to the tumors. We examined the tumors for CD31 immunostaining, a marker for endothelial cells; the number of CD31 positive cells were significantly reduced in CoREST1 depleted tumors (Figure 2.3 C). These results were supported by additional immunofluorescence studies with F4/80, a marker for macrophages, which showed significant reduction in F4/80 positive staining between control and shCoREST1 tumors (Figure 2.3 D). These xenograft studies suggest CoREST1 is important for tumorigenesis and may regulate communication of the breast cancer cells with the surrounding stroma, specifically angiogenesis and tumor-mediated inflammation to influence tumor growth.

We hypothesized that CoREST1, a nuclear factor, might regulate the MDA-MB-231 secretome to influence tumor/stroma communication. We therefore analyzed conditioned media collected from control and shCoREST1-231 cells using a human angiogenesis antibody array (R&D Systems, MN). The

array allowed for simultaneous evaluation of 55 secreted factors associated with angiogenesis to determine if any of these were affected by knockdown of CoREST1 (Figure 2.4). We observed a dramatic downregulation of several pro angiogenic factors like VEGF-A, pro inflammatory factors like MCP1 and CXCL16 and anti angiogenic factors like TSP1 in the secretome of the CoREST1 depleted MDA MB 231 cells (Figure 2.5).

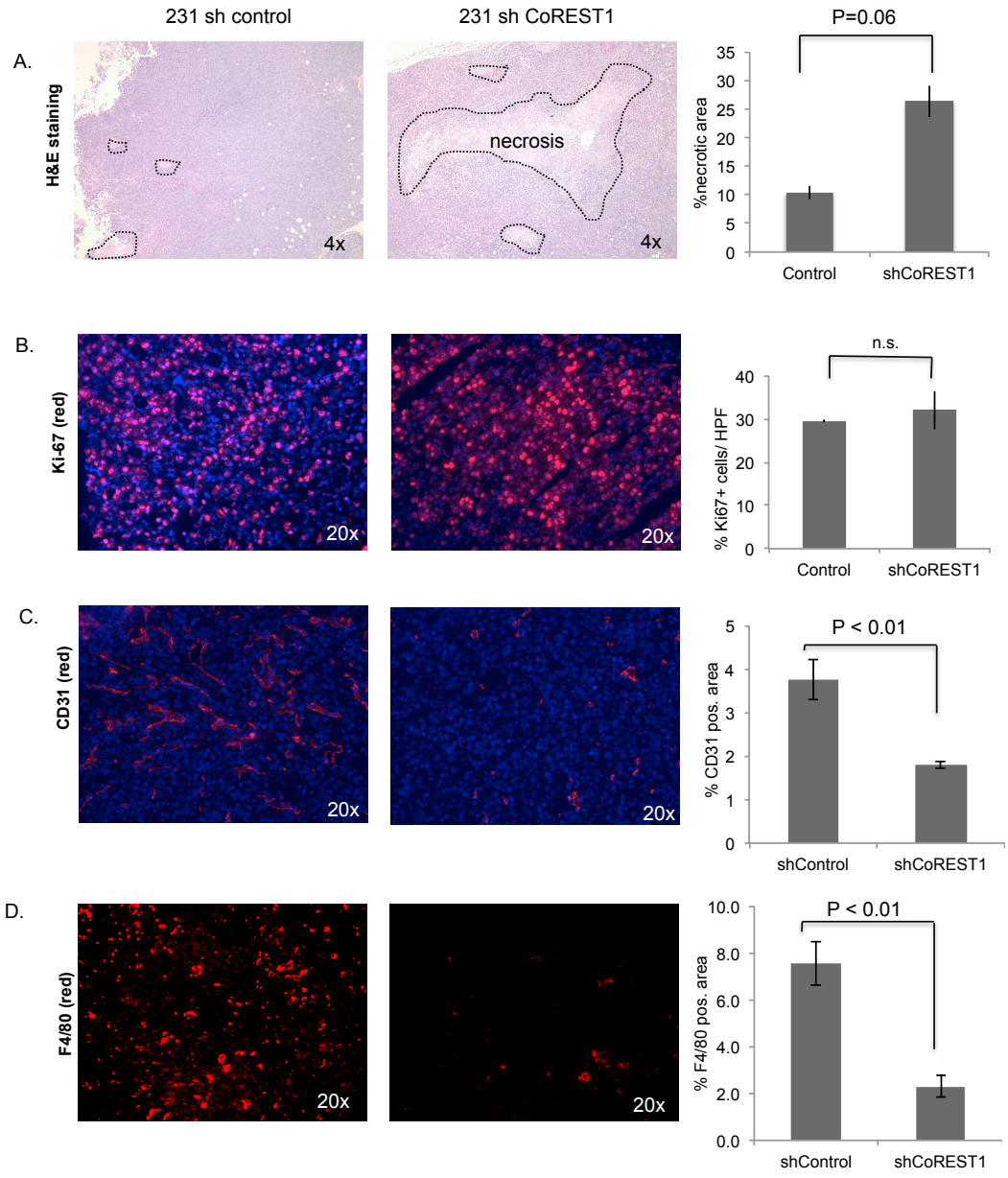


Figure 2.3. Depletion of CoREST1 in MDA MB 231 tumor cells affects both cell autonomous and non cell autonomous mechanisms of tumor formation.

(A) Necrotic index analysis by H&E staining in shCoREST1-231 tumors compared to control tumors. Images at 4x (B) Examination of degree of proliferation in control and shCoREST1-231 tumors by Ki67 staining. Images at 20x. Representative images were quantified using ImageJ. An average of five

high power fields (HPFs) of DAPI positive nuclei were used to obtain measurements; with a minimum of 3 independent tumors per group (C) Examination of differences in blood vessel formation in control and shCoREST1-231 tumors by CD31 staining and quantification. Images at 20x Representative images were quantified using ImageJ. An average of five high power fields were used to obtain measurements; with a minimum of 3 independent tumors per group. DAPI staining was used to ensure that equal numbers of tumor cells were counted for each HPF. (D) Examination of differences in macrophage infiltration in control and shCoREST1-231 tumors by F4/80 staining and quantification. Images at 20x. Representative images were quantified using ImageJ. An average of five high power fields of DAPI positive nuclei were used to obtain measurements; with a minimum of 3 independent tumors per group.

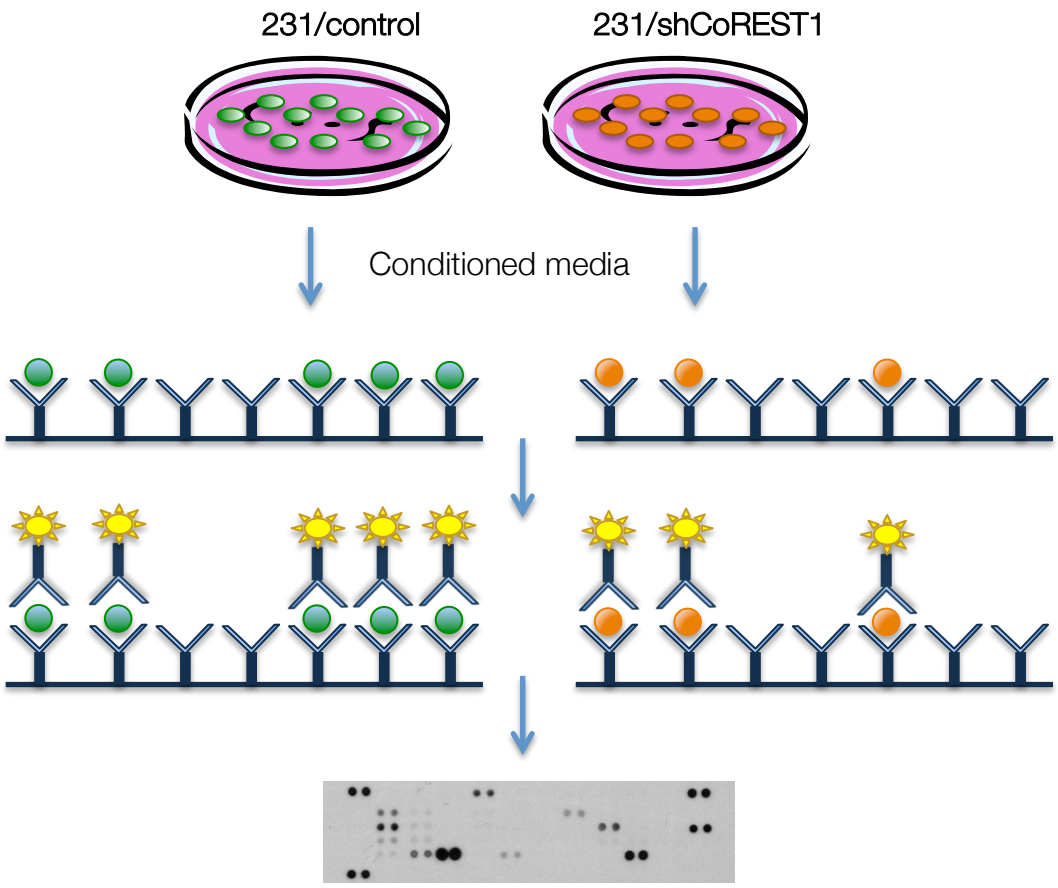


Figure 2.4. Schematic representation of the secretome assay used as a discovery tool to probe for changes in angiogenic factors upon depletion of CoREST1.

Conditioned media collected from both groups was used to probe commercially available membranes (R & D Biosciences) that were pre spotted with 55 different factors in the angiogenic/inflammatory pathway. The assay was carried out as per manufacturer's protocol, with appropriate controls and visualized using chemiluminiscent methods.

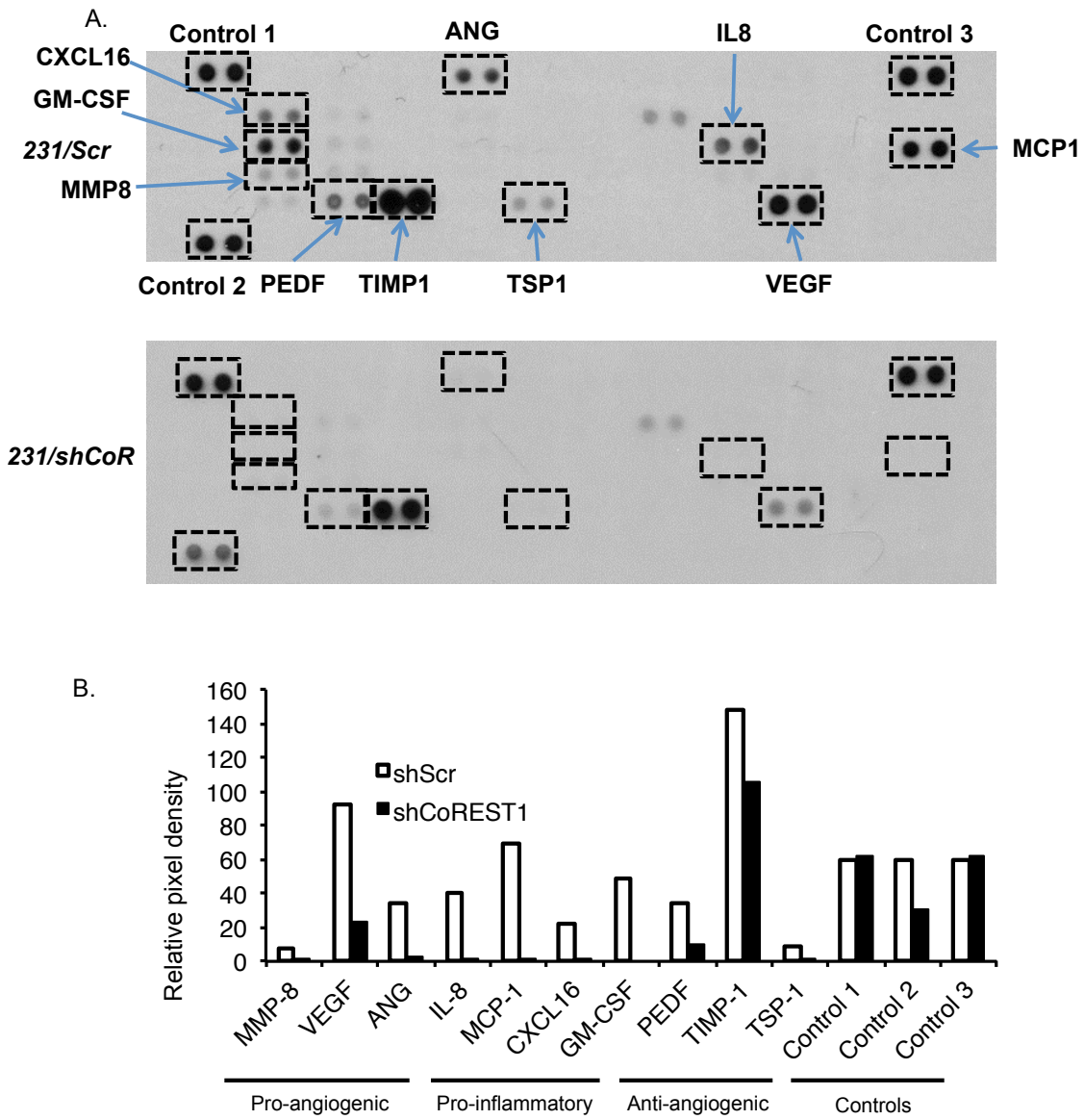


Figure 2.5. Depletion of CoREST1 changes the angiogenic secretome of MDA MB 231 cells.

(A) Immunoblot images of the human angiogenesis arrays (R&D Biosystems). Conditioned media collected overnight from control and shCoREST1-MDA MB-231 cells was incubated with membranes (B) Quantification of the relative pixel density for changes in pro-angiogenic, pro-inflammatory and anti-angiogenic

factors secreted by shCoREST1-231 cells. Controls are as indicated. Control 1 and 2 are positive controls, and control 3 is background or negative control. The assay was considered successful as 2 out of 3 controls worked as per manufacturer's protocols.

Given that CoREST1 acts to coordinate the activities of several histone modifying enzymes, we asked if the changes in the levels of secreted proteins could be due to changes in mRNA of these factors. RT-qPCR analysis indicated that the changes seen in the MDA MB 231 secretome were reflected at the transcriptional level, where mRNA of several pro-angiogenic and pro-inflammatory factors including MCP1, PEDF and VEGF was reduced upon CoREST1 depletion (Figure 2.6). This was a surprising finding as CoREST1 is most well documented as a corepressor (Abrajano et al., 2009b; Andres et al., 1999; Gocke and Yu, 2008; Gomez et al., 2008; Ouyang et al., 2009; Qureshi et al., 2010; Saijo et al., 2009; Xiao et al., 2010). While these effects may be indirect and related to CoREST1-mediated repression, LSD1 which is commonly found in a repressor complex with CoREST1 can function as an activator in some contexts (Bennani-Baiti, 2012), raising the possibility that CoREST1 could also assume activator function in certain contexts.

Since tumor angiogenesis/inflammation and hypoxia are interdependent, we examined if levels of master regulator of hypoxia, HIF1a were affected by depletion of CoREST1. Examination of mRNA and protein HIF1a levels in the presence and absence of CoREST1 revealed no significant differences indicating that CoREST1 does not regulate HIF1a at the transcriptional or translational level (Figure 2.9).

In order to confirm that the CoREST1-dependent changes in the MDA-MB-231 secretome were functional, we investigated the impact of human umbilical

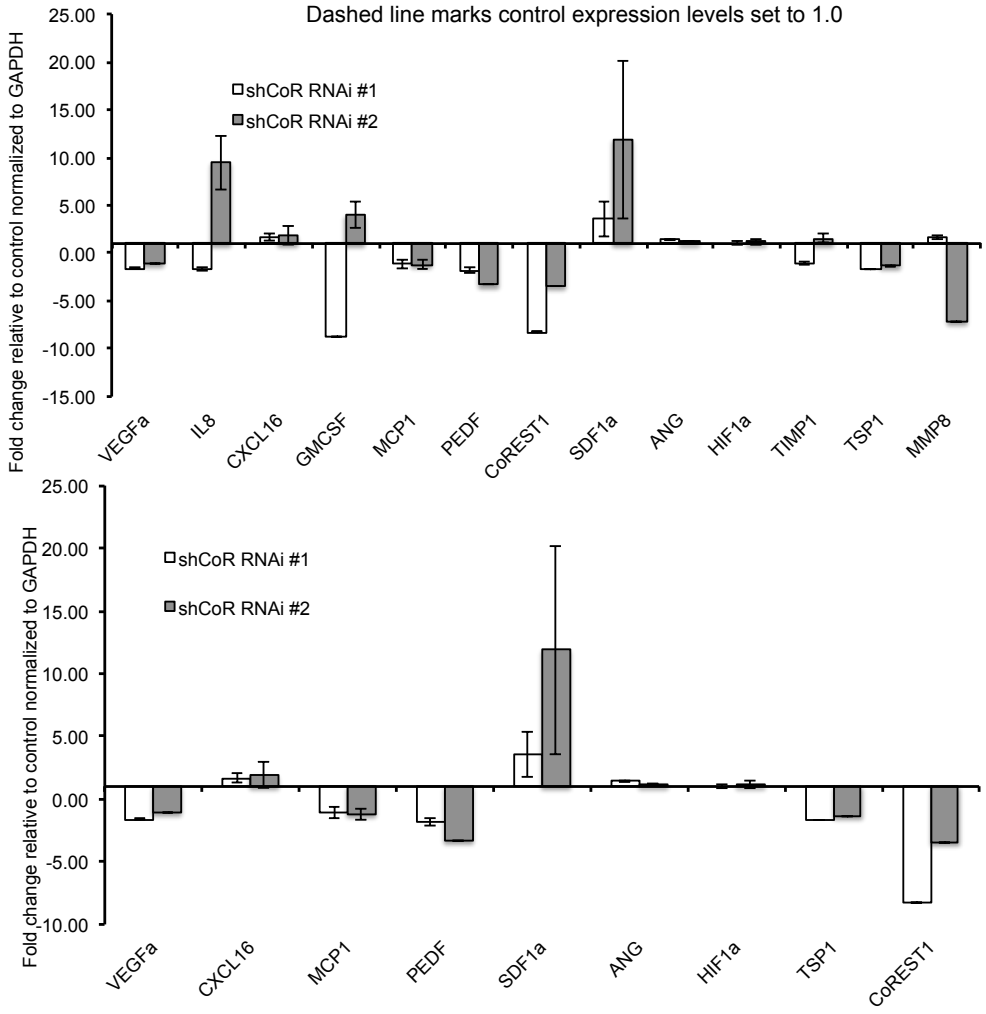


Figure 2.6 Depletion of CoREST1 in MDA MB 231 cells results in decrease in mRNA levels of several angiogenic factors.

(A) Gene expression analysis of candidate angiogenic factors in shCoREST1 MDA MB 231 cells. Candidates were selected based on a positive signal in the secretome assay (Figure 2.4). Results were validated in a second cell line generated by using an independent lentiviral construct against CoREST1 to identify CoREST1 specific gene expression changes. Results shown in (B). (B) Gene expression analysis of angiogenic factors that showed the same trends in

gene expression using shCoREST1 MDA MB 231 cells generated with two independent RNAi constructs against CoREST1.

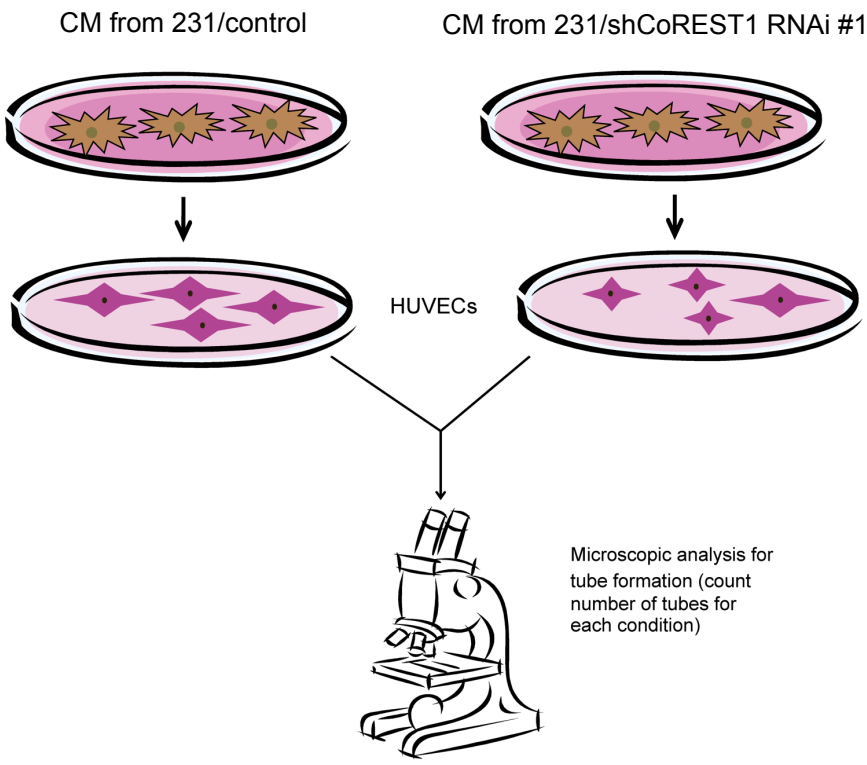


Figure 2.7: Schematic of HUVEC tube formation assay.

Conditioned media (CM) collected from control and shCoREST1-MDA MB 231 cells was collected for 16-18 hours and mixed with human umbilical vein endothelial cells and plated on a basement membrane. Cells were imaged after after 4 hours of incubation at 37 C. Positive control included basal media spiked with FGF, a potent pro angiogenic factor; negative control included basal media only. Basal media comprises of EBM media (Lonza) + 0.1% BSA). Results shown in Figure 2.8 A.

vein endothelial cells (HUVECs) to form tubes on a basement membrane that recapitulate capillary formation in tissues as robust tube formation is a surrogate marker for angiogenesis (Figure 2.7). We exposed the HUVECS to conditioned media from control and shCoREST1 MDA MB 231 cells and measured tube formation. As shown in Figure 2.8 A, conditioned media from CoREST1 knockdown cells had a significantly reduced ability to promote tube formation. Conditioned media from CoREST1 knockdown cells was also used in a cell migration/wound healing assay that assessed the ability of the HUVECs to migrate in response to wounding. Secreted factors from shCoREST1 cells had significantly reduced wound healing activity, but no significant differences in their effect on proliferation of HUVEC cells (Figure 2.8 B, C). Together, these data suggest that CoREST1 plays an important biological role in regulating the expression of genes in the angiogenic and inflammatory repertoire.

2.4 Discussion:

2.4.1 CoREST1 and the tumor microenvironment

Secretome analysis (Figure 2.5), gene expression analyses (Figure 2.6) and functional assays with the HUVECs (Figure 2.8) support the hypothesis that CoREST1 regulates transcription of angiogenic genes (including a subset of hypoxia inducible genes) resulting in an enhanced angiogenic response and larger, more vascularized tumors. Additionally, our studies identify an uncharacterized role for CoREST1 in tumor angiogenesis and the tumor

microenvironment. There is a growing body of literature that highlights how epigenetic aberrations in tumor cells can result in tumor progression and maintenance. Our studies revealed an unanticipated influence of CoREST1 in the tumor cells exerting a profound non-cell autonomous effect on the surrounding tumor stroma, resulting in an altered stromal response.

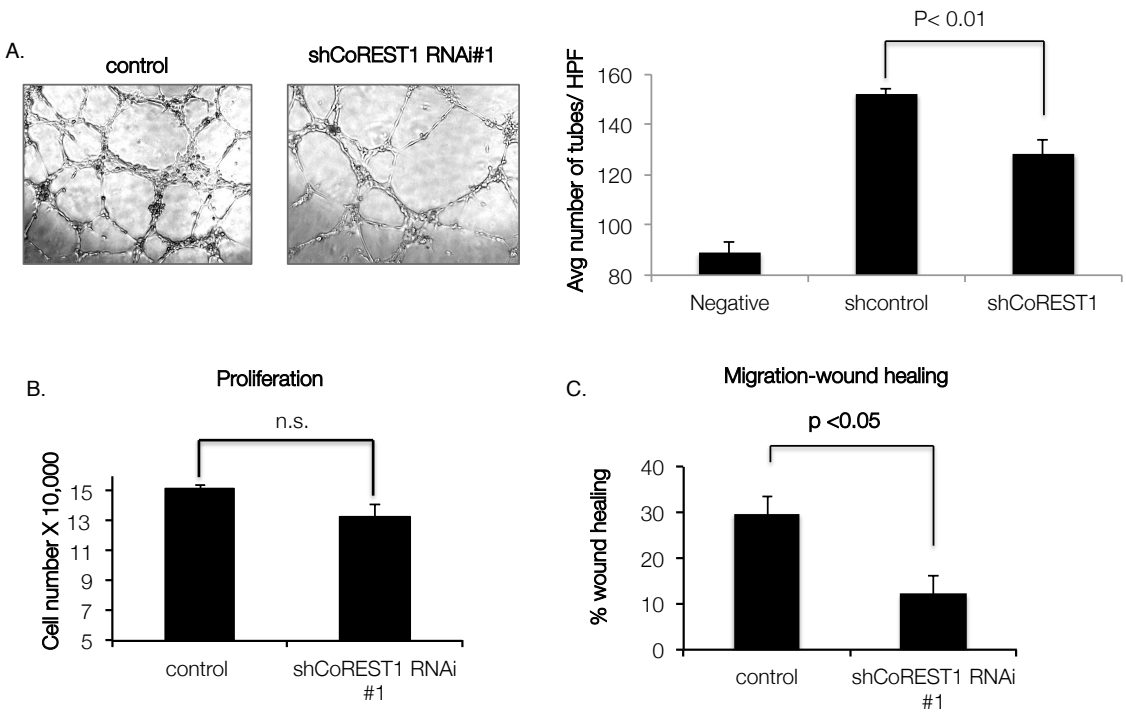


Figure 2.8: Secreted factors from CoREST1 depleted MDA MB 231s cells have decreased ability to promote tube formation and wound healing/cell migration but not proliferation in HUVECs.

(A) Left: Phase contrast images of tube formation assays (FGF treated, control CM, shCoREST1 #1 CM, and shCoREST1 #1 CM spiked with FGF) Right: Quantitation of number of tubes per high power field. Measurements were and average of 5 high power fields for each condition tested. (B) Proliferation of HUVEC cells in response to treatment with control and shCoREST1 CM. 50,000 cells were plated in respective CM and counted with an automatic cell counter after 72 hours. 12 replicates were plated for each condition; measurements are an average of three independent experiments. (C) Wound healing/cell migration

of HUVEC cells in response to treatment with control and shCoREST1 CM. 300,000 cells were plated overnight and then wounded with a pipette tip. They were imaged at Time 0 and exposed to conditioned media from control and shCoREST1 CM for 6 hours and imaged again. ImageJ software was used to calculate width of wound sites and further calculate % wound healing. Measurements were an average of 10 replicates per condition and 3 independent experiments.

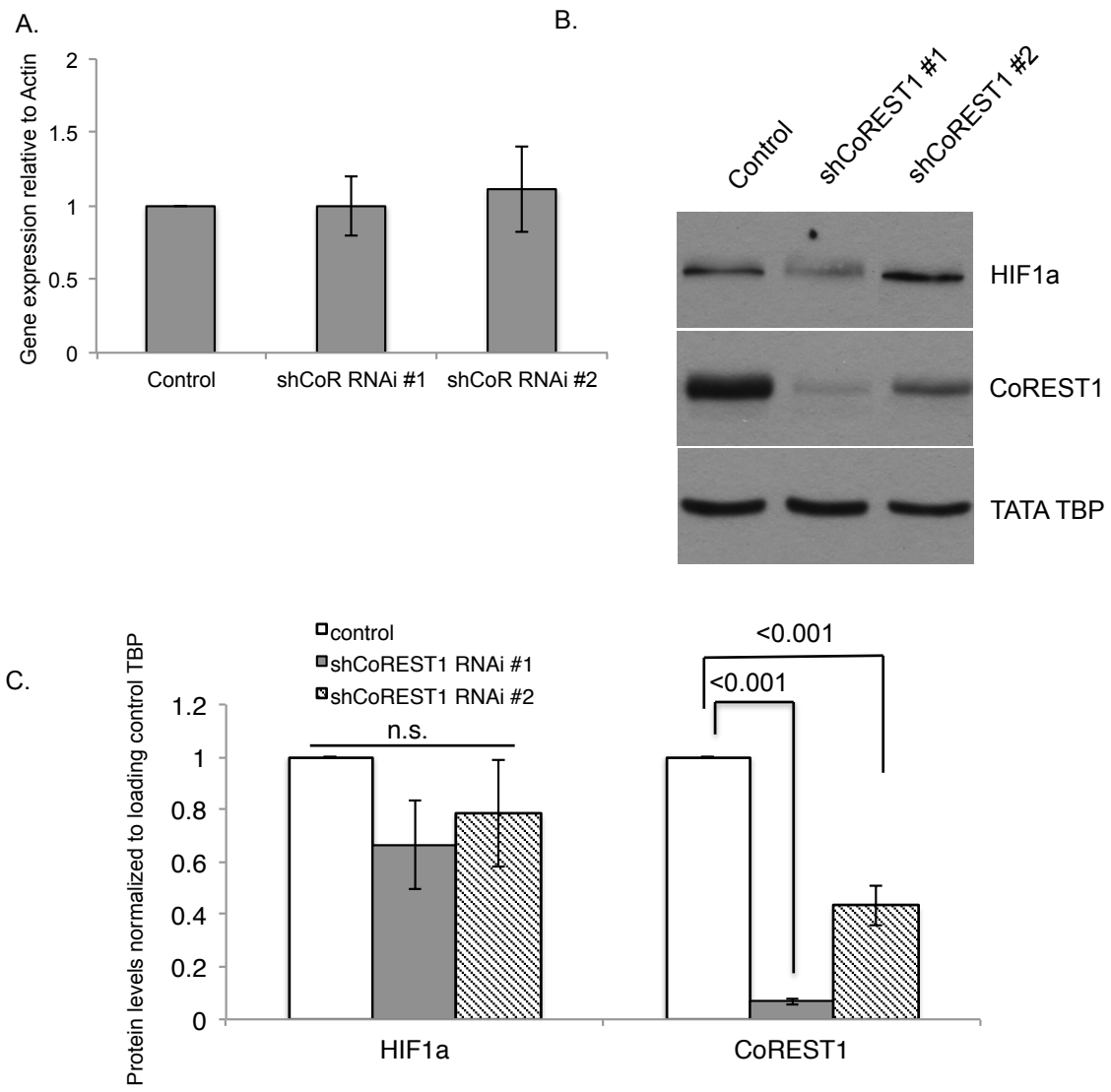


Figure 2.9: Effect of CoREST1 depletion on HIF1a levels in MDA MB 231 cells. HIF1a (A) mRNA levels (B) protein levels in MDA MB 231 cells (C) Quantitation of HIF1a and CoREST1 protein levels in nuclear lysates in two independent shCoREST1- MDA MB 231 cell lines (60 ug of nuclear lysate per lane in (B).

The xenograft breast cancer model helped us understand the role of CoREST1 in tumor stroma interactions and its effect on non-cell autonomous mechanisms of tumor growth. Ablation of CoREST1 in MDA MB 231 tumor cells results in the formation of significantly smaller tumors (weight and volume) in mouse xenograft experiments, in addition to lower tumor incidence. On examining the tumors histologically, they displayed no changes in proliferation (by Ki67 staining) but significant reduction in both CD31 positive and F4/80 positive staining. CD31 and F4/80 are classical markers of endothelial cells and macrophages respectively, suggesting that lack of CoREST1 in the tumor cells results in potentially fewer endothelial cells in the tumors, as well as reduced macrophage infiltration.

Cancer cells secrete an array of cytokines and chemokines that then attract endothelial cells as well as inflammatory cells like macrophages to the site of the primary tumor (Figure 1.6). Tumor secreted cytokines can directly attract endothelial cells for neoangiogenesis, or attract inflammatory cells that then secrete chemoattractants which signal to endothelial cells for homing. Both protein and mRNA levels of monocyte chemoattractant protein-1 (MCP1), one of the key chemokines that regulates infiltration of macrophages and monocytes (Deshmane et al., 2009) were reduced in shCoREST1 tumors. This data in addition to the F4/80 immunofluorescence staining suggests that CoREST1 may influence levels of inflammatory factors, which in turn influences the angiogenic capability of the tumor cells.

Knockdown of CoREST1 in MDA MB 231 tumor cells resulted in a dramatic downregulation of pro-angiogenic, pro-inflammatory and anti-angiogenic factors, at both RNA and protein level. Of the factors that shCoREST1-231 cells secrete at reduced levels, of note are key regulator of angiogenic growth, VEGFA, and pro inflammatory factors like MCP1 and CXCL16. Additionally, anti-angiogenic factors like PEDF were also downregulated. Interestingly, VEGF, VEGF receptor Flt-1 and MCP1 are hypoxia responsive genes, highlighting the interdependence of angiogenesis and the hypoxic response (Forsythe et al., 1996; Galindo et al., 2001; Gerber et al., 1997).

Treatment of HUVEC cells with conditioned media from shCoREST1 231 cells showed defects in HUVEC migration and their ability to organize into endothelial tubes in vitro. This suggested that the tumor cells downregulate some positive factors in their secretome that could hinder the endothelial cells from being recruited to the site of the tumor formation. However, the marked decrease in macrophage infiltration in the CoREST1 depleted tumors implies that their ability to secrete cytokines that attract inflammatory cells is also compromised, suggesting that both direct and indirect mechanisms of endothelial cell homing to tumors maybe regulated by CoREST1. Taken together, our studies reveal an unanticipated biological role for CoREST1 in the tumor microenvironment (Figure 2.10).

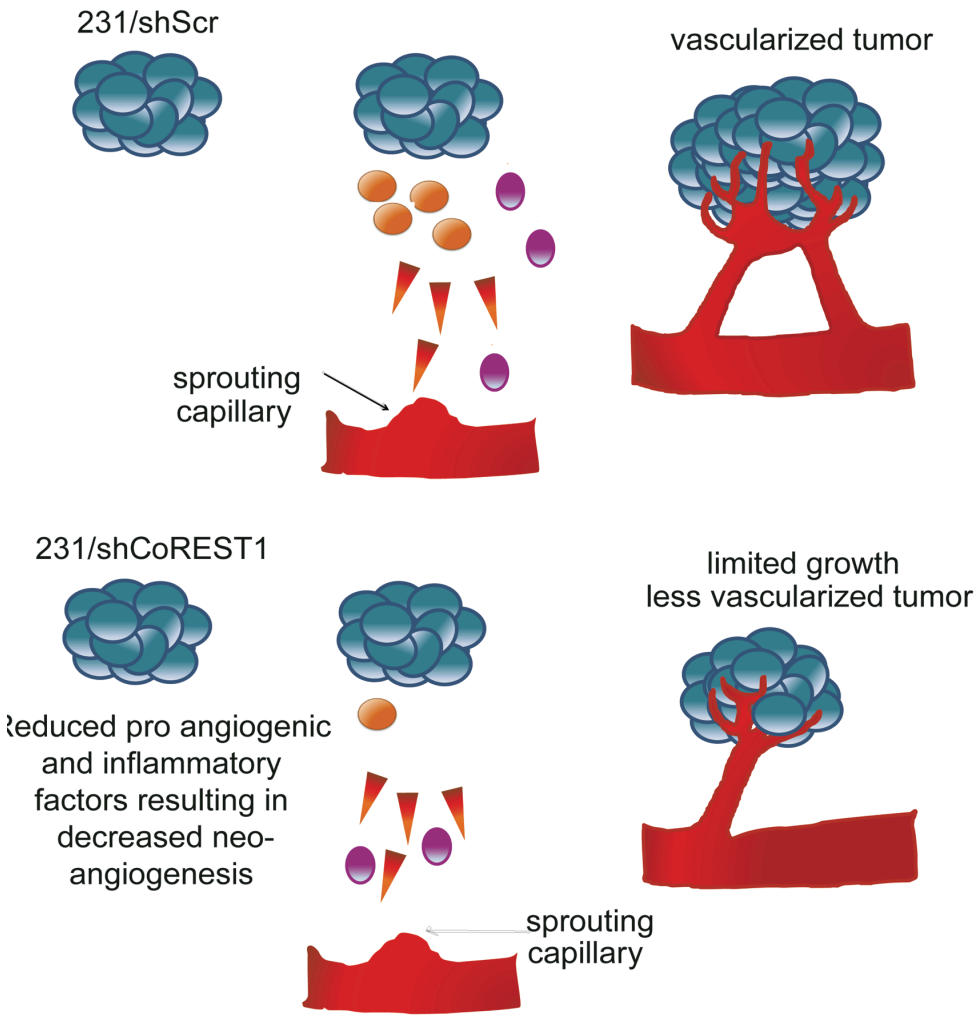


Figure 2.10.: Model for biological role of CoREST1 in tumor angiogenesis and tumor/stroma interactions.

Reduced pro-angiogenic and pro-inflammatory factors secreted by shCoREST1 tumors result in limited tumor growth and less vascularized tumors.

2.4.2 CoREST1 as a putative epigenetic modifier

CoREST1 is most well documented as a corepressor and is found in complexes with known epigenetic modifiers like LSD1, HDAC1/2 and SIRT1 (Abrajano et al., 2009b; Andres et al., 1999; Gocke and Yu, 2008; Gomez et al., 2008; Ouyang et al., 2009; Qureshi et al., 2010; Saijo et al., 2009; Xiao et al., 2010). Previous reports suggest that knockdown of LSD1 results in decreased proliferation (Huang et al., 2012; Pollock et al.; Sun et al., 2010) and tumor growth (Schulte et al., 2009), however knockdown of CoREST1 in MDA MB 231s did not change the ability of the cells to proliferate in vitro. These observations imply a potential LSD1-independent role for CoREST1 or a CoREST1 independent function for LSD1.

Our data indicate that the CoREST1 mediated changes in gene expression occurs at the transcriptional level, favoring CoREST1 function as a direct or indirect activator (Figure 4.2). Despite their canonical roles as repressors, both LSD1 and CoREST1 have been reported to function as an activator in some contexts opening up the possibilities for several models (Bennani-Baiti, 2012; Domanitskaya and Schupbach, 2012). For example, CoREST1 can act as a direct activator for pro-angiogenic and pro-inflammatory genes, which would explain why levels of these factors are reduced on CoREST1 knockdown. Alternatively, CoREST1 may function as a repressor for intermediate repressory factors resulting in angiogenic gene activation (Figure 4.2); in this model,

knockdown of CoREST1 results in depression of intermediate repressor that can then silence angiogenic genes, leading to a dampened angiogenic response.

Together, our current data support a role for CoREST1 in tumor promoted inflammation and angiogenesis. A genome wide analysis of CoREST1 target genes using microarrays would shed light on other genes that could be part of commonly regulated pathways in addition to angiogenesis and tumor mediated inflammation. For a more detailed discussion on future studies, see Chapter IV.

Chapter III

LSD1 co-operates with Slug in normal breast cell differentiation

3. 1 Abstract:

The transcription factor Slug has a role in mammary epithelial cell differentiation (Come et al., 2004; Nassour et al., 2012) and elevated expression of Slug is also observed in ER negative, basal-type BRCA1 associated breast cancer (Alves et al., 2009b; Proia et al., 2011). LSD1 is a known epigenetic regulator that directly interacts with proteins like Slug that contain a SNAG domain (Chowdhury et al., 2013; Lin et al., 2010b). Based on our observation of an interaction between LSD1 and Slug, we hypothesized that an LSD1/Slug complex contributes to breast cell fate determination by altering chromatin structure and repressing transcription of specific genes associated with the luminal epithelial differentiation program. To test this hypothesis, LSD1 and Slug were knocked down in normal human mammary epithelial cells (HMECs) via lentiviral hairpins to create stable cell lines. Gene expression profile analyses by RT-qPCR and microarray identified common LSD1 and Slug target genes. Analysis of endogenous protein complexes by coimmunoprecipitation revealed that LSD1, Slug and CoREST3 but not CoREST1, formed a complex in the HMEC lines tested. ChIP studies demonstrated that LSD1 and Slug are recruited to luminal genes including CK18. Consistent with the observed gene expression changes, a more pronounced luminal phenotype was observed upon LSD1 or Slug knockdown using FACS analyses. Taken together, these data support the view that elevated LSD1/Slug activity favors basal cell populations and the maintenance of a basal like state. A better understanding of the factors and programs that promote basal cell fate

may provide a foundation for treatment of basal-like breast cancers that are largely refractory to current treatments.

3.2 Introduction

3.2.1 EMT in development and cancer

Epithelial and mesenchymal cells are in a dynamic flux via the processes of epithelial to mesenchymal transition (EMT) and mesenchymal to epithelial transition (MET), during cellular differentiation and organogenesis. EMT is a critical process in embryogenesis such as blastula and neural crest formation, (Nakaya and Sheng, 2013) and improper EMT results in developmental defects. EMT is tightly regulated during wound healing as well as during morphogenesis, where it results in cell fate decisions, however in tumorigenesis the process is less constrained. Broadly speaking, EMT is defined by downregulation of epithelial markers, like E-cadherin, and an increased expression of mesenchymal markers, including vimentin and fibronectin, resulting in an increase in cell migration and invasion (Micalizzi et al., 2010).

Tumors also display plasticity of cellular states, where EMT can allow for metastasis from the primary tumor highlighting the importance of EMT in neoplastic progression. Common pathways have been identified in normal development and cancer progression, suggesting that tumor cells hijack these developmental pathways. Misexpression of transcription factors including Snail/Slug and Twist, in addition to inappropriate activation of pathways like TGF-β and Wnt pathways are observed in breast cancer, and result in poor prognostic outcomes (Micalizzi et al., 2010).

3.2.2 Slug and Snail in breast cell differentiation

Zinc-finger transcription factors SNAIL (Snai1) and SLUG (Snai2) are part of the Snail superfamily that regulates processes of development and cellular differentiation and also tumorigenesis. Slug is expressed in basal cells during mammary gland morphogenesis and is upregulated in a variety of cancers, including breast cancer (Alves et al., 2009a; Nassour et al., 2012). The Snail family of transcription factors is involved in tumor progression and invasion by mechanisms including repression of E-cadherin, a key EMT regulator and suppressor of tumor invasion (Peinado et al., 2007). Slug and Snail expression was also observed in tumors that metastasized supporting the rationale for the correlations between high Slug levels and aggressive cancers (Alves et al., 2009b; Come et al., 2004).

3.2.3 LSD1 interacts with SNAG domain containing proteins

LSD1/CoREST1 complexes interact with SNAG domain containing transcription factors like Gfi-1, Snail and Slug (Chowdhury et al., 2013; Ferrari-Amorotti et al., 2013; Lin et al., 2010b). Snai1 and LSD1 directly bind to each other and CoREST1 stabilizes this complex. Expression of SNAIL suppresses E-cadherin expression and induces EMT in breast cancer cells in vitro, suggesting that SNAIL has an integral role in EMT and breast cancer metastasis (Banck et al., 2009; Huang et al., 2012; Lin et al., 2013; Lin et al., 2010b; Murray-Stewart et al., 2013; Vasilatos et al., 2013). The LSD1/CoREST1 complex interacts with

Snail (Snai1) via the SNAG domain in Snail; this interaction recruits LSD1 to Snail target genes to repress EMT genes like E-cadherin.

While Slug is well known for its involvement in tumor invasion and metastasis, the role of Slug during normal mammary gland development and in stem cell function/maintenance is poorly described. Additionally, mechanisms by which Slug regulates transcriptional programs to maintain cellular states are still poorly understood. Here we investigate a role for a Slug/LSD1 complex in breast epithelial cell differentiation.

3.2 Results

Previous affinity purification mass spectrometry and coimmunoprecipitation experiments with tagged Slug protein in 293T cells identified LSD1 and CoREST1 as potential Slug interactors (Proia, data not shown). This finding and other reports of LSD1 and Slug/Snail interactions (Ferrari-Amorotti et al., 2013; Lin et al., 2010b) led us to hypothesize that increased levels of LSD1 complex activity represses transcription of specific genes associated with the luminal epithelial differentiation program. We tested the role of LSD/Slug in gene expression and cell fate by knocking down LSD1 and Slug in two patient-derived, normal, human mammary epithelial cell lines (Figure 3.1) and CoREST1 in two tumor-derived breast cancer cell lines (Supplementary Figure 2) followed by characterization and identification of LSD1/CoREST1 target genes.

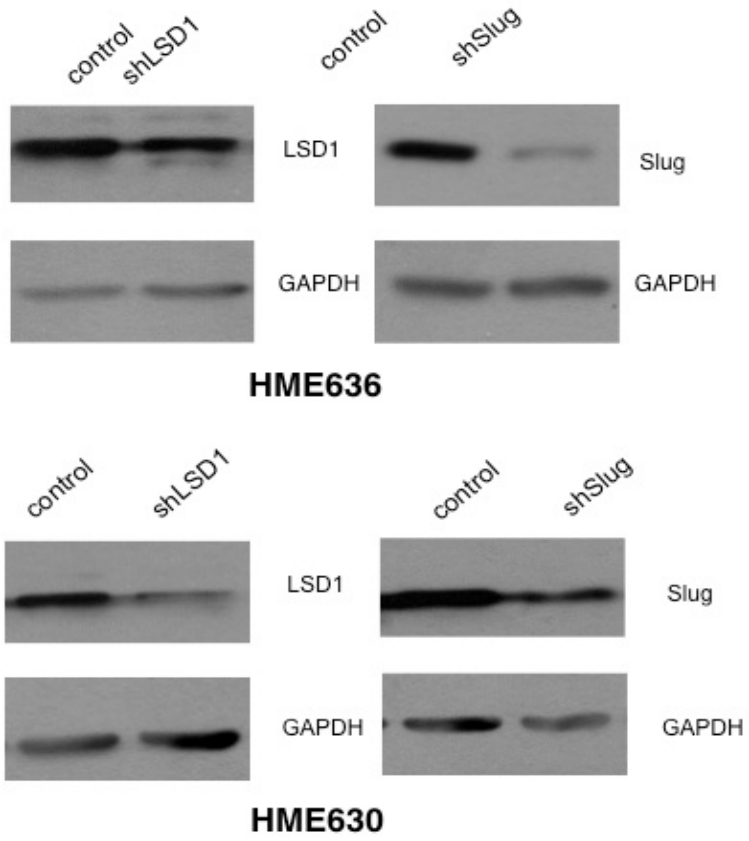


Figure 3.1: Levels of LSD1 and Slug in shLSD1 and shSlug HMECs.

Representative samples of RIPA extracted whole cell lysates from two patient derived, non-tumorigenic, human mammary epithelial cell lines-- HME636 (top) and HME630 (bottom) were tested for knockdown of protein using Western Blot. shSlug data provided by Sarah Phillips.

Knockdown of LSD1 and Slug displayed similar gene signatures, upregulation of luminal genes like CK18, CK19 and Muc1, and downregulation of basal genes like Vimentin (Figure 3.2). Although the trends for these changes were consistent between the two patient derived cell lines, there were individual differences in expression levels of genes like Bmi1 and E cadherin, underscoring the heterogeneity of these patient derived lines. We further analyzed shLSD1 and shSlug HMECs using a customized PCR super array assaying luminal and basal genes, important in breast cell differentiation (SABiosciences, Valencia, CA). Luminal genes including CK8, CK18, ERBB3 and EPCAM were upregulated upon LSD1 and Slug depletion. Genes of basal origin like SMA (ACTA2) and the stem cell marker, NANOG were downregulated (Figure 3.3). Results from the super array validated the data from our RT-qPCR experiments and further support the idea that Slug and LSD1 function together to regulate common genes involved in luminal differentiation in mammary epithelial cells. Data from endogenous immunoprecipitation of Slug confirmed association with LSD1, KAP1 and RCOR3 in HMECs (Figure 3.4). Surprisingly, LSD1 co-immunoprecipitated CoREST1, but not Slug or CoREST3 suggesting that Slug LSD1 complexes may contain CoREST3 rather than CoREST1 (Figure 3.4, bottom).

To gain a broader view of the genes regulated by LSD1 and Slug in these breast cells, we carried out microarray analysis. Microarray data analyses indicated that approximately 30% of all LSD1 target genes were also Slug target genes (16.2% of upregulated genes and 15.1% downregulated genes). GO analyses show that

common target genes that are repressed by Slug and LSD1 (mRNA levels upregulated on knockdown) are genes involved in epithelial cell differentiation. Similarly genes at which Slug and LSD1 function as activators, (mRNA levels downregulated on knockdown) are genes involved in DNA replication and repair. Candidate gene analyses by RT-qPCR showed similar trends in upregulation of certain luminal genes involved in epithelial differentiation (Figure 3.7).

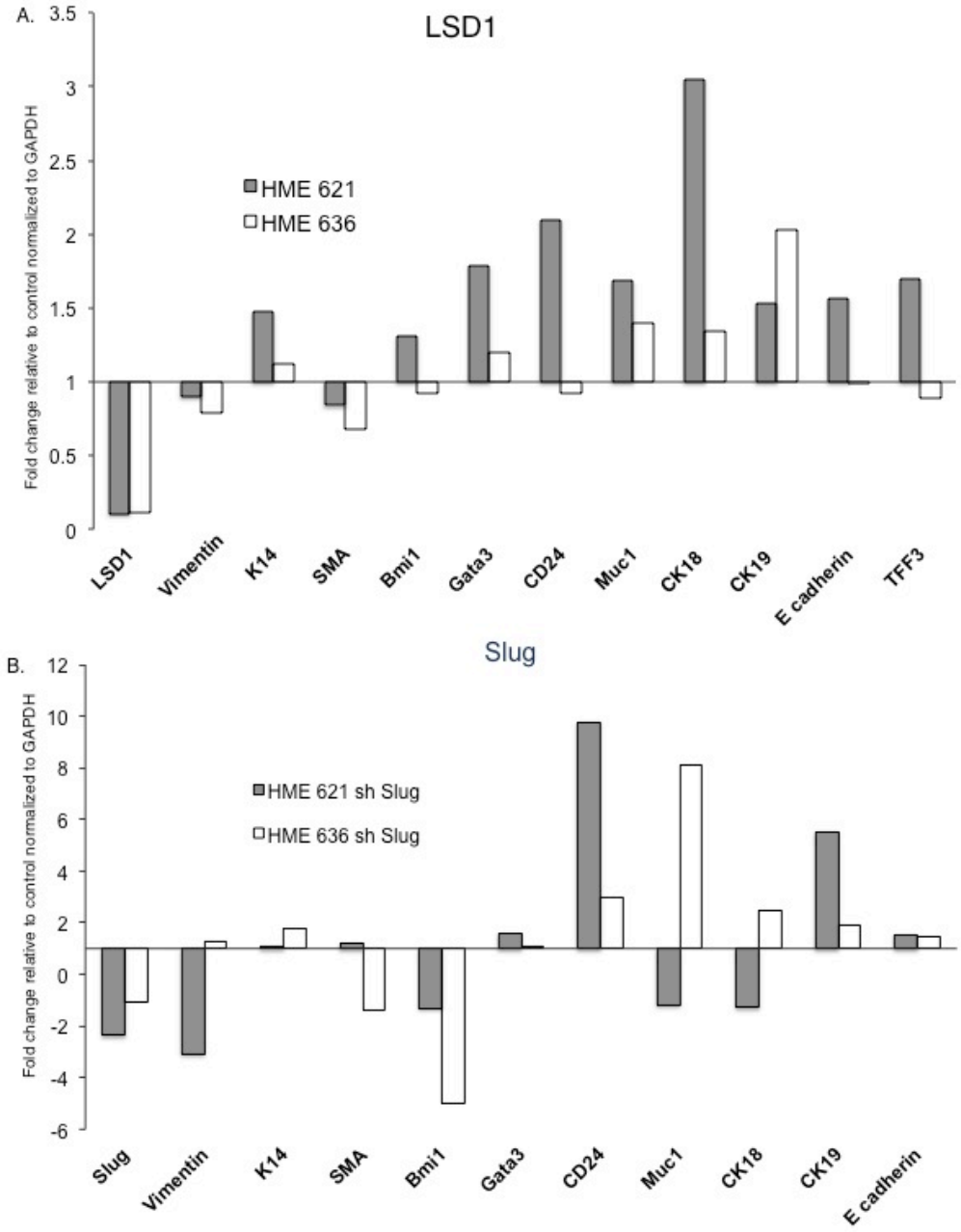


Figure 3.2: mRNA levels of select genes of luminal (CD24, Muc1, CK18, CK19, E-cadherin, basal (Vimentin, SK14, SMA) and stem origin (Bmi1, Gata3) in (A) shLSD1 and (B) shSlug patient derived human mammary epithelial cell lines (patient #HME621 and #HME636) using RT-qPCR. Gene expression is calculated by the ddCT method and results were normalized to the housekeeping

gene, GAPDH. Graphical data depicts control cell line gene expression levels to 1 to calculate fold change. shSlug data provided by Sarah Phillips.

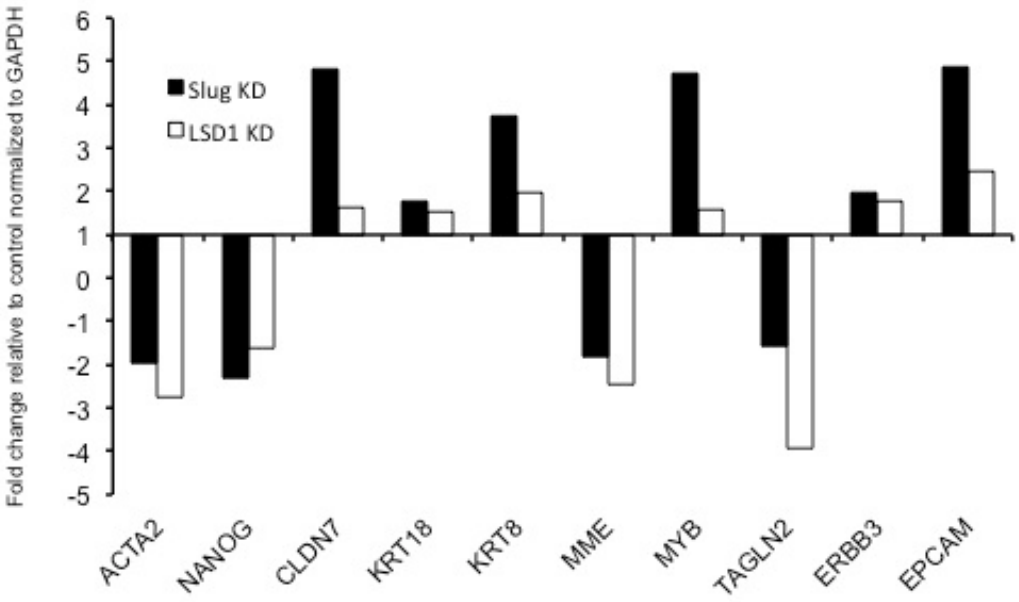


Figure 3.3: RT-qPCR results from shLSD1 and shSlug HME636 cell line depicting upregulation of LSD1/Slug common target genes.

mRNA from shLSD1 and shSlug HME cells lines was used in a super array assay with 84 genes related to luminal, basal and stem cell origin (from SABiosciences/Life Technologies). The super array was used as a discovery tool to select candidate LSD1/Slug common target genes. (Luminal: KRT18, ERBB3, EPCAM, KRT8, CLDN7; Basal: MME, MYB, ACTA2; Stem: NANOG). Gene expression is calculated by the ddCT method and results were normalized to housekeeping gene, GAPDH. Graphical data depicts control cell line gene expression levels to 1 to calculate fold change. shSlug data provided by Sarah Phillips.

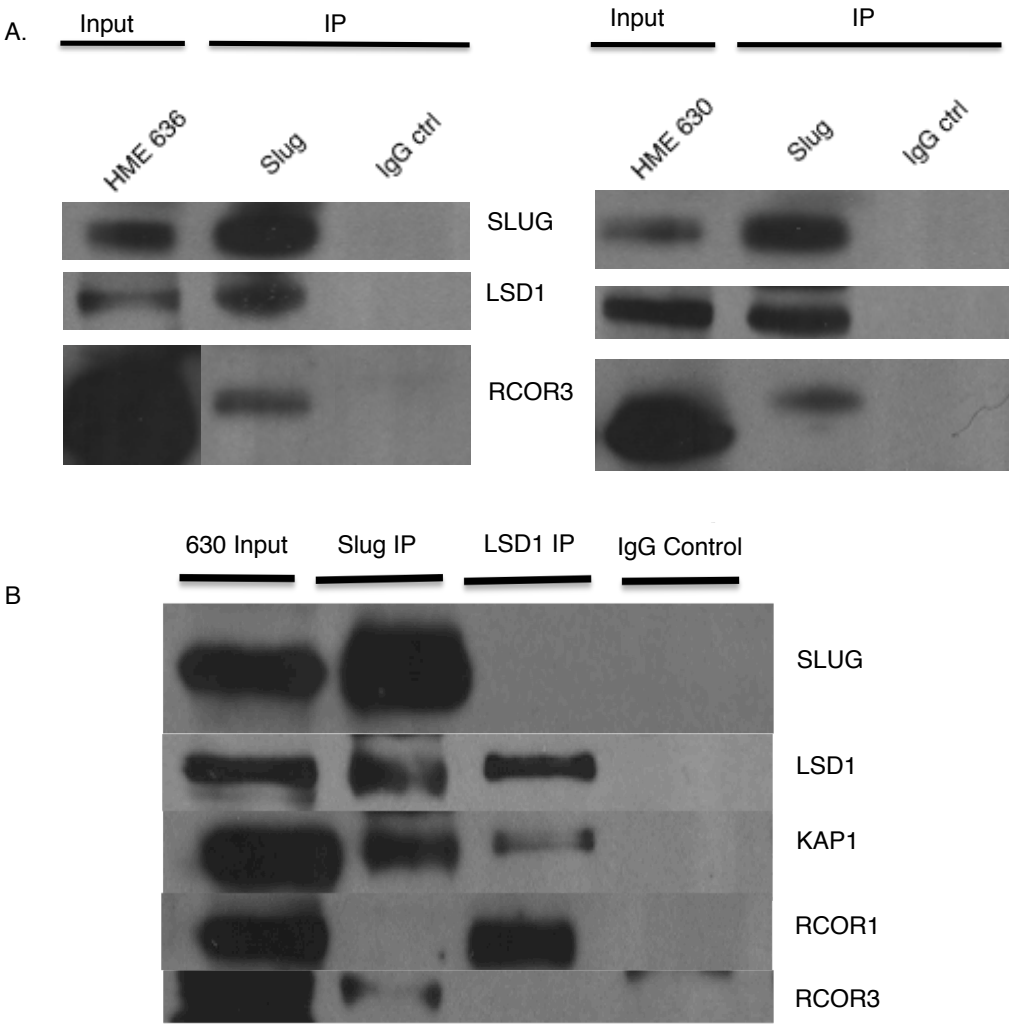


Figure 3.4: Coimmunoprecipitation of endogenous LSD1 and Slug complexes in HME630s and HME636s.

Whole cell lysates from HME630s and HME636s were incubated with LSD1, Slug or IgG. Pull downs were immunoblotted for KAP1, RCOR1 (CoREST1), RCOR3 (CoREST3), LSD1 and Slug. Data provided by Sarah Phillips.

In order to demonstrate that these changes in gene expression were reflected at the level of cell surface markers, we performed FACS analyses for expression of CD24 (luminal), EPCAM (luminal) and CD49f (basal) markers. Use of these markers allowed us to stain and analyze cell surface protein expression levels of luminal and basal population before and after LSD1 and Slug knockdown in HMECs. FACS analyses on control and LSD1 and Slug depleted HMECs showed increased EPCAM and CD24 expression and decreased CD49f expression (Figure 3.5 and 3.6). The finding that depletion of LSD1 or Slug leads to upregulation of luminal populations and downregulation of basal/stem populations is consistent with the observed changes in mRNA levels. These findings support roles for both LSD1 and Slug in determining cell fate.

Finally, chromatin immunoprecipitation experiments demonstrated that LSD1 and Slug were directly bound at the promoters of luminal genes, including CK18, CK14 and CD24 (Figure 3.8). We also examined CoREST1 occupancy at these promoters and, while we observed CoREST1 occupancy at CK18 and CK14, CoREST1 did not localize to the CD24 promoter. The finding that not all LSD1/Slug targets tested showed CoREST1 localization at their promoters supports the idea that different flavors of complexes are recruited to specific target promoters, resulting in unique gene expression patterns that contribute to cell fate.

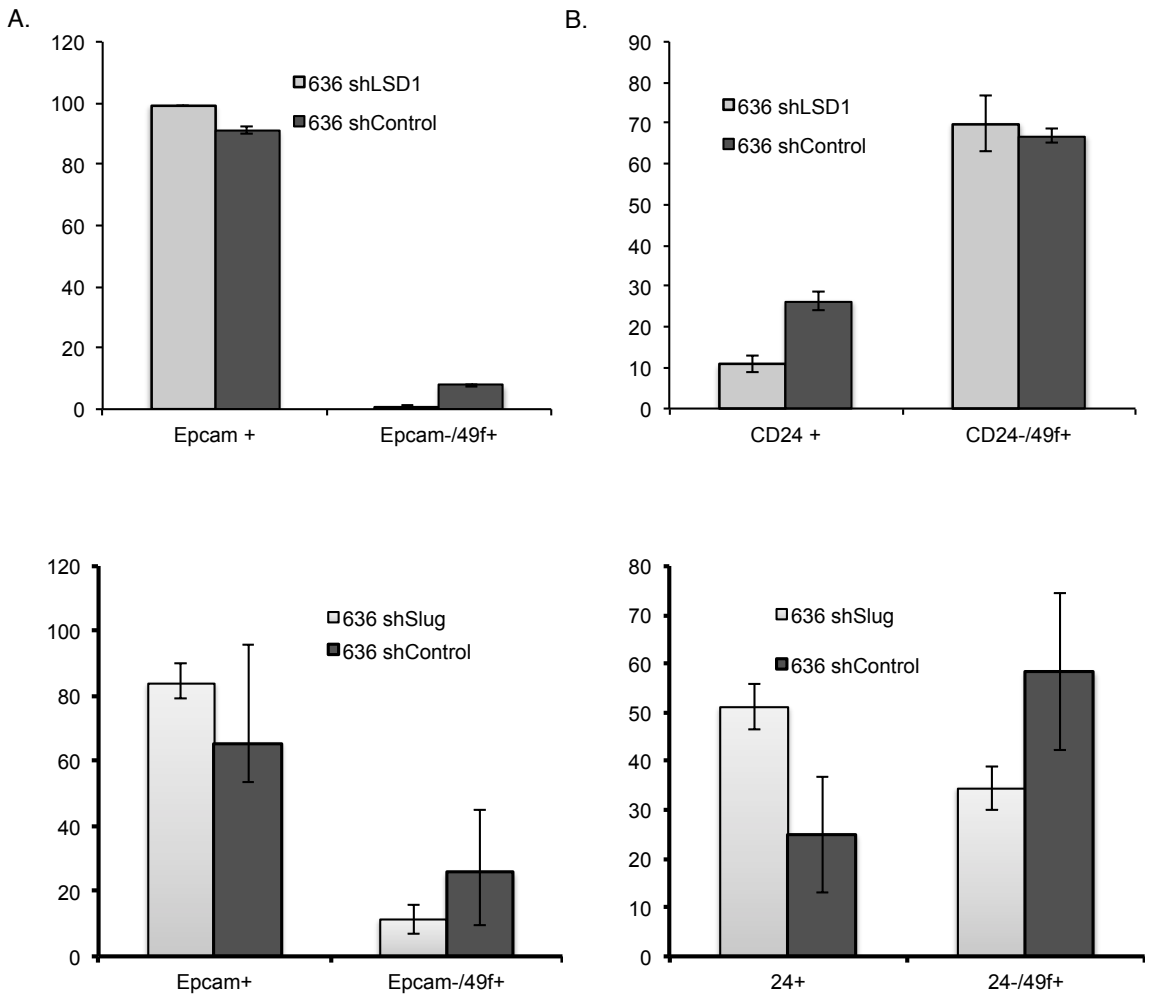


Figure 3.5: Cell surface staining of shLSD1 and shSlug HME636s using CD24, Epcam and CD49f followed by cell sorting analyses.

shLSD1 and shSlug HMECs were stained with CD24, Epcam and CD49f. Cells were then sorted into luminal and non luminal populations (luminal: $CD24^{+}/EpCAM^{+}/CD49f^{+)}$, basal: $CD24^{-}/EpCAM^{-}/CD49f^{+}$ and stem: $CD24^{-}/EpCAM^{+}/CD49f^{+}$. Measurements are averages of 3 independent experiments. In collaboration with Sarah Phillips.

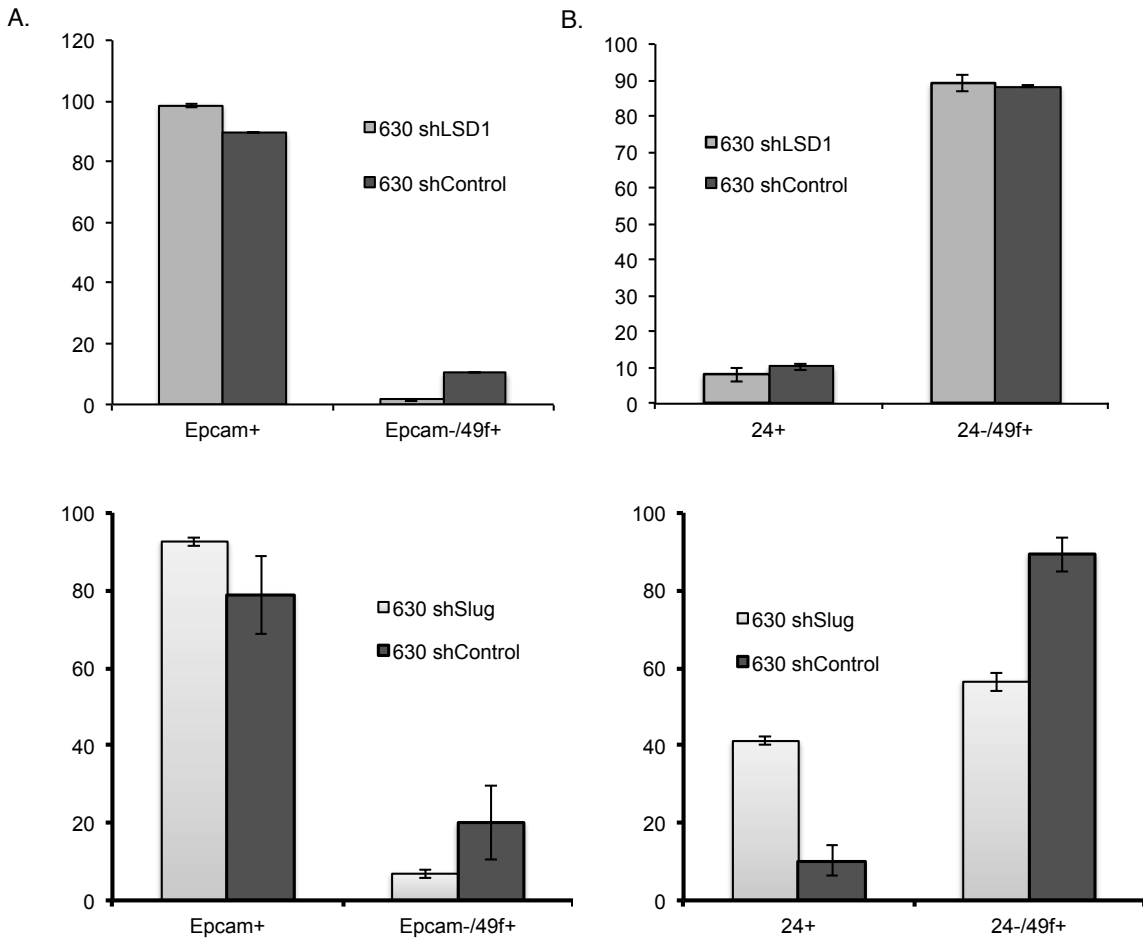


Figure 3.6: Cell surface staining of shLSD1 and shSlug HME630s using CD24, Epcam and CD49f followed by cell sorting analyses.

shLSD1 and shSlug HMECs were stained with CD24, Epcam and CD49f. Cells were then sorted into luminal and non luminal populations (luminal: $CD24^{+}/EpCAM^{+}/CD49f^{+)}$, basal: $CD24^{-}/EpCAM^{-}/CD49f^{+}$ and stem: $CD24^{-}/EpCAM^{+}/CD49f^{+}$. Measurements are averages of 3 independent experiments. In collaboration with Sarah Phillips.

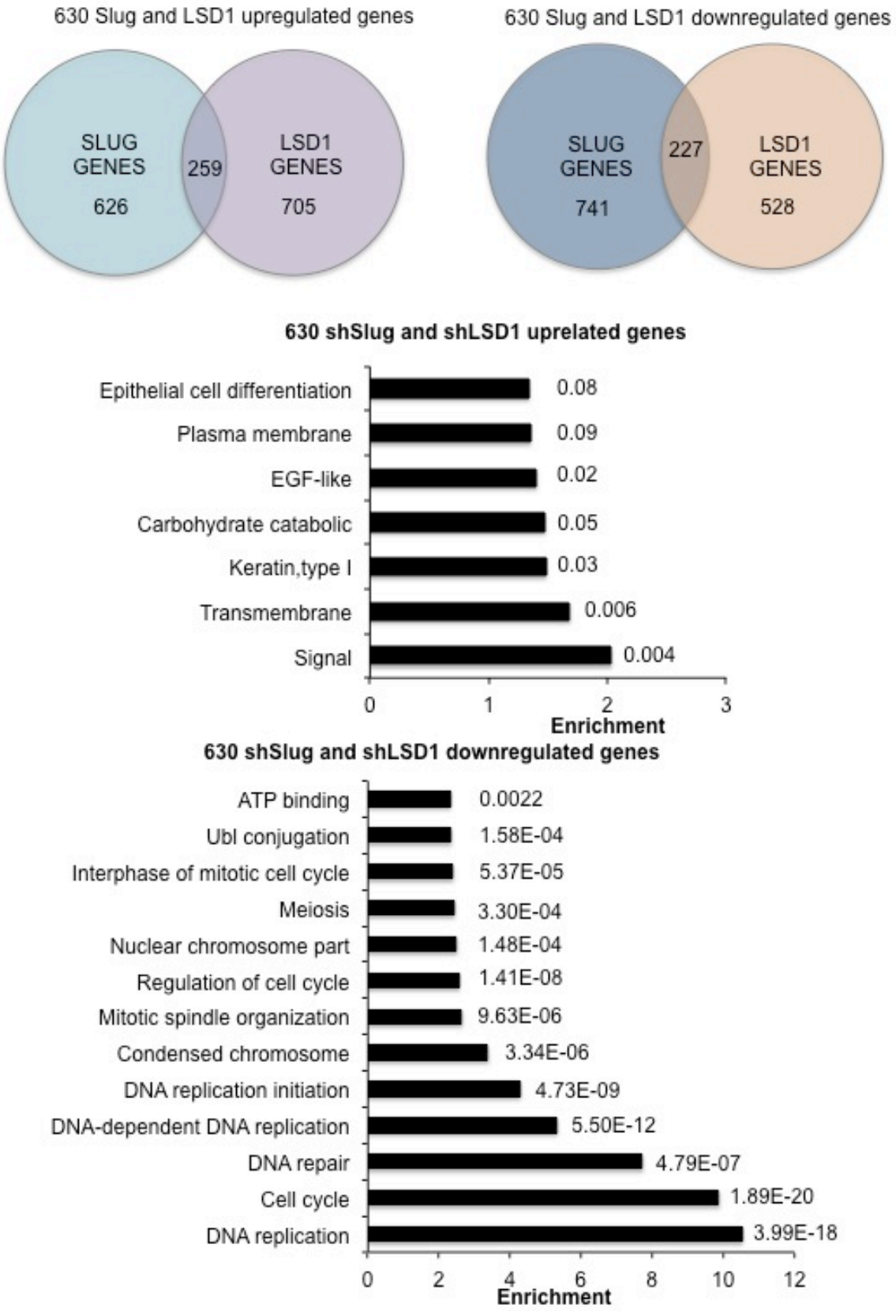

630 Slug and LSD1 upregulated genes
SLUG GENES
626
259
LSD1 GENES
705
630 Slug and LSD1 downregulated genes
SLUG GENES
741
227
LSD1 GENES
528
630 shSlug and shLSD1 uprelated genes
Epithelial cell differentiation
0.08
Plasma membrane
0.09
EGF-like
0.02
Carbohydrate catabolic
0.05
Keratin,type I
0.03
Transmembrane
0.006
Signal
0.004
0
1
2
3
Enrichment
630 shSlug and shLSD1 downregulated genes
ATP binding
0.0022
Ubl conjugation
1.58E-04
Interphase of mitotic cell cycle
5.37E-05
Meiosis
3.30E-04
Nuclear chromosome part
1.48E-04
Regulation of cell cycle
1.41E-08
Mitotic spindle organization
9.63E-06
Condensed chromosome
3.34E-06
DNA replication initiation
4.73E-09
DNA-dependent DNA replication
5.50E-12
DNA repair
4.79E-07
Cell cycle
1.89E-20
DNA replication
3.99E-18
0
2
4
6
8
10
12
Enrichment

Figure 3.7: Microarray and Gene Ontology analyses of shLSD1 and shSlug in patient derived epithelial cell line, HME630.

(A) Number of shLSD1 and shSlug target genes upregulated and overlap between them (B) Number of shLSD1 and shSlug target genes downregulated and overlap between them (C) Gene Ontology analyses for upregulated and downregulated genes in shLSD1 and shSlug HMEs. Graphical representation of significant GO categories in upregulated and downregulated common LSD1/Slug target genes. Data provided by Sarah Phillips.

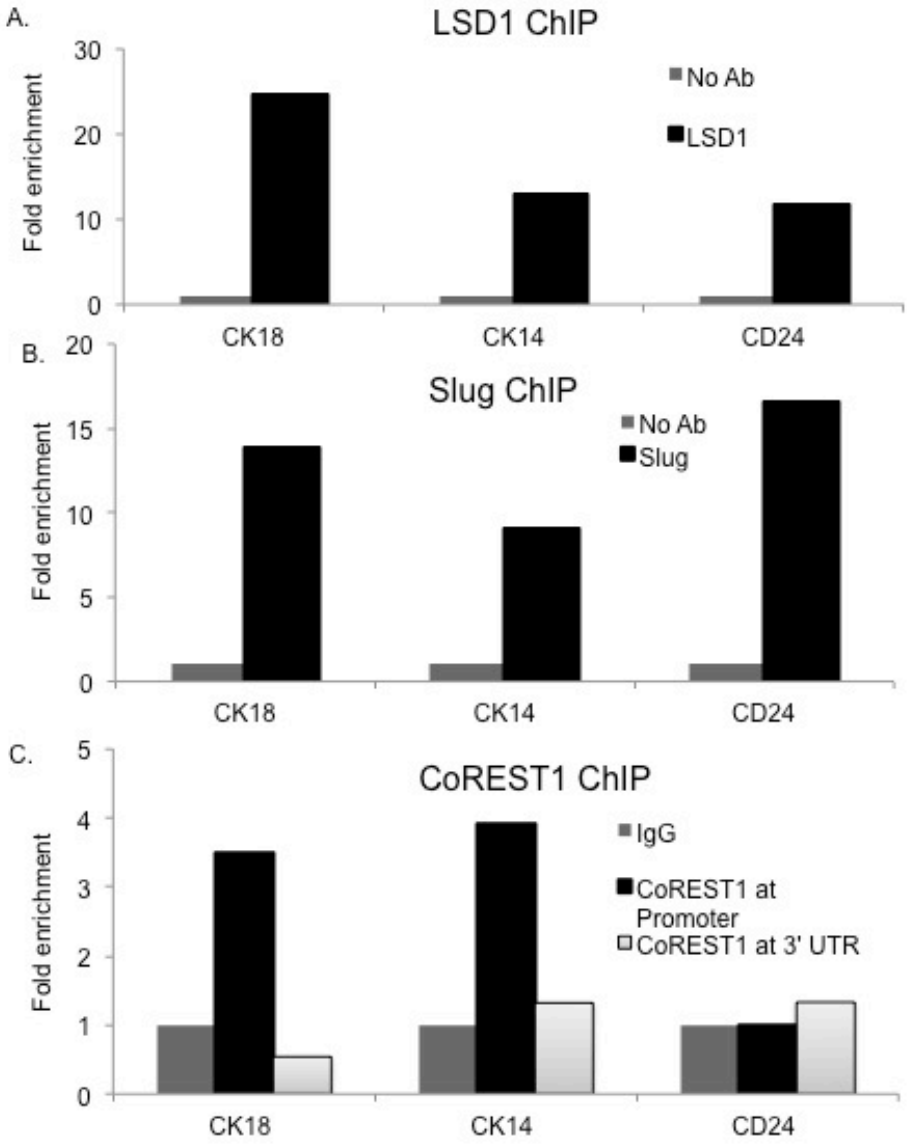


Figure 3.8: The LSD1/Slug complex is directly recruited to common target genes in patient-derived HME636s.

Chromatin immunoprecipitations were carried out as described in Chapter V. Crosslinked sonicated lysates were incubated with LSD1, Slug and CoREST1 antibodies or IgG and purified DNA was used in qPCR reactions. Fold enrichment

was calculated by normalizing values relative to % input and then setting signal for IgG at 1.0. (A) LSD1 ChIP (B) Slug ChIP (C) CoREST1 ChIP at target genes, CK18, CK14 and CD24. In collaboration with Sarah Phillips.

3.4 Discussion

Our results augment the understanding of the importance of Slug in mammary epithelial differentiation and expand our knowledge on the role of LSD1 and Slug in maintaining undifferentiated or basal like cell states.

3.4.1 Slug and LSD1 in development

The role of Slug has been described in mammary gland development as well as tumor progression, but most of the developmental studies to date have been done in MCF10A cell culture models (Lindeman and Visvader, 2011; Proia et al., 2011). Evidence suggests a role for Slug in mammary epithelial differentiation and tubulogenesis as it regulates mammary cell proliferation, apoptosis and migration (Come et al., 2004; Nassour et al., 2012; Proia et al., 2011). Slug is localized to the proliferating basal niche during mammary gland development and is expressed in basal/mammary stem cells (Guo et al., 2012; Nassour et al., 2012). A previous report by Nassour et al, 2012 and our own data support the hypothesis that Slug functions to restrict epithelial cell differentiation.

As discussed in Chapter I; Section 1.4.1, LSD1 plays an important role in development and differentiation. In our current studies we have tried to elucidate a mechanism by which LSD and Slug control genes in the luminal differentiation program. The data presented here show that LSD1 and Slug have many common target genes, bind to common promoters and play similar roles in determining cell phenotype. Our data are consistent with previous reports, and suggest that

LSD1 plays a role in Slug mediated repression (Ferrari-Amorotti et al., 2013; Wu et al., 2012).

3.4.2 Mechanism for LSD1 mediated Slug repression

Slug is essential for recruitment of LSD1 at Slug target promoters:

Our findings, as well as recent data from other labs, support the view that Slug bound to specific promoter elements (E boxes) recruits LSD1 to repress gene expression. LSD1 binds directly to the SNAG domain of Snail and Slug and contributes to repression of luminal genes like E-cadherin (Lin et al., 2010a). LSD1 is also essential for Snai-mediated repression of E-cadherin because it facilitates H3K4 demethylation that promotes recruitment of histone methyltransferases G9a and Suv39H1 and H3/H4 deacetylation that can facilitate binding of PRC2 to methylate H3K27 (Lin et al., 2013). Depletion of LSD1 in MCF10A cells results in partial derepression of the E cadherin promoter, highlighting the importance of LSD1 in Snai-mediated transcriptional regulation (Lin et al., 2010a).

LSD1 and Slug activity also mediate repression of the BRCA1 promoter, and high levels of Slug correlate with low levels of BRCA1 expression in basal tumors (Wu et al., 2012). Our collaborators have confirmed by ChIP that LSD1 and Slug occupy the proximal promoters of luminal epithelial genes such as EpCAM, CDH1 and MUC1 in MCF10A cells. Importantly, they found that LSD1

was no longer recruited to the promoters of these genes in the absence of Slug (data not shown) (Phillips et al, in preparation).

Role of LSD1 enzymatic activity in Slug mediated repression:

Since we started exploring the role of LSD1 and Slug in breast cell biology, other groups have identified that blocking Slug-LSD1 interactions using LSD1 inhibitors, Parnate or a competitive inhibitor peptide, TAT-SNAG, inhibited Slug dependent functions like derepression of E-cadherin, motility and invasion but not proliferation of cancer cells. LSD1 inhibition increased expression of genes associated with epithelial differentiation and structure like occludin and desmoplakin and; congruent with our own data, downregulation of mesenchymal markers like vimentin (Ferrari-Amorotti et al., 2013). Evidence also suggests that LSD1 inhibitors like PG1114 lead to global changes in histone demethylation as well as changes in gene expression in in vitro cell based assays (Zhu et al., 2012). While 30% of the genes tested were both Slug and LSD1 targets, there is a large group of LSD1 genes that are Slug independent and vice versa, suggesting that other transcription factors involved in epithelial cell biology recruit LSD1 to regulate promoters. Together, these data support a model in which Slug binding to promoters can recruit LSD1 and result in repression of luminal genes. Examining changes in histone modifications including H3K4me1/2 and H3K9me1/2 at Slug/LSD1 target genes like E-cadherin, CK18 and Muc1 will provide evidence that LSD1 is both physically as well as enzymatically important for Slug mediated gene expression.

3.4.3 LSD1 and Slug in cell fate determination

Developing breast epithelium expresses high levels of Slug and reports discussed in section 3.4.1 highlight the importance of Slug in mammary morphogenesis. Slug also plays a critical role in cell fate decisions, demonstrated by the fact that overexpression of Slug in luminal epithelial cells converted luminal progenitor cells, but not differentiated cells to form mammary stem cells with long term self renewing capability (Guo et al., 2012). Consistent with the published literature, results from the Gene Ontology and microarray analyses using shLSD1 and shSlug HMECs (Figure 3.5) indicate upregulation of epithelial differentiation genes, with a simultaneous downregulation of basal/stem differentiation genes suggesting a broader role for LSD1 and Slug in epithelial cell biology beyond mammary gland formation (Phillips et al., in preparation). These changes in gene expression were consistent with the observed increase in $EpCAM^{hi}/CD49f^{-}$ luminal cells, decrease in the proportion of $EpCAM^{-}/CD49f^{+}$ basal cells and decreased basal populations after LSD1 and Slug knockdown (Figure 3.5 and 3.6). Additionally, 15.1% of the genes that were down regulated in the shLSD1 samples were also downregulated in shSlug cells, suggesting that LSD1 and Slug have a direct of indirect activator function at these promoters. More experiments will be needed to identify if this gene activation requires LSD1 and Slug to function in a single complex, or if they are in separate complexes but acting in co-ordination.

Genetic deletion of Slug in a tissue specific manner in mammary glands using a conditional knockout mouse model showed increased levels of luminal marker, EPCAM in histological studies consistent with observations made in cell culture (Phillips et al., in preparation). These studies revealed an increased number of EpCAM+ luminal cells in Slug-/- mammary glands. Cell transition state modeling studies suggest that Slug is responsible for maintaining basal cell state, and loss of Slug results in reprogramming of basal cells into more luminally differentiated cells, with no changes in stem cell populations (Phillips et al., in preparation). Collectively, our data thus far indicate a mechanistic role for LSD1 in Slug-mediated maintenance of basal cell states.

Chapter IV

Discussion and Future Directions

In this work, we have investigated the role of CoREST1 in tumor/stroma interactions, specifically tumor angiogenesis and inflammation. We have also explored the role of LSD1 in breast epithelial cell biology. Our findings support a pro-tumorigenic role for CoREST1 in breast cancer cells and describe an LSD1/Slug protein complex that represses luminal genes and acts to influence cell fate. These findings have important implications and raise additional questions, some of which are addressed below.

4.1 Biological role for CoREST1 in tumor angiogenesis and the tumor microenvironment

Our findings reveal an unanticipated biological role of CoREST1 in interactions between the tumor and its microenvironment, including tumor angiogenesis and tumor mediated inflammatory responses. Reduced expression of CoREST1 in tumor cells has a striking influence in non-cell autonomous angiogenic and inflammatory interactions with the surrounding tumor microenvironment. While CoREST1 has been shown to be important in developmental pathways including hematopoeitic and neuronal lineages (Andres et al., 1999; Ballas et al., 2001; Ballas et al., 2005; Saleque et al., 2007), further in vivo studies and animal models are needed to fully understand the biological role of CoREST1 in development. Our study is the first to indicate a biological role for CoREST1 in tumorigenesis and tumor/stroma interactions. Our cell culture and in vivo data significantly add to the understanding of CoREST1 beyond its described biochemical functions.

In addition to the effects on pro-angiogenic and pro-inflammatory genes, we found that knockdown of CoREST1 in two basal breast cancer cell lines, MDA MB 231 and SUM149 showed a signature of upregulation of luminal genes and downregulation of basal genes (Supplementary Figure 2). Interestingly, a study that comprehensively analyzed gene expression patterns of basal (CD44+) versus luminal (CD24+) cells sorted from breast epithelial tissue isolated during reduction mammoplasty and primary breast tumors showed differences in expression of genes important in angiogenesis and inflammation in addition to previously described differences in basal and luminal genes (Shipitsin et al., 2007). Notably, these distinct cell populations showed significant differences in gene expression levels of targets such as VEGF, IL8, MCP1 and PEDF suggesting that gene expression programs of cells that favor a basal state promote a pro-angiogenic and pro-inflammatory environment in normal breast epithelia as well as breast tumors (Shipitsin et al., 2007). In our own work we observe that depletion of CoREST1 in MDA MB 231s show upregulation of luminal genes and downregulation of basal genes, in addition to downregulation of angiogenic and inflammatory genes (Figure 2.6 and Supplementary Figure 2).

Together, these results support the idea that CoREST1 complexes that regulate gene expression in normal developmental processes may be responsible for tumor mediated angiogenesis and inflammation. We hypothesize that CoREST1 complexes that maintain a basal cell state also contribute to pro-angiogenic and pro inflammatory gene profiles. We further hypothesize that cell

fate dictates the nature of tumors that arise in the event of a tumorigenic event and that basal cancers are more aggressive and have poor prognosis because the basal cell state favors pro- angiogenic and pro-inflammatory gene expression programs, and CoREST1 plays a role in these programs. Our work shows that LSD1 regulates basal/luminal fate in human mammary epithelial cells suggesting that LSD1 may play role in regulating angiogenic and inflammatory programs too.

Further, there is evidence to indicate that inflammatory processes contribute to proliferation and survival of tumor cells, increased angiogenesis and resistance to chemotherapeutics. Additional tumor mediated inflammation can result in genomic instability through accumulation of inflammatory mediators resulting in random genetic alterations, providing tumors with further survival advantage (Colotta et al., 2009). Tumors secrete chemokines that recruit tumor associated macrophages. Infiltration of macrophages is associated with neoangiogenesis and poor prognosis (Balkwill et al., 2005; Murdoch et al., 2008). Since our observations show significant changes in inflammatory mediators as well infiltration of macrophages, an important future direction will be further exploration on the biological and mechanistic role of CoREST1/LSD1 in mediating tumor inflammatory responses.

4.1.1. Nature of CoREST1 containing complexes

Current studies indicate that the biological activity of CoREST1 depends on CoREST1 interaction in larger complexes, but it is not clear if the full range of

multiprotein complexes containing CoREST1 has been described. The current literature describes several flavors of CoREST1 containing complexes that have been discussed in detail in Chapters I and II. Our data support the idea of cell type specific complexes that play distinct roles in tumor biology and breast cell differentiation. Appendix 1 lists factors identified in CoREST1 complexes in our studies, some of which have been previously identified. A majority of the complexes described thus far have been identified in biochemical assays, either in 293T or HeLa cells. While these studies bring to light valuable information on interacting partners, they have certain caveats, one of which is that they do not account for the cell type specific formation of complexes. For example, CoREST1 interacts with HDAC2 but not HDAC1 in cortical neurons demonstrating specific interactions in different cell lineages and how this specificity contributes to different physiological outcomes (Guan et al., 2009). This cell type specificity is suggested in our own work, where LSD1 is found in Slug-containing complexes with CoREST3 but not CoREST1 in HMEs (see section 4.2.1). However, we would not have been able to dissect out these specific interactions based on our affinity purification studies in 293T cells alone.

Notably, CoREST1 plays diverse roles in these complexes including functioning as a scaffold, regulating localization and as well as activity of cofactors and, depending on cellular context, can result in a variety of physiological outputs. The biochemical functions of CoREST1 range from mediating tissue specific gene expression (Abrajano et al., 2010; Guan et al.,

2009) to associating with oncogenic transcription factors such as ZNF217 (Banck et al., 2009). Based on the composition of the core complexes identified, CoREST1 complexes are widely implicated in epigenetic regulation. Biochemically, CoREST1 acts as a molecular beacon to recruit histone-modifying complexes by direct interaction (Ouyang et al., 2009) or by indirectly coordinating chromatin_complexes with different activities by binding to scaffolds like non-coding RNA HOTAIR (Tsai et al., 2010). Other biochemical functions include regulating the stability and enzymatic activities of associated epigenetic enzymes, as shown for both LSD1 and HDACs (Lee et al., 2005; Ouyang et al., 2009; Shi et al., 2005).

Since CoREST1 does not have DNA binding activity, we sought to identify DNA binding transcription factors that interact with CoREST1 to gain a better understanding of the biological activities of CoREST1. We performed double affinity purification mass spectrometry analysis of CoREST1 associated factors in 293T cells (Appendix 1). Our analyses identified DNA binding factors including TRIM28/KAP1, RREB1, ZNF516 and enzymes such as PARP1. Additional experiments are needed to explore the role of these interactors in CoREST1 mediated biology. Gene expression analyses to identify CoREST1 target genes will help to further identify already known protein complexes and transcription factors that regulate them. These gene expression profiles and CoREST1 interactors are likely cell type specific, hence, the cells for these analyses should be chosen accordingly.

4.1.2 CoREST1 complexes in angiogenesis

While our studies described in Chapter II revealed a biological role for CoREST1 in tumor angiogenesis and the microenvironment, the nature of the CoREST1 complex that mediates this function, and the mechanism by which this occurs remains to be elucidated . As discussed above, the Shipitsin et al study supports the hypothesis that CoREST1 mediated gene expression important in developmental angiogenesis may also play a role in tumor mediated angiogenesis and inflammation (Shipitsin et al., 2007).

Biochemically, the role of CoREST1 in tumor/stroma interactions likely requires CoREST1 dependent recruitment of histone modifying enzymes (notably LSD1 and/or HDAC1/2). Analyzing histone marks including H3K4me1/2 and H3K4ac at specific CoREST1-regulated promoters such as VEGF, MCP1 and PEDF will test this model. Increased levels of these histone modifications in the CoREST1 depleted cells would imply that CoREST1 complex associated epigenetic modifications are responsible for the changes in gene expression at these promoters. ENCODE database analyses in other cell lines such as K562 show increased density of histone marks including H3K4me3 and H3K27Ac at the VEGFA promoter. These data mining analyses can allow for fine-tuned querying at specific promoters allowing us a better understand the chromatin marks and therefore understand the epigenetic enzymes regulating these promoters.

There are several reports that suggest that HDACs may be involved in angiogenesis. For example, the HDAC inhibitor TSA reduced both basal and hypoxia-induced HIF-1α protein accumulation but not HIF-1α mRNA levels, and both protein and mRNA levels of VEGF in cancer cells (Kang et al., 2012). Second-generation HDAC inhibitors also resulted in downregulation of HIF-1α and HIF-1α-related genes in MDA MB 231 cells and displayed antiproliferative effects that were more pronounced under hypoxic conditions (Naldini et al., 2012). These reports suggest that high levels and/or activity of HDACs support hypoxia driven gene expression.

On the other hand, studies also suggest that some classes of HDACs such as HDAC 2, 3 and 5 may repress expression of angiogenic genes. For example, HDAC2 and HDAC3 are recruited to the VEGF promoter by transcription factor KLF4 to suppress VEGF expression and HDACs 1, 2 and 3 are differentially expressed in different molecular subtypes of breast cancer suggesting a role for these factors in cell fate determination (Muller et al., 2013; Ray et al., 2013).

There is one study that describes a correlation between LSD1 levels and VEGFA downregulation in prostate cancer. Depletion of LSD1 in prostate cancer cells led to reduction in VEGF mRNA levels, similar to what we observed (Figure 2.6). Interestingly, these results were not recapitulated by LSD1 inhibitor treatment suggesting that the role of LSD1 in VEGF regulation may extend beyond its enzymatic activity. The nature of this LSD1 complex and how it

regulates VEGFA, the clinical relevance of this positive correlation in prostate cancer and the nature of other LSD1 regulated angiogenesis genes are not addressed in this study, but definitely merit investigation (Kashyap et al., 2013). In contrast, another study in MCF7 cells suggested that the LSD1/NuRD complex maybe important in suppressing the angiogenic response via suppression of CCL14 (Li et al., 2011). Thus, there are conflicting reports concerning role of LSD1 in tumor angiogenesis and additional studies are required to determine whether CoREST1 may be functioning together with or independently of LSD1 in playing a pro-angiogenic role.

CoREST1 depletion in MDA MB 231s did not show global changes in H3K4me2 levels suggesting that global LSD1 activity levels were not compromised on depletion of CoREST1 in these cells (Figure 4.1). Examining histone marks at specific promoters will better help identify the epigenetic factors that CoREST1 co ordinates at these promoters. Coimmunoprecipitation of CoREST1-LSD1 endogenous complexes in MDA MB 231s were unsuccessful. CoREST1 pulldowns did not show presence of LSD1 in immunoblots and while LSD1 IPs showed the presence of CoREST1 (protein at the predicted molecular weight for CoREST1) we were not able to obtain successful immunoblot data for LSD1 (data not shown). Immunoblotting of whole cell lysates for CoREST1 and LSD1 demonstrated that the antibodies used in the CoIP experiments were able to recognize the targeted proteins. Together, this could imply that (1) the total amount of LSD1 bound CoREST1 in MDA MB 231 cells is very low (2) LSD1-

CoREST1 complexes are transient and need to be purified under special conditions, (3) LSD1-CoREST1 complexes may form only after the cells are exposed to certain stimuli (for example, hypoxia).

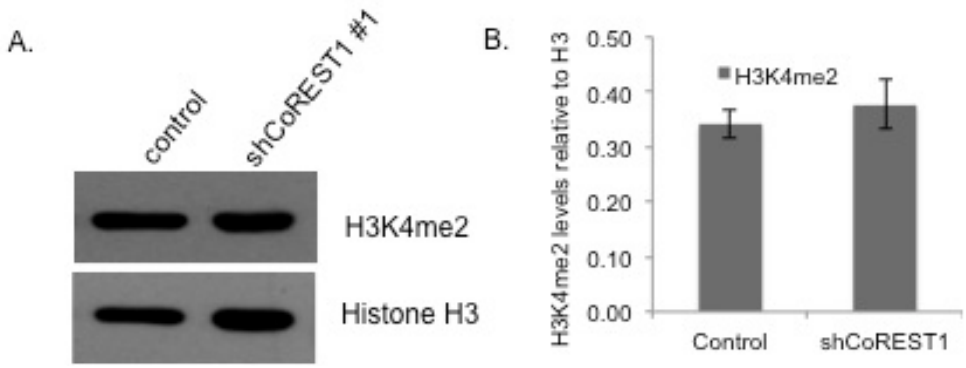


Figure 4.1: Global H3K4me2 levels do not change upon CoREST1 depletion in MDA MB 231 cells.

(A) Histones were extracted from control and shCoREST1-MDA MB 231 cells as described in Chapter V and 5 ug of histone extracts were analyzed using SDS PAGE (15% gel). (B) Quantitation of H3K4me2 signal relative to histone H3 levels (loading controls) using ImageJ software. Results are an average of 3 independent histone extraction experiments.

Additionally, as discussed in Chapter II, the CoREST1 complex could influence expression of angiogenic and inflammatory genes by direct or indirect mechanisms (for models see Figure 4.2). Mechanistic studies and microarray analyses will help elucidate the mechanism by which CoREST1 regulates the expression of these genes. CoREST1 may function as an activator by directly binding to the regulatory regions of angiogenic and inflammatory genes whose expression is reduced upon CoREST1 depletion.

While our current data does not provide evidence supporting a CoREST1-LSD1 activator complex in tumor angiogenesis, we have no hard evidence to disprove this either. Though the proliferation defect upon knockdown of LSD1 in MDA MB 231 cells prevented us from generating stable shLSD1 cells, additional studies using short-term depletion with siRNA or inhibiting LSD1 activity and assaying the cells for the expression of the candidate genes tested in Figure 2.6 may be informative. CoREST1 may also recruit novel DNA binding factors specifically important in the context of tumor angiogenesis and inflammation. ChIP analyses will elucidate if the altered angiogenic and inflammatory genes are direct CoREST1 targets. Furthermore, it is important to note that CoREST1 may be recruited to these promoters under certain conditions only. For example, we know that HIF1a occupancy at VEGF is hypoxia dependent; it is possible that CoREST1 is robustly recruited to the VEGF promoter only under hypoxic conditions.

Genome wide gene expression analyses of control and shCoREST1 MDA MB 231 cells will also give us a more holistic and unbiased view of CoREST1 mediated effects on gene expression, informing us of additional CoREST1 dependent genes that may serve as intermediate activators or repressors to angiogenic and inflammatory genes, as well as additional potential angiogenesis and inflammatory genes that may be important in vivo. These results will help elucidate the CoREST1 complexes and mechanisms involved in tumor angiogenesis as well as inflammation.

4.1.3 Role of CoREST1 in tumor angiogenesis and beyond

Gene expression and Gene Ontology analyses will give us an unbiased and broader view of CoREST1 regulated genes, which can then help tease apart the links between cell fate, angiogenesis, tumor mediated inflammation and the hypoxic response. Further, there is a need for animal models that can help recapitulate the functional roles of CoREST1 in tumor biology. Histological and biochemical analysis of different molecular subtypes of human tumors will shed further light on CoREST1 expression levels, localization within the tumors etc. In addition to more sophisticated animal models, studies on CoREST1 in different breast cancer cell lines and other tumor types will inform us whether these CoREST1 dependent changes in angiogenesis genes that we observed are specific to MDA MB 231s or breast cancer cell lines. It would also be interesting to explore the role of CoREST1 in the tumor stroma itself to see if it has any autocrine functions in the stroma.

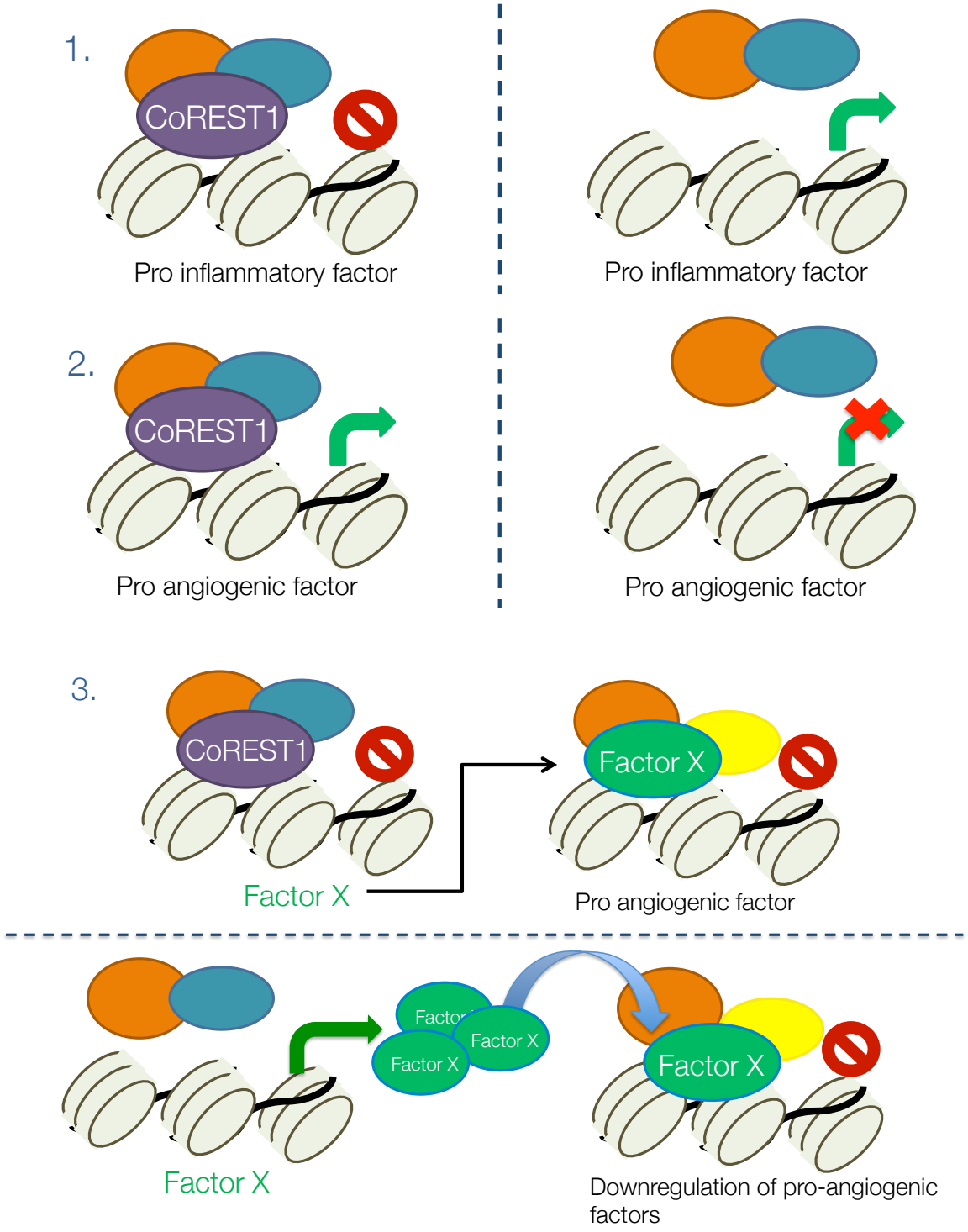


Figure 4.2: Different models of CoREST1 mediated gene expression.

Our current data support these models. (A) Model for CoREST1 as a direct activator. (B) CoREST1 as an indirect activator (by acting as an activator of an

activator) of pro-angiogenic, anti-angiogenic genes and pro-inflammatory genes. (C) CoREST1 as an indirect activator (by acting as a repressor of a repressor) of pro-angiogenic, anti-angiogenic genes and pro-inflammatory genes.

4.1.4 Role of CoREST1 in hypoxia

Our studies have revealed a role for CoREST1 in tumor angiogenesis. Solid tumors adapt in response to hypoxia by altering their angiogenic response, metabolism and survival or cell death. HIF1a is a master regulator of oxygen sensing and response to hypoxia and can promote tumorigenesis by facilitating neoangiogenesis, cell motility and invasion (Rankin and Giaccia, 2008). Increased levels of HIF1a activity are commonly observed in many cancers, via enhanced transcription and translation of HIF1a or increased stability and activity of HIF1a protein. The fact that different mechanisms by which cancers activate HIF and that HIF target genes are involved in angiogenesis, makes the HIF pathway a viable therapeutic target (Pugh and Ratcliffe, 2003). Experiments in which HIF1 was genetically ablated or inhibited in cancer cells resulted in a marked reduction in tumor angiogenesis and growth. (Hopfl et al., 2002). Since our xenograft experiments with CoREST1 knockdown demonstrated a similar phenotype, we hypothesize that CoREST1 plays a role in mediating the effects of HIF1a. We did not observe a significant and reproducible change in HIF1a mRNA or protein levels upon CoREST1 knockdown, signifying that CoREST1 does not alter HIF1 transcription or translation (Figure 2.8); however effects of CoREST1 knockdown on HIF1 stability still remain to be tested and it would be worth comparing HIF1 levels in CoREST1 KD cells in conditions of hypoxia as well. Additionally, CoREST1 could be affecting HIF1a localization or HIF1a

posttranslational modifications such as hydroxylation, both of which need to be investigated in our model system.

One of the ways in which HIF1a can regulate neoangiogenesis is by directly regulating a key angiogenic response gene, VEGFA (Cascio et al., 2010; Semenza, 2000). High expression levels of HIF1a and VEGF are associated with more aggressive tumors (Jensen et al., 2006); not only can HIF1a directly activate VEGF but it can also activate transcription of VEGFR-1 (Forsythe et al., 1996). VEGF is indispensable during normal development established by the fact that loss of VEGF in knockout mouse models is embryonic lethal (Ferrara et al., 1996). Inhibition of Flt-1/ VEGF/ autocrine signaling by abrogating HIF1a activity resulted in decreased in vitro endothelial cell proliferation and tube formation and significantly reduced in vivo tumor angiogenesis and reduced tumor growth (Tang et al., 2004). Together, these studies highlight the role of the HIF1a regulated VEGF in tumor angiogenesis.

Studies with glioblastoma xenografts suggest that VEGF upregulation can both be HIF1a dependent as well as independent (Hendriksen et al., 2009). It would be interesting for us to examine protein lysates from our own xenograft studies and determine levels of VEGF expression. RNA analysis from these tumors (control and shCoREST1-231) will also give us a clue if VEGF mRNA levels are altered in shCoREST1-derived tumors, potentially shedding light on the role of CoREST1 in HIF1a mediated regulation of angiogenesis. Preliminary CoIP data suggest that HIF1a and CoREST1 may be part of a protein complex, but

these interactions need to be further verified (Figure 4.3). If this can be confirmed, additional studies on the composition of the complex and ChIP studies to look at promoter occupancy would further identify the mechanism by which HIF1a interacts with CoREST1 to modulate gene expression of HIF1a target genes like VEGF and MCP1.

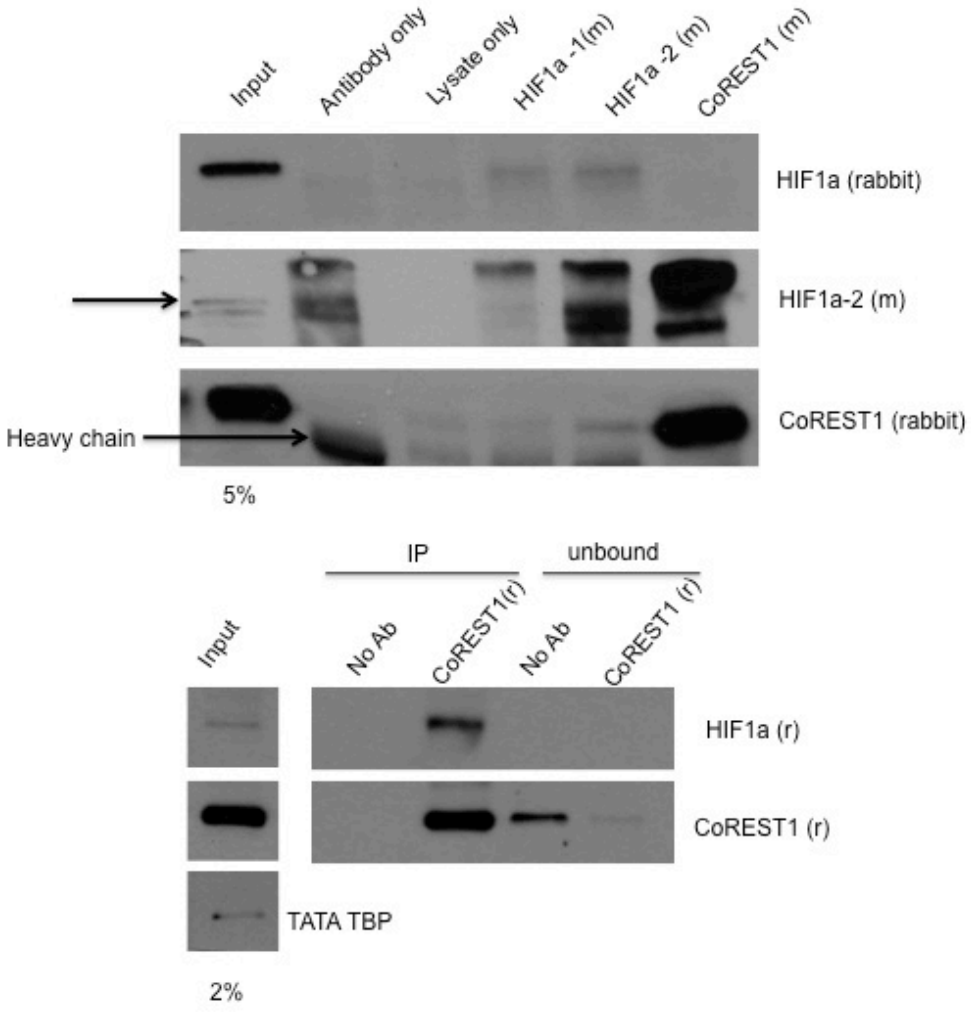


Figure 4.3: Endogeneous coimmunoprecipitations (CoIPs) from MDA MB 231 nuclear extracts suggest that CoREST1 and HIF1a may interact.

Nuclear lysates were used in CoIPs and input was loaded as described. 1 mg of nuclear lysate was used in each IP. No antibody (beads only) or IgG was used as negative control as indicated. Mouse antibodies were used in IPs and rabbit or mouse antibodies were used in immunoblotting as described. Top panel shows reverse CoIPs with CoREST1 and HIF1a (rabbit and mouse antibodies used as

indicated). Rabbit HIF1a blots show a light band at the appropriate molecular weight. The signal to noise ratio with the mouse HIF1a utilizing mouse antibodies in the IP reactions prevents a meaningful conclusion. Bottom panel demonstrates an interaction between CoREST1 and HIF1a, however the lack of an appropriate IgG control does not allow for a conclusive result.

Previous ChIP studies indicate that HIF1a is directly recruited to the VEGFA promoter hypoxia responsive element in breast cancer cells (Forsythe et al., 1996). CoREST1 ChIP at the VEGF promoter, and HIF1a/CoREST1 ChIPs at additional CoREST1 target genes will help elucidate if CoREST1 and HIF1a occupy similar promoters, lending support to the existence of CoREST1/HIF1a complex. Microarray analysis of shCoREST1 MDA MB 231 cells under hypoxic conditions will reveal how CoREST1 affects the hypoxic response, and if there is a large overlap between HIF1a and CoREST1 target genes.

4.1.5 Angiogenic therapies and the future

Although anti-angiogenic therapies are promising they have not been fully successful in increasing disease free survival in breast cancer. Resistance to anti-angiogenic therapies can occur because of redundancy of biological systems; when one pathway is abrogated, often another signaling pathway can compensate. For example, anti VEGFR-2 therapies failed in the clinic because factors like FGF and PDGF compensated for the loss of VEGF signaling (Casanovas et al., 2005; Fernando et al., 2008). This acquired resistance can be partially overcome by targeting downstream signaling nodes like mTOR and MAPK where multiple pathways converge (Weis and Cheresh, 2011). Additionally, tumor-associated endothelial cells can secrete pro-angiogenic growth factors to compensate for factors that are downregulated by anti-angiogenic therapies, further lending support to the idea that combination therapies are more likely to succeed than monotherapies (Johannessen et al.,

2013). For example, studies show that inhibiting another histone demethylase, JMJD1A along with bevacizumab and sunitinib treatment further enhanced their therapeutic activity (Osawa et al., 2013).

In early studies, anti-angiogenic therapies in breast cancer demonstrated improved response rates and progression free survival, but clinical trials showed no improvement in overall survival compared to chemotherapy alone. Bevacizumab (Avastin®), a monoclonal antibody against VEGFA, decreased risk of disease progression in breast cancer (Miller et al., 2007), but did not improve overall survival, which is a gold standard in metastatic breast cancer, causing it to be discontinued as a treatment of metastatic breast cancer by the FDA. While the rationale for using anti-angiogenic therapies to treat breast cancer is strong, research still needs to be done to optimize the therapeutic indices of these drugs for more sophisticated and targeted therapies, with fewer side effects.

In addition, since VEGF is expressed in early stages of tumor development, anti VEGF treatments may be more beneficial in early stage disease, rather than metastatic disease, where tumors have already activated compensatory pathways for VEGF signaling (Miller et al., 2005; Relf et al., 1997). Further, in order for anti-angiogenic therapies to be effective it is important to consider other biomarkers that can help stratify patients. For example, a study retrospectively discovered that single nucleotide polymorphisms in the *VEGF* gene can predict patients who are more likely to clinically benefit (including overall survival) as well as those who are at risk for hypertension(Schneider et

al., 2008). Evidence also suggests that HER2 status and hormone receptor status can determine if patients will respond well to anti-angiogenic therapies (Alameddine et al., 2013). Clinical trials focused on patient stratification, understanding resistance mechanisms and combinatorial therapies including simultaneous inhibition of angiogenic targets like HIF1a seem to be the most promising approaches (Mackey et al., 2012). Thus, understanding regulators of angiogenesis and their mechanism of action and targets will help us create future generation combination angiogenic therapies with greater efficacy in promoting disease free as well as overall survival in patients. More specifically, understanding CoREST1 function and mechanism in angiogenesis could lead to therapies that specifically target tumor cells and not the surrounding stroma, with benefits like higher efficacy and fewer side effects.

4.2. The Slug-LSD1 histone modifier complex regulates epithelial gene expression programs

Our data demonstrate a coordinated role for LSD1 in Slug mediated repression of luminal genes. Our collaborators have found that LSD1 is not recruited to gene promoters in the absence of Slug, underscoring an essential role for Slug in recruiting an LSD1 containing histone-modifying complex to common Slug/LSD1 target genes. Together, LSD1 and Slug repress luminal genes, maintaining a basal cell state. Genetic deletions of Slug in mouse models support the role of Slug in basal cell fate determination, and molecular and

mechanistic data demonstrate that LSD1 is involved in this process of cell fate determination.

4.2.1. The Slug/LSD1 complex

Affinity purification analyses in 293T cells expressing tagged Slug protein identified LSD1 and CoREST1 as potential Slug interactors (Theresa Proia's thesis, data not shown). These results were confirmed by CoIP analyses of overexpressed Slug in 293T cells. Additional candidate binding partners CoREST3 and KAP1 were also identified in Slug affinity purification studies. Affinity purification analyses using tagged CoREST1 also identified LSD1 and KAP1 as CoREST1 interactors. Previous reports suggest that LSD1 is found in CoREST1 and KAP1 complexes and CoREST3 is part of the CoREST1 complex (Lee et al., 2005; Macfarlan et al., 2011).

Endogenous CoIP experiments demonstrated the presence of complexes that contain Slug, LSD1, KAP1 and CoREST3 in human mammary epithelial cells (Figure 3.4 A). In order to tease out if Slug formed a large complex with all of these factors or if it was in separate complexes, reverse CoIPs were performed (Figure 3.4 B and data not shown). LSD1 coimmunoprecipitated KAP1 and CoREST1 as expected, but not CoREST3. This may imply that LSD1-CoREST1 and LSD1-KAP1 complexes are more abundant in breast epithelial cells than LSD-CoREST3 complexes. Slug was able to pull down LSD1 and CoREST3, however, reciprocal IPs with LSD1 did not show presence of Slug, which maybe a stoichiometric issue where only a small fraction of total LSD1 present binds to

Slug in HMECs. No interactions were observed between CoREST1 and Slug in HMECs (data not shown) suggesting that CoREST3, which is closely related to CoREST1, may be present in a CoREST1 independent complex in HMECs. Since the factors we are studying are predominantly nuclear, using nuclear lysates in the IPs may increase the probability of detecting endogenous complexes. If the complexes being studied are transient, methods like crosslinking might aid in detection as well. Additional experiments will be required to identify the other proteins in the LSD1/Slug complex.

4.2.2. Linking the role of Slug/LSD1 in differentiation to cancer

The data thus far demonstrate that Slug and LSD1 co-operate to maintain cells in a basal cell state. As described earlier, high levels of LSD1 and Slug are found in tumors that are more undifferentiated, or basal like, and have poor prognosis (Lim et al., 2010; Proia et al., 2011), supporting the hypothesis of an LSD1-Slug complex that maintains basal or stem cell like state. Slug expression is necessary for increased basal-like phenotypes prior to and following neoplastic transformation. This is supported by the fact that cells from BRCA mut/+ patients, which express high levels of Slug, show downregulation of luminal genes (including various hormone-related genes including progesterone and estrogen beta receptors), and upregulation of genes associated with progenitor or basal cells (Proia et al., 2011).

In addition to being classified based on ER (estrogen receptor) status, tumors can be classified based on the cell surface markers they express, for

example, luminal tumors express CK18 and CK19 etc and basal tumors express Slug, CK14 and SMA. The type of cell determines the nature of the tumor i.e. cells from the luminal lineage that give rise to tumors with luminal characteristics, are less aggressive and have better prognosis; cells from the basal lineage give rise to tumors with basal characteristics and are more aggressive and refractory to treatment (Birnbaum et al., 2004).

Our data raises a number of interesting questions including:

1. Does loss of Slug protein cause an increase in luminal type tumors?
2. Does treatment with LSD1 inhibitors change the molecular phenotype of a basal-like tumor to luminal type?
3. Does LSD1 confer any functions in Slug mediated cell fate determination beyond histone modifying activities, such as, but not limited to, acting as a scaffold and aiding in protein stability of other complex members?

Taken together, these additional studies will advance understanding of the molecular and epigenetic pathways associated with breast epithelial biology and a potential role for LSD1 as an oncogene in basal type breast cancers.

Chapter V

Materials and Methods

5.1 Plasmids, cell lines and stable lines

MDA MB 231, SUM149 and 293T cells were from and cultured in conditions recommended by ATCC. HMECs were isolated as previously described (Proia, 2011) and cultured in MEGM (Lonza) supplemented with bovine pituitary extract (BPE), insulin (5 µg/mL), EGF (10 ng/mL) and hydrocortisone (1 µg/mL). These cells were immortalized with the catalytic subunit of human telomerase (hTERT).

The VSV-G-pseudotyped lentiviral vectors were generated by transient cotransfection of the vector construct with the VSV-G-expressing construct pCMV- VSVG (Miyoshi et al., 1998) and the packaging construct pCMV DR8.2Dvpr (Gupta et al., 2005; Miyoshi et al., 1998) generously provided by Inder Verma, into 293T cells together with FuGENE 6 transfection reagent (Roche). Lentiviral shRNA constructs targeting CoREST1 LSD1 and Slug (Sigma-Aldrich MISSION shRNA TRCN0000147958, TRCN0000418894; respectively) were prepared as previously described (Gupta et al., 2005). 293T cells were used to produce the viral particles using accessory plasmids that aid in viral replication. Target cells were exposed to virus for 16 hours followed by a 24-hour recovery period in native media. Finally, cells were subjected to puromycin selection (1 ug/mL puromycin) to get shRNA positive cells. Cells were periodically challenged with puromycin to ensure that the constructs remained integrated.

5.2 RNA and protein extractions

RNA was extracted using TRIzol or QIAzol followed by the Qiagen RNeasy® Mini kit according to manufacturer's protocol.

Protein extracts were made in RIPA buffer (unless otherwise specified) supplemented with protease cocktail inhibitors (Roche, cOmplete EDTA free, Cat No. 1187358000). Cells were resuspended in RIPA and incubated on ice for 30 minutes with intermittent vortexing. Lysates are then passed through Qiashredder spin columns 2-3x (at 14000 rpm) and then quantitated using Biorad DC Protein assay.

5.3 Histone assays

Cells were washed in PBS and suspended in 0.5% Triton X 100 (supplemented with protease inhibitors, sodium butyrate and PMSF) to lyse the cells. After multiple washes in Triton buffer, cells were extracted with 0.2 N HCl overnight at 4 C. Samples were pelleted and supernatant was subjected to TCA precipitation. Histones were precipitated in the pellet fraction, which was subjected to two acetone washes. After air-drying, the pellet was resuspended in sterile dH2O and quantitated using the Biorad DC Protein assay.

5.4 RT q-PCR and cDNA preparation

cDNA was prepared using the BioRad iScript cDNA kit as per manufacturer's protocols. 1 ug of RNA was used for a 40 ul cDNA reaction. qPCR was carried out with SYBR Green (BioRad) using a BioRad CFX96. For the list of primers used please refer to Table 4.

5.5 Western Blotting

Cultured cells were harvested by trypsinization, pelleted and incubated in RIPA buffer supplemented with protease and phosphatase inhibitors (Roche) to obtain whole cell lysates (or nuclear lysates, when applicable). Cellular debris was separated by centrifugation at 13,000rpm for 10min. 30-60µg of the whole cell lysate was used per sample. Western blotting was performed according to the manufacturer's protocol (BioRad). Briefly, 10% and/or 4- 15% gels (depending on the kDa size of the proteins) and 10X SDS running buffer were used for SDS-PAGE electrophoresis. 0.2 µm nitrocellulose membrane was used for protein transfer. Membranes were incubated overnight at 4°C with primary antibodies diluted in 5% bovine serum albumin or milk in TBS-T. Secondary antibodies were applied for 1hr at room temperature. Antibodies used can be found in Table 3.

5.6 Immunoprecipitations

Cells were lysed in IP buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100) supplemented with protease and phosphatase inhibitors (Roche). For immunoprecipitation assays, protein lysates (800-1000 µg) were combined with appropriate amounts of antibody and 20 µl of Dynabeads A/G beads (Invitrogen; 10003D, 10001D). Following an overnight incubation at 4°C, magnetic beads were extensively washed in RIPA buffer, resuspended in SDS sample buffer (125 Mm Tris pH 6.8, 2.5% SDS, 10% glycerol, 2.5% 2-mercaptoethanol, 0.01% bromo-phenol-blue) and loaded on to a

protein gel. For antibodies, refer to Table 3.

5.7 Mammary fat pad injections and histology

1 X 10^6 cells were injected into the fourth fat pad of 8 to 10 week old NOD SCID mice. Animals were monitored over a period of 7-8 weeks and sacrificed when the size of their tumors reached a diameter of 2 cm. Tumor measurements were recorded approximately twice a week and used to generate a growth curve. Cell growths of greater than 3 mm were considered tumors. Tumor weights were also recorded. Excised tumor tissue was used for RNA and protein analysis along with paraffin embedding and cryofreezing for histological analysis.

5.8 Immunofluorescence

Frozen sections were thawed at room temperature, fixed and treated with 0.1% Triton X-100. Samples were incubated overnight at 4°C with primary antibodies diluted in 1.5% goat serum in PBS. Fluorescently labeled secondary antibodies were applied for 1 hr at room temperature. Cells were counterstained with DAPI. A Nikon Eclipse 80t microscope and SPOT camera were used for analyzing and photographing the stained sections. For antibodies refer to Table 3.

5.9 Semi quantitative angiogenesis arrays

We used a commercially available human angiogenesis antibody array (R&D Biosystems).

5.10 HUVEC assays

Tube formation: Conditioned media was collected from control and shCoREST1-231 cells by exposing them to basal serum free media (EBM +0.1% BSA) for a period of 16-18 hours. Conditioned media was when sterile filtered to remove debris and HUVEC cells were co-mixed with conditioned media and plated on a basement membrane. Cells were then observed over a period of 4-5 hours for tube formation with imaging carried out during the maximal tube formation window (4-4.5 hours). Positive controls included proangiogenic factors like FGF and serum free media was used as a negative control.

Proliferation: Conditioned media was collected from control and shCoREST1-231 cells by exposing them to basal serum free media (EBM +0.1% BSA) for a period of 16-18 hours. Conditioned media was when sterile filtered to remove debris. 50,000 HUVECs/ per well (of a 24 well plate) were seeded 6 hours prior to exposing them to conditioned media. Cells were allowed to proliferate for 72 hours and then counted using counted using the BioRad TC10 Cell Counter. Each condition was seeded in ten replicates; n=3 using conditioned media collected from three independent experiments; serum free media was used as a negative control.

Migration/wound healing: Conditioned media was collected from control and shCoREST1-231 cells by exposing them to basal serum free media (EBM +0.1% BSA) for a period of 16-18 hours. Conditioned media was when sterile filtered to remove debris. 300,000 HUVECs/ per well (of a 12 well plate) were seeded overnight. Confluent cells were wounded with a 20-200 ul pipette tip and washed

with PBS and imaged. Cells were exposed to conditioned media for 6 hours and imaged again. ImageJ software was used to calculate % wound healing. Each condition was seeded in ten replicates; n=3 using conditioned media collected from three independent experiments.

5.11 Proliferation assays

Cells were plated at the same density in multiple replicates and trypsinized and counted using the BioRad TC10 Cell Counter at 2, 4 and 6 days.

5.12 ChIP assays

Crosslinking and cell lysis: 1% formaldehyde in PBS was used to crosslink cells for 15 minutes with continuous shaking and quenched with glycine (final concentration: 0.125 M). Cells were rinsed in PBS and scraped off the plates. Pellets were resuspended in complete Szak's RIPA buffer (proteased and HDAC inhibitors added)

Sonication: Lysates were sonicated to result in DNA ranging from 200-600 bp (Branson Sonifier macro tip; 23 X 25s cycles with 1 min cooling between pulses; constant duty, output 0.4). Sonicated lysates were supplemented with Triton X-100 (final concentration 1%) and centrifuged at 12000 rpm (4 C) to pellet debris. Lysates are quantified and normalized to 1mg/ml in Szak's RIPA.

Immunoprecipitation: Lysates were pre cleared for 2 hours (4C) with 50% bead slurry (Protein G Sepharose Plus, Millipore). Bead slurry was prepared overnight

with 1mg/mL BSA and salmon sperm DNA. Lysates were incubated overnight with appropriate amount of antisera or non reactive IgG where applicable. Beads were added after overnight incubation of antisera and allowed to form complexes for 1 hour at 4C. Beads were then subjected to washes (2x RIPA, 4x Szak's IP wash buffer, 2x RIPA (all at 4 C) and 2x final washes in TE buffer.

Elution and DNA precipitation: Samples were eluted in 1.5x Talianidis Elution buffer at 65 C for 10 minutes and supplemented with NaCl (200 uM final concentration). Eluted complexes were reverse crosslinked overnight at 65C and then RNAse (37 C) and Proteinase K (55 C) treated. DNA was extracted using Phenol:Chloroform:Isoamyl alcohol (25:24:1) and precipitated with NaCl and ethanol at -80 C for 90 minutes. Pellets were washed in 70% ethanol and resuspended in RNAse free water. DNA samples were used in qRT PCR reactions.

5.13 Flow Cytometry and FACS

Nonconfluent cultures of MCF10A and immortalized HME cells were trypsinized into single-cell suspension, counted, washed with PBS, and stained with antibodies specific for human cell CD24 (PE), EpCAM (APC) (BD Biosciences), and CD49f (FITC). Antibody-bound cells were washed and resuspended at 1x 10^6 cells/ml in 2% FBS in PBS and run on a FACS Calibur flow cytometer (BD Biosciences) or sorted on a BD Influx FACS sorter (BD Biosciences). Flow cytometry data was analyzed with the Flowjo software package (TreeStar).

Table 1: Different classes of histone modifications (Kouzarides, 2007)

Different Classes of Modifications Identified on Histones		
Chromatin Modifications	Residues Modified	Functions Regulated
Acetylation	K-ac	Transcription, Repair, Replication, Condensation
Methylation (lysines)	K-me1 K-me2 K-me3	Transcription, Repair
Methylation (arginines)	R-me1 R-me2a R-me2s	Transcription
Phosphorylation	S-ph T-ph	Transcription, Repair, Condensation
Ubiquitylation	K-ub	Transcription, Repair
Sumoylation	K-su	Transcription

Table 2: Factors in the angiogenesis pathway

All factors in this table are altered after knockdown of CoREST1.

Factor	Role in angiogenesis
VEGF	Mediates increased vascular permeability, induces angiogenesis, vasculogenesis and endothelial cell growth, promotes cell migration, and inhibits apoptosis. Targeted in cancer therapies.
IL8	IL8 is member of the CXC chemokine family and a major mediator of the inflammatory response and is secreted by several cell types. It functions as a chemoattractant, and a potent angiogenic factor
SDF1a	This gene encodes a stromal cell-derived alpha chemokine member and along with its receptor CXCR4, can activate lymphocytes and has been implicated in the metastasis of breast cancer
GM-CSF	Cytokine that stimulates the growth and differentiation of hematopoietic precursor cells from various lineages, including granulocytes, macrophages, eosinophils and erythrocytes
CXCL16	Acts as a scavenger receptor on macrophages, which specifically binds to OxLDL (oxidized low density lipoprotein), suggesting that it may be involved in pathophysiology such as atherogenesis (By similarity). Induces a strong chemotactic response. Induces calcium mobilization
ANG	Binds to actin on the surface of endothelial cells; once bound, angiogenin is endocytosed and translocated to the nucleus. Angiogenin induces vascularization of normal and malignant tissues. Angiogenic activity is regulated by interaction with RNH1 in vivo

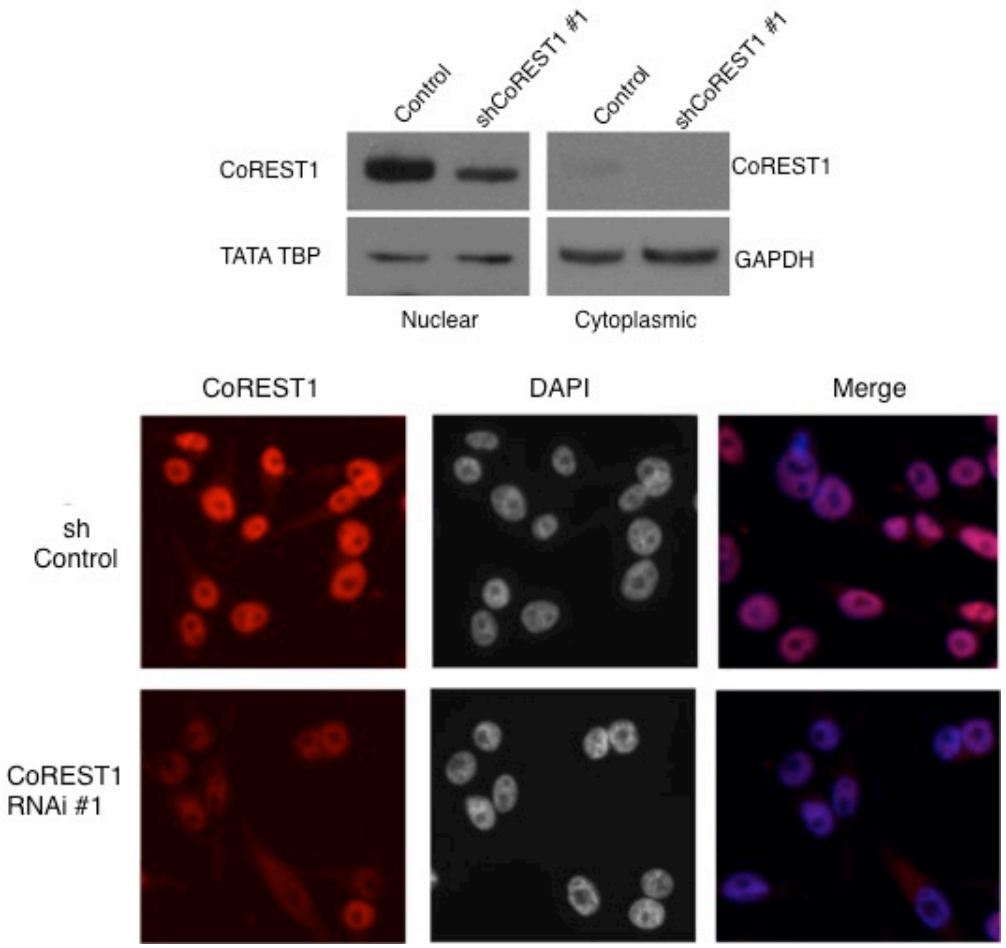
Table 3: List of antibodies used in Chapter II and Chapter III

Antibody	Application	Dilution	Source
CoREST1	WB, IP	1/2500 (5%BSA), 10ug	Millipore, Catalog number: 07-455
HIF1a	WB	1:2000 (5% BSA)	Novus Biologicals, Catalog number: rabbit (NB100-449); mouse (NB100-105)
H3K4me2	WB	1:2000 (5% BSA)	Abcam, Catalog number: 32356
Slug	WB, IP, ChIP	1:500 (5% BSA), 10 ug, 10ug	CST, Catalog number: C19G7 rabbit mAb
GAPDH	WB	1:10,000 (5% milk, BSA)	Santacruz, Catalog number:
TATA-TBP	WB	1:2000 (5% milk)	Abcam, Catalog number: 1TBP18
H3	WB	1:1000 (5%BSA)	CST, Catalog number: 9715
SIRT1	WB	5% BSA or gelatin	Millipore, Catalog Number: 04-1557
CD31	IF	1:200 (1.5% goat serum	BD Pharminigen Catalog Number: 550274
Ki67	IF	1:200 (1.5% goat serum	Abcam, Catalog Number: 15580-100
F4/80	IF	1:200 (1.5% goat serum	eBioscience, Catalog Number: 17-4801-80

Table 4: List of primers used in qRT PCR

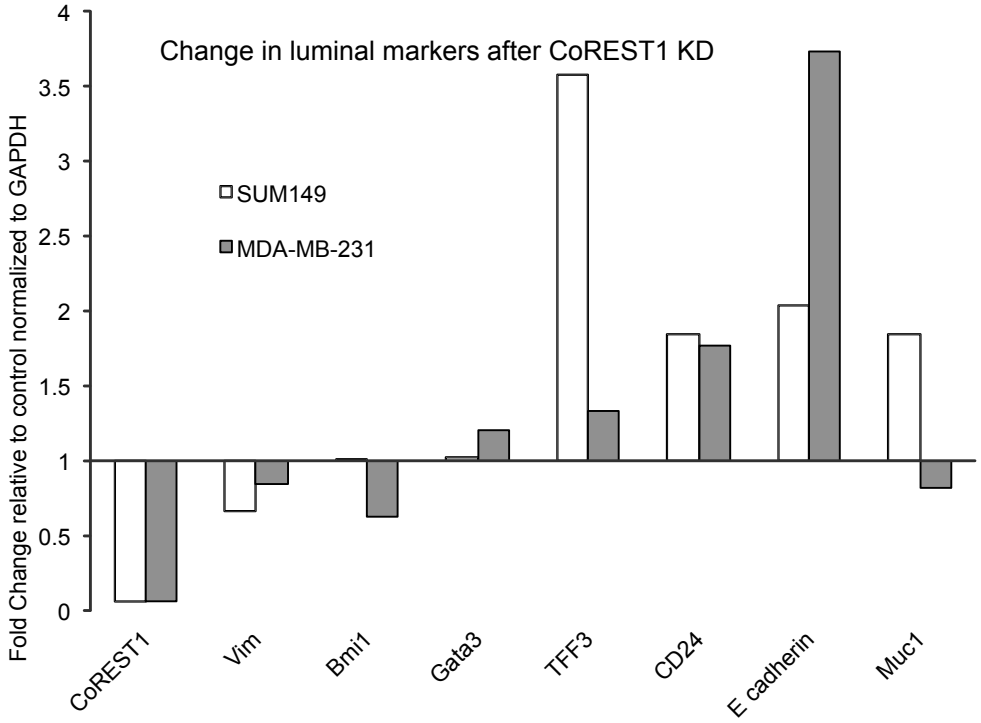
Name of target	Primer sequence
ANG	F: ACACTTCCTGACCCAGCACT R: CCGTCTCCTCATGATGCTTT
Bmi1	F:AGCCATTTTGATTGCTGTTTGA R: CCGCTTTTAGGCATACAGATTGTA
CD24	F: TGAAGAACATGTGAGAGGTTTGAC R: GAAAACTGAATCTCCATTCCACAA
CK14	F: CATGAGTGTGGAAGCCGACAT R: GCCTCTCAGGGATTCATCTC
CK18	F: TGATGACACCAATATCACACGAC R: TACCTCCACGGTCAACCCA
CK19	F: ACCAAGTTTGAGACGGAACAG R: CCCTCAGCGTACTGATTTCCT
CoREST1	F: ACTTCCAGAGGTCATTCAG R: CTTCGGGCATCTTAATGG
CXCL16	F: AAGCCATTGAGACACCAGCTG R: ACCTCGCTCTGACTCCCAGA
GATA3	F: GCGGGCTCTATCACAAAATGA R: GCTCTCCTGGCTGCAGACAGC
GAPDH	F: CGGATTTGGTCGTATTGGGC R: TGGAAGATGGTGATGGGATTTC
GMCSF	F: GGCGTCTCCTGAACCTGAGT R: GGGGATGACAAGCAGAAAGT

HIF1a	F: CAATACCCTATGTAGTTGTGGAAGTTTATG R: ACCAACAGGGTAGGCAGAACATT
IL8	F: CTGCGCCAACACAGAAATTA R: TGAATTCTCAGCCCTCTTCA
LSD1	F: CAAGTGTCAATTTGTTCGGG R: TTCTTTGGGCTGAGGTACTG
MCP1	F: GTCTCTGCCGCCCTTCTGT R: TTGCATCTGGCTGAGCGAG
MMP8	F: TGGACCCAATGGAATCCTTGC R: ATAGCCACTCAGAGCCCAGTA
PEDF	F: TCCAATGCAGAGGAGTAGCA R: TGTGCAGGCTTAGAGGGACT
SDF1a	F: TGAGAGCTCGCTTTGAGTGA R: CACCAGGACCTTCTGTGGAT
SMA	F: CAGGGCTGTTTTCCCATCCAT R: GCCATGTTCTATCGGGTACTTC
TFF3	F: AGAAAAACTGTCTGGGAGCTTG R: CTCATTTATGCACCGTTGTTTG
TIMP1	F: CACCAGAGAACCCACCATGGC R: CACTCTGCAGTTTGCAGG
TSP1	F: TTGTCTTTGGAACCACACCA R: CTGGACAGCTCATCACAGGA
VEGFA	F: GCAGAATCATCACGAAGTGG R: GCATGGTGATGTTGGACTCC



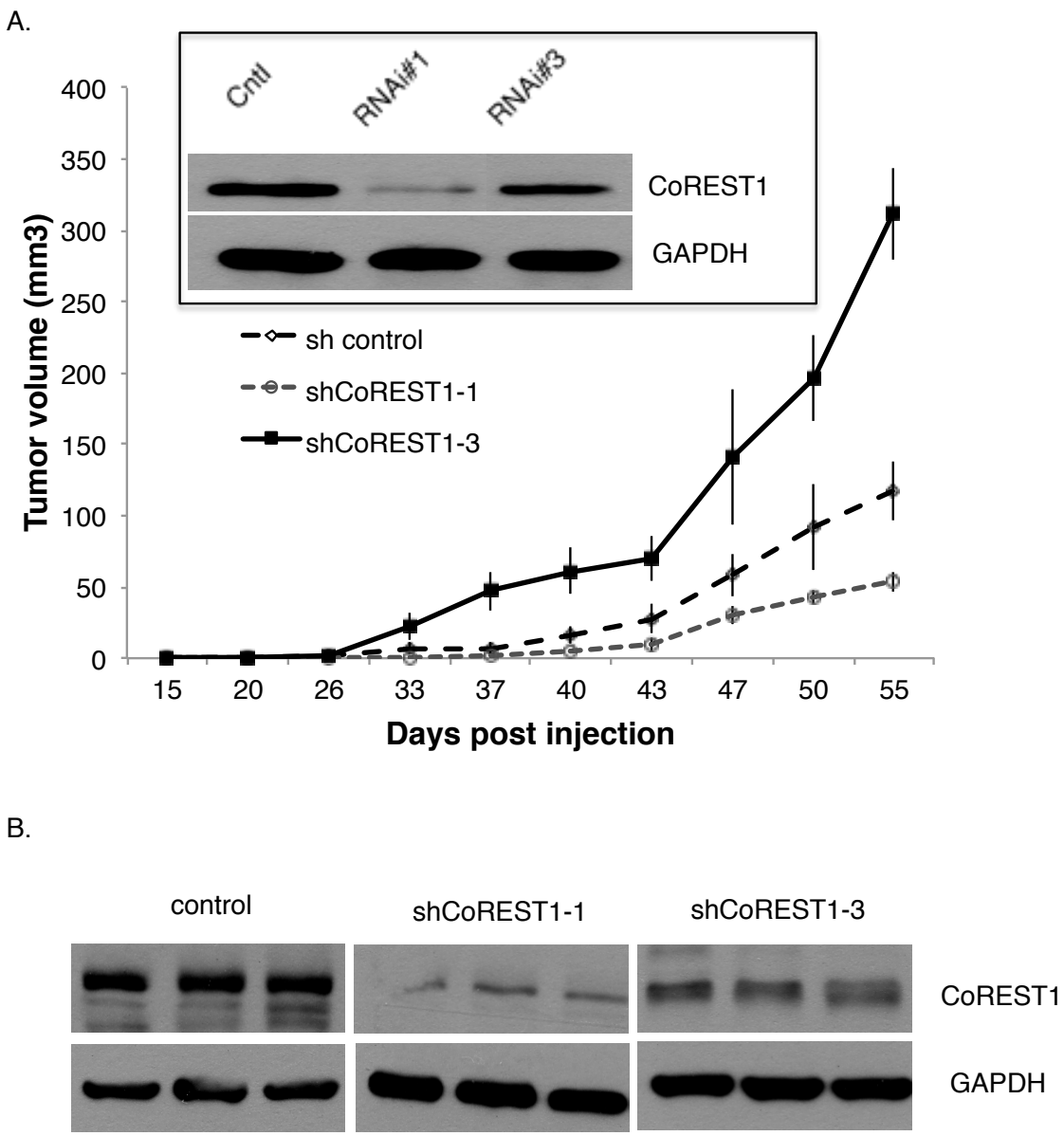
Supplementary Figure 1: CoREST1 is predominantly nuclear in the MDA MB 231 cells.

(A) Nuclear fractionation of MDA MB 231 cells followed by western blot analysis for CoREST1. TATA- TBP as nuclear loading control and GAPDH as cytoplasmic loading control. (B) Immunocytochemisty analyses on control and shCoREST1 RNAi #1 cells. CoREST1 (red); DAPI (black and white) and merge showing that CoREST1 mostly localizes to the nucleus.



Supplementary Figure 2: Depletion of CoREST1 in MDA MB 231 and SUM149 breast cancer cells results in a characteristic gene expression profile.

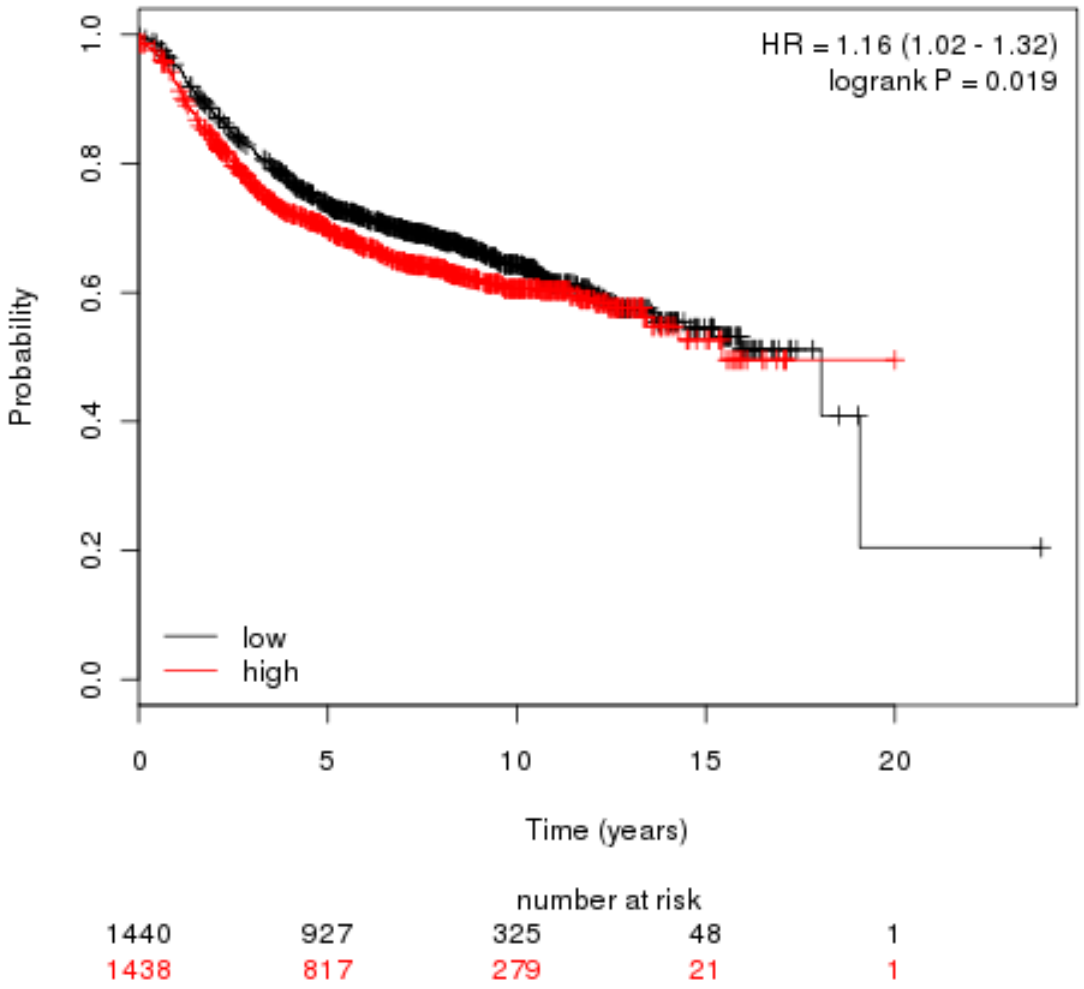
mRNA from shCoREST1 MDA MB 231s and SUM149s was used to assay candidate genes. RT-qPCR results were analyzed using the ddCT method and values were normalized to housekeeping gene, GAPDH. Fold change was calculated by normalizing gene expression values for the control cell lines to 1.0.



Supplementary Figure 3: Dose dependent role of CoREST1 in tumor formation.

(A) Tumor burden curve using RNAi construct used in figure 2.1 (shCoREST1-1) and another RNAi construct shCoREST1-3 (n=6 for each group). Inset shows levels of CoREST1 knockdown in MDA MB 231 cells prior to injection. Tumor burden for shCoREST1-3 was higher than control and shCoREST1-1.

(B) CoREST1 levels in excised tumors. Representative samples shown for each group. CoREST1 levels in shCoREST1-3 appear to be elevated in comparison to levels in injected cells. Increased CoREST1 levels corresponded with increased tumor burden.



Supplementary Figure 4: Kaplan Meier curve for CoREST1 expression (KMPlotter)

High levels of CoREST1 (red) corresponded with poor prognosis (recurrence free survival) when compared to lower levels of CoREST1 (black) in a cohort of 2878 patients. CoREST1 levels calculated using gene expression data and patient cohorts from multiple databases (Gyorffy et al., 2010)

Appendices

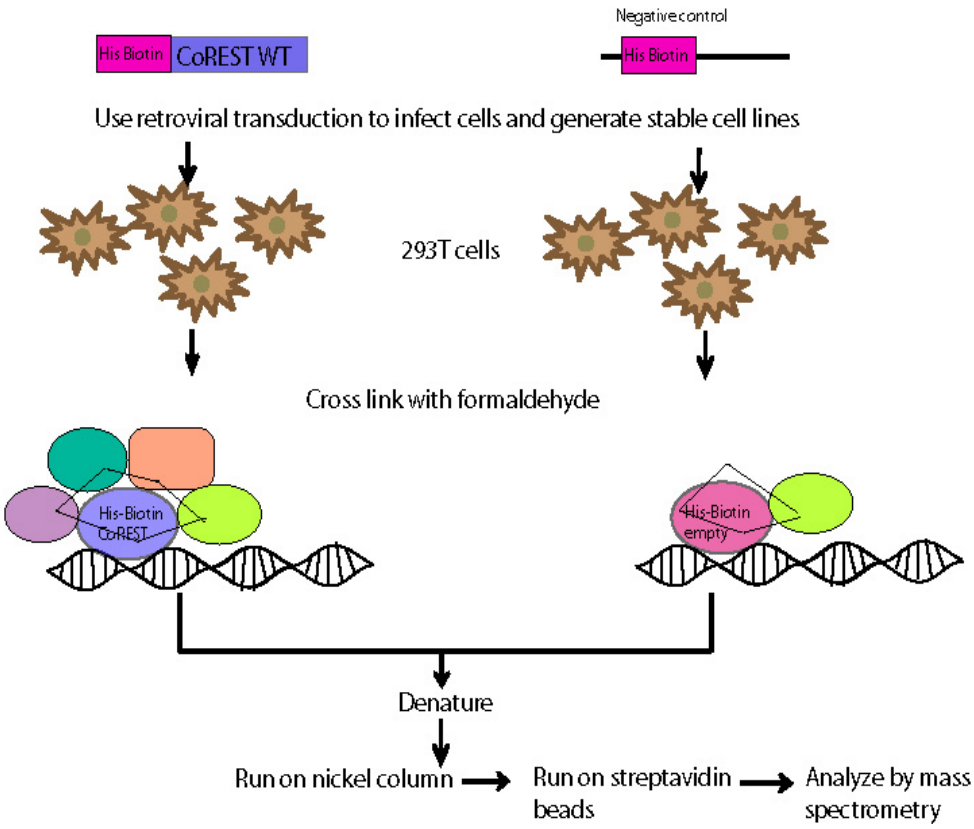
Appendix 1: Peptides found in the Mass Spectrometry Analysis using crosslinking, doubled tagged affinity purification in His- Biotin CoREST1

Symbol	Name of Peptide	GO term associations	Number of peptides, % protein coverage
ACTA2*	Smooth muscle Actin	regulation of blood pressure, vascular smooth muscle contraction, actin cytoskeleton, smooth muscle contractile fiber	12, 19.2%, 7.9%
ALB	Albumin	transport, extracellular space	2, 5.1%
ANXA2P2	Putative annexin A2-like protein	basement membrane, melanosome, calcium binding	6, 22.5%
APEH	Acylamino-acid-releasing enzyme	proteolysis, serine endopeptidase activity	2, 4.5%
BRAF35	HMG20B, SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily E member 1-related	blood coagulation, chromatin modification, cell cycle, transcripton regulation,	1, 3.8 %
CAPN1	Calpain 1 catalytic subunit	calcium dependent endopeptidase activity	2, 4%
CoREST1	Co repressor of REST-1		78, 29.9%,20.8%, 26%, 54.7%, 23.5%
GAPDH	Glyceraldehyde 3 Phosphate dehydrogenase	carbohydrate metabolism, response to IFN gamma response	4, 20.3%

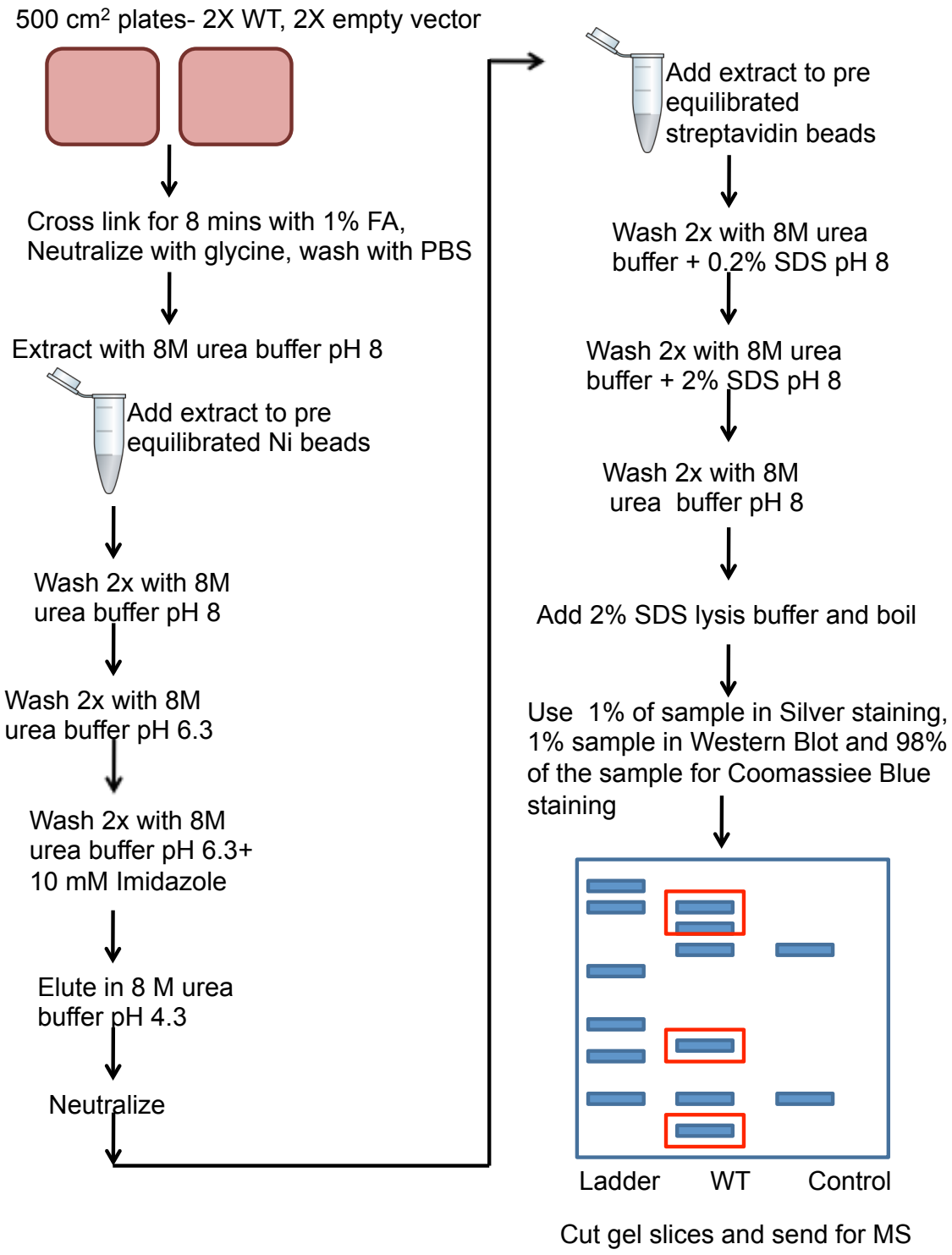
HAL	Histidine Ammonia Lyase	Ammonia lyase activity	12, 3.4%, 19.6%
HDAC1	Histone deacetylase 1	blood coagulation, chromatin modification, chromatin regulation,	7, 10%, 5.1%
HDAC2	Histone deacetylase 2	development-neurons, cardiac, chromatin modification, chromatin regulation	10, 5.2%, 7.7%
HIST1H4J	Histone H4	chromatin organization, nucleosome assembly, telomere maintenance	4, 40.1%
HIST2H2BE*	Histone H2B type 2-E		6, 25%
HSP90AA2	Putative heat shock protein HSP 90-alpha A2	protein folding, stress response	2, 6%
HSPA5	78 kDa glucose-regulated protein	stress response, blood coagulation, protein folding	3, 6.5%
HSPA7	Putative heat shock 70 kDa protein 7	protein folding, ATP binding	
HSPA8*	Heat shock cognate 71 kDa protein	ATP binding	10, 3.8%, 14.4%
KIAA0182	GSE, Genetic suppressor element 1	protein binding	8, 8%
LSD1	Lysine demethylase 1	blood goagulation, cell proliferation, negative regulator of transcription, H3K4 methylation, H3K9 methylation	38, 21.9%, 26.8%
LTF	lactotransferrin	iron transport, humoral response	6, 11%
MMP9	Matrix metallopeptidase 9	extracellular matrix, macrophage differentiation	3, 5.4%
MPO	Myeloperoxidase	chromatin binding	20, 3.1%, 14.2%

PARP1	Poly (ADP-Ribose) Polymerase 1	DNA binding, transcription factor binding	9, 6.4%, 4.8%
RREB1	Ras Responsive Element Binding Protein 1	DNA binding	2, 2.9%
TGM3	Transglutaminase 3	GTPAse activity	3, 5.6%
TRIM28	Tripartite Motif Containing 28/KAP1	DNA binding, transcription factor binding	6, 2.6%, 6.5%
VIM	Vimentin	protein binding	9, 8.8%, 11.9%
XRCC6	X-ray repair complementing defective repair in Chinese hamster cells 6	DNA binding	1, 2%
ZNF217	Zinc Finger Protein 217	protein binding, transcriptional regulation	1, 1.1%
ZNF516	Zinc Finger Protein 516	nucleic acid binding, DNA binding	3, 2.9%

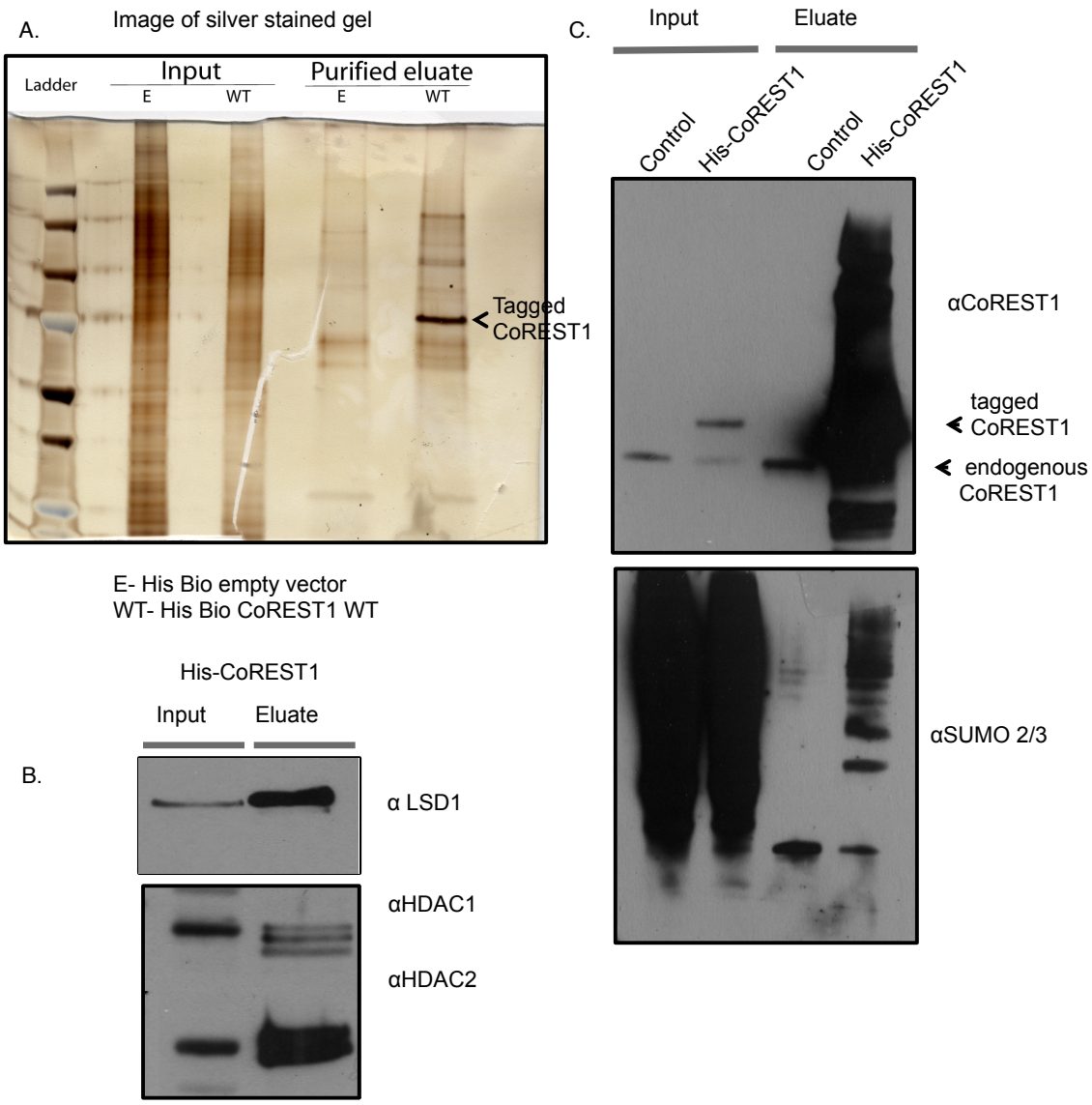
When multiple % values for protein coverage are present they indicate coverage of peptides in different gel slices. Total peptide number is a sum of all peptides found in different slices. Highlighted peptides have been previously described as part of CoREST complexes. * are genes that are common contaminants in affinity purification and mass spectrometry studies (Mellacheruvu et al., 2013).



Appendix 2: Schematic representation of crosslinked double-tagged affinity purification using His-Biotin tagged CoREST1 or control empty tagged vector.



Appendix 3: Schematic representation of step-by-step protocol for the double affinity purification strategy used with over expressed His- Biotin tagged CoREST1.



Appendix 4: (A) Image of silver stained gel of purified eluate from crosslinked, doubled affinity purification using His-Biotin-CoREST1 (B) Western blot for known CoREST1 complex members to ensure that purification was successful (C) Presence of tagged CoREST1 interactors, including presence of modified substrates like SUMO modified substrates (also known to interact with CoREST1 via SUMO)

References

1. (2003). Decitabine: 2'-deoxy-5-azacytidine, Aza dC, DAC, dezocitidine, NSC 127716. Drugs in R&D *4*, 352-358.
2. Abrajano, J.J., Qureshi, I.A., Gokhan, S., Molero, A.E., Zheng, D., Bergman, A., and Mehler, M.F. (2010). Corepressor for element-1-silencing transcription factor preferentially mediates gene networks underlying neural stem cell fate decisions. Proceedings of the National Academy of Sciences of the United States of America *107*, 16685-16690.
3. Abrajano, J.J., Qureshi, I.A., Gokhan, S., Zheng, D., Bergman, A., and Mehler, M.F. (2009a). Differential deployment of REST and CoREST promotes glial subtype specification and oligodendrocyte lineage maturation. PloS one *4*, e7665.
4. Abrajano, J.J., Qureshi, I.A., Gokhan, S., Zheng, D., Bergman, A., and Mehler, M.F. (2009b). REST and CoREST modulate neuronal subtype specification, maturation and maintenance. PloS one *4*, e7936.
5. Agger, K., Christensen, J., Cloos, P.A., and Helin, K. (2008). The emerging functions of histone demethylases. Current opinion in genetics & development *18*, 159-168.
6. Alameddine, R.S., Otrock, Z.K., Awada, A., and Shamseddine, A. (2013). Crosstalk between HER2 signaling and angiogenesis in breast cancer: molecular basis, clinical applications and challenges. Current opinion in oncology *25*, 313-324.
7. Althoff, K., Beckers, A., Odersky, A., Mestdagh, P., Koster, J., Bray, I.M., Bryan, K., Vandesompele, J., Speleman, F., Stallings, R.L.*, et al.* (2013). MiR-137 functions as a tumor suppressor in neuroblastoma by downregulating KDM1A. International journal of cancer Journal international du cancer.
8. Alves, C.C., Carneiro, F., Hoefler, H., and Becker, K.F. (2009a). Role of the epithelial-mesenchymal transition regulator Slug in primary human cancers. Front Biosci (Landmark Ed) *14*, 3035-3050.
9. Alves, C.C., Carneiro, F., Hoefler, H., and Becker, K.F. (2009b). Role of the epithelial-mesenchymal transition regulator Slug in primary human cancers. Frontiers in bioscience : a journal and virtual library *14*, 3035-3050.
10. Amente, S., Lania, L., and Majello, B. (2013). The histone LSD1 demethylase in stemness and cancer transcription programs. Biochimica et biophysica acta *1829*, 981-986.

11. Andres, M.E., Burger, C., Peral-Rubio, M.J., Battaglioli, E., Anderson, M.E., Grimes, J., Dallman, J., Ballas, N., and Mandel, G. (1999). CoREST: a functional corepressor required for regulation of neural-specific gene expression. Proceedings of the National Academy of Sciences of the United States of America *96*, 9873-9878.
12. Attardi, L.D. (2005). The role of p53-mediated apoptosis as a crucial anti-tumor response to genomic instability: lessons from mouse models. Mutation research *569*, 145-157.
13. Baer, C., Claus, R., and Plass, C. (2013). Genome-wide epigenetic regulation of miRNAs in cancer. Cancer research *73*, 473-477.
14. Balkwill, F., Charles, K.A., and Mantovani, A. (2005). Smoldering and polarized inflammation in the initiation and promotion of malignant disease. Cancer cell *7*, 211-217.
15. Ballas, N., Battaglioli, E., Atouf, F., Andres, M.E., Chenoweth, J., Anderson, M.E., Burger, C., Moniwa, M., Davie, J.R., Bowers, W.J.*, et al.* (2001). Regulation of neuronal traits by a novel transcriptional complex. Neuron *31*, 353-365.
16. Ballas, N., Grunseich, C., Lu, D.D., Speh, J.C., and Mandel, G. (2005). REST and its corepressors mediate plasticity of neuronal gene chromatin throughout neurogenesis. Cell *121*, 645-657.
17. Banck, M.S., Li, S., Nishio, H., Wang, C., Beutler, A.S., and Walsh, M.J. (2009). The ZNF217 oncogene is a candidate organizer of repressive histone modifiers. Epigenetics : official journal of the DNA Methylation Society *4*, 100-106.
18. Barlesi, F., Giaccone, G., Gallegos-Ruiz, M.I., Loundou, A., Span, S.W., Lefesvre, P., Kruyt, F.A., and Rodriguez, J.A. (2007). Global histone modifications predict prognosis of resected non small-cell lung cancer. Journal of clinical oncology : official journal of the American Society of Clinical Oncology *25*, 4358-4364.
19. Baron, R., Binda, C., Tortorici, M., McCammon, J.A., and Mattevi, A. (2011). Molecular mimicry and ligand recognition in binding and catalysis by the histone demethylase LSD1-CoREST complex. Structure *19*, 212-220.
20. Behbahani, T.E., Kahl, P., von der Gathen, J., Heukamp, L.C., Baumann, C., Gutgemann, I., Walter, B., Hofstadter, F., Bastian, P.J., von Ruecker, A.*, et al.* (2012). Alterations of global histone H4K20 methylation during prostate carcinogenesis. BMC urology *12*, 5.
21. Bennani-Baiti, I.M. (2012). Integration of ERalpha-PELP1-HER2 signaling by LSD1 (KDM1A/AOF2) offers combinatorial therapeutic opportunities to

circumventing hormone resistance in breast cancer. Breast cancer research : BCR *14*, 112.
22. Berger, S.L., Kouzarides, T., Shiekhattar, R., and Shilatifard, A. (2009). An operational definition of epigenetics. Genes & development *23*, 781-783.
23. Bird, A. (2002). DNA methylation patterns and epigenetic memory. Genes & development *16*, 6-21.
24. Birnbaum, D., Bertucci, F., Ginestier, C., Tagett, R., Jacquemier, J., and Charafe-Jauffret, E. (2004). Basal and luminal breast cancers: basic or luminous? (review). International journal of oncology *25*, 249-258.
25. Bisagni, G., Musolino, A., Panebianco, M., De Matteis, A., Nuzzo, F., Ardizzoni, A., Gori, S., Gamucci, T., Passalacqua, R., Gnoni, R.*, et al.* (2013). The Breast Avastin Trial: phase II study of bevacizumab maintenance therapy after induction chemotherapy with docetaxel and capecitabine for the first-line treatment of patients with locally recurrent or metastatic breast cancer. Cancer chemotherapy and pharmacology *71*, 1051-1057.
26. Biswas, D., Milne, T.A., Basrur, V., Kim, J., Elenitoba-Johnson, K.S., Allis, C.D., and Roeder, R.G. (2011). Function of leukemogenic mixed lineage leukemia 1 (MLL) fusion proteins through distinct partner protein complexes. Proceedings of the National Academy of Sciences of the United States of America *108*, 15751-15756.
27. Butler, J.M., Kobayashi, H., and Rafii, S. (2010). Instructive role of the vascular niche in promoting tumour growth and tissue repair by angiocrine factors. Nature reviews Cancer *10*, 138-146.
28. Casanovas, O., Hicklin, D.J., Bergers, G., and Hanahan, D. (2005). Drug resistance by evasion of antiangiogenic targeting of VEGF signaling in late-stage pancreatic islet tumors. Cancer cell *8*, 299-309.
29. Cascio, S., D'Andrea, A., Ferla, R., Surmacz, E., Gulotta, E., Amodeo, V., Bazan, V., Gebbia, N., and Russo, A. (2010). miR-20b modulates VEGF expression by targeting HIF-1 alpha and STAT3 in MCF-7 breast cancer cells. Journal of cellular physiology *224*, 242-249.
30. Ceballos-Chavez, M., Rivero, S., Garcia-Gutierrez, P., Rodriguez-Paredes, M., Garcia-Dominguez, M., Bhattacharya, S., and Reyes, J.C. (2012). Control of neuronal differentiation by sumoylation of BRAF35, a subunit of the LSD1-CoREST histone demethylase complex. Proceedings of the National Academy of Sciences of the United States of America *109*, 8085-8090.

31. Cedar, H., and Bergman, Y. (2009). Linking DNA methylation and histone modification: patterns and paradigms. Nature reviews Genetics *10*, 295-304.
32. Chinnadurai, G. (2002). CtBP, an unconventional transcriptional corepressor in development and oncogenesis. Molecular cell *9*, 213-224.
33. Chowdhury, A.H., Ramroop, J.R., Upadhyay, G., Sengupta, A., Andrzejczyk, A., and Saleque, S. (2013). Differential transcriptional regulation of meis1 by Gfi1b and its co-factors LSD1 and CoREST. PloS one *8*, e53666.
34. Clarke, R. (1996). Human breast cancer cell line xenografts as models of breast cancer. The immunobiologies of recipient mice and the characteristics of several tumorigenic cell lines. Breast cancer research and treatment *39*, 69-86.
35. Clavarezza, M., Turazza, M., Aitini, E., Saracchini, S., Garrone, O., Durando, A., De Placido, S., Bisagni, G., Levaggi, A., Bighin, C.*, et al.* (2013). Phase II open-label study of bevacizumab combined with neoadjuvant anthracycline and taxane therapy for locally advanced breast cancer. Breast *22*, 470-475.
36. Colotta, F., Allavena, P., Sica, A., Garlanda, C., and Mantovani, A. (2009). Cancer-related inflammation, the seventh hallmark of cancer: links to genetic instability. Carcinogenesis *30*, 1073-1081.
37. Come, C., Arnoux, V., Bibeau, F., and Savagner, P. (2004). Roles of the transcription factors snail and slug during mammary morphogenesis and breast carcinoma progression. Journal of mammary gland biology and neoplasia *9*, 183-193.
38. Connolly, R., and Stearns, V. (2012). Epigenetics as a therapeutic target in breast cancer. Journal of mammary gland biology and neoplasia *17*, 191-204.
39. Costa, F.F. (2008). Non-coding RNAs, epigenetics and complexity. Gene *410*, 9-17.
40. Coussens, L.M., and Werb, Z. (2002). Inflammation and cancer. Nature *420*, 860-867.
41. Cowger, J.J., Zhao, Q., Isovic, M., and Torchia, J. (2007). Biochemical characterization of the zinc-finger protein 217 transcriptional repressor complex: identification of a ZNF217 consensus recognition sequence. Oncogene *26*, 3378-3386.
42. Curigliano, G., Pivot, X., Cortes, J., Elias, A., Cesari, R., Khosravan, R., Collier, M., Huang, X., Cataruozolo, P.E., Kern, K.A.*, et al.* (2013). Randomized phase II study of sunitinib versus standard of care for

patients with previously treated advanced triple-negative breast cancer. Breast.
43. Curtis, B.J., Zraly, C.B., and Dingwall, A.K. (2013). Drosophila LSD1-CoREST demethylase complex regulates DPP/TGFbeta signaling during wing development. Genesis *51*, 16-31.
44. Dallman, J.E., Allopenna, J., Bassett, A., Travers, A., and Mandel, G. (2004). A conserved role but different partners for the transcriptional corepressor CoREST in fly and mammalian nervous system formation. The Journal of neuroscience : the official journal of the Society for Neuroscience *24*, 7186-7193.
45. Dawson, M.A., and Kouzarides, T. (2012). Cancer epigenetics: from mechanism to therapy. Cell *150*, 12-27.
46. de Visser, K.E., Eichten, A., and Coussens, L.M. (2006). Paradoxical roles of the immune system during cancer development. Nature reviews Cancer *6*, 24-37.
47. Deshmane, S.L., Kremlev, S., Amini, S., and Sawaya, B.E. (2009). Monocyte chemoattractant protein-1 (MCP-1): an overview. Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research *29*, 313-326.
48. Domanitskaya, E., and Schupbach, T. (2012). CoREST acts as a positive regulator of Notch signaling in the follicle cells of Drosophila melanogaster. Journal of cell science *125*, 399-410.
49. Dunn, G.P., Old, L.J., and Schreiber, R.D. (2004). The immunobiology of cancer immunosurveillance and immunoediting. Immunity *21*, 137-148.
50. Efroni, S., Duttagupta, R., Cheng, J., Dehghani, H., Hoeppner, D.J., Dash, C., Bazett-Jones, D.P., Le Grice, S., McKay, R.D., Buetow, K.H.*, et al.* (2008). Global transcription in pluripotent embryonic stem cells. Cell stem cell *2*, 437-447.
51. Ellinger, J., Kahl, P., von der Gathen, J., Rogenhofer, S., Heukamp, L.C., Gutgemann, I., Walter, B., Hofstadter, F., Buttner, R., Muller, S.C.*, et al.* (2010). Global levels of histone modifications predict prostate cancer recurrence. The Prostate *70*, 61-69.
52. Elsheikh, S.E., Green, A.R., Rakha, E.A., Powe, D.G., Ahmed, R.A., Collins, H.M., Soria, D., Garibaldi, J.M., Paish, C.E., Ammar, A.A.*, et al.* (2009). Global histone modifications in breast cancer correlate with tumor phenotypes, prognostic factors, and patient outcome. Cancer research *69*, 3802-3809.
53. Esteve, P.O., Chin, H.G., Benner, J., Feehery, G.R., Samaranayake, M., Horwitz, G.A., Jacobsen, S.E., and Pradhan, S. (2009). Regulation of

DNMT1 stability through SET7-mediated lysine methylation in mammalian cells. Proceedings of the National Academy of Sciences of the United States of America *106*, 5076-5081.

54. Fabbri, M., and Calin, G.A. (2010). Epigenetics and miRNAs in human cancer. Advances in genetics *70*, 87-99.
55. Fang, R., Barbera, A.J., Xu, Y., Rutenberg, M., Leonor, T., Bi, Q., Lan, F., Mei, P., Yuan, G.C., Lian, C.*, et al.* (2010). Human LSD2/KDM1b/AOF1 regulates gene transcription by modulating intragenic H3K4me2 methylation. Molecular cell *39*, 222-233.
56. Feinberg, A.P., and Vogelstein, B. (1983). Hypomethylation distinguishes genes of some human cancers from their normal counterparts. Nature *301*, 89-92.
57. Fernando, N.T., Koch, M., Rothrock, C., Gollogly, L.K., D'Amore, P.A., Ryeom, S., and Yoon, S.S. (2008). Tumor escape from endogenous, extracellular matrix-associated angiogenesis inhibitors by up-regulation of multiple proangiogenic factors. Clinical cancer research : an official journal of the American Association for Cancer Research *14*, 1529-1539.
58. Ferrara, N., Carver-Moore, K., Chen, H., Dowd, M., Lu, L., O'Shea, K.S., Powell-Braxton, L., Hillan, K.J., and Moore, M.W. (1996). Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. Nature *380*, 439-442.
59. Ferrari-Amorotti, G., Fragliasso, V., Esteki, R., Prudente, Z., Soliera, A.R., Cattelani, S., Manzotti, G., Grisendi, G., Dominici, M., Pieraccioli, M.*, et al.* (2013). Inhibiting interactions of lysine demethylase LSD1 with snail/slug blocks cancer cell invasion. Cancer research *73*, 235-245.
60. Folkman, J. (1971). Tumor angiogenesis: therapeutic implications. The New England journal of medicine *285*, 1182-1186.
61. Fong, P.C., Boss, D.S., Yap, T.A., Tutt, A., Wu, P., Mergui-Roelvink, M., Mortimer, P., Swaisland, H., Lau, A., O'Connor, M.J.*, et al.* (2009). Inhibition of poly(ADP-ribose) polymerase in tumors from BRCA mutation carriers. The New England journal of medicine *361*, 123-134.
62. Forneris, F., Binda, C., Adamo, A., Battaglioli, E., and Mattevi, A. (2007). Structural basis of LSD1-CoREST selectivity in histone H3 recognition. The Journal of biological chemistry *282*, 20070-20074.
63. Forsythe, J.A., Jiang, B.H., Iyer, N.V., Agani, F., Leung, S.W., Koos, R.D., and Semenza, G.L. (1996). Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. Molecular and cellular biology *16*, 4604-4613.

64. Foster, C.T., Dovey, O.M., Lezina, L., Luo, J.L., Gant, T.W., Barlev, N., Bradley, A., and Cowley, S.M. (2010). Lysine-specific demethylase 1 regulates the embryonic transcriptome and CoREST stability. Molecular and cellular biology *30*, 4851-4863.
65. Fraga, M.F., Ballestar, E., Villar-Garea, A., Boix-Chornet, M., Espada, J., Schotta, G., Bonaldi, T., Haydon, C., Ropero, S., Petrie, K.*, et al.* (2005). Loss of acetylation at Lys16 and trimethylation at Lys20 of histone H4 is a common hallmark of human cancer. Nature genetics *37*, 391-400.
66. Galindo, M., Santiago, B., Alcami, J., Rivero, M., Martin-Serrano, J., and Pablos, J.L. (2001). Hypoxia induces expression of the chemokines monocyte chemoattractant protein-1 (MCP-1) and IL-8 in human dermal fibroblasts. Clinical and experimental immunology *123*, 36-41.
67. Gerber, H.P., Condorelli, F., Park, J., and Ferrara, N. (1997). Differential transcriptional regulation of the two vascular endothelial growth factor receptor genes. Flt-1, but not Flk-1/KDR, is up-regulated by hypoxia. The Journal of biological chemistry *272*, 23659-23667.
68. Gianni, L., Romieu, G.H., Lichinitser, M., Serrano, S.V., Mansutti, M., Pivot, X., Mariani, P., Andre, F., Chan, A., Lipatov, O.*, et al.* (2013). AVEREL: a randomized phase III Trial evaluating bevacizumab in combination with docetaxel and trastuzumab as first-line therapy for HER2-positive locally recurrent/metastatic breast cancer. Journal of clinical oncology : official journal of the American Society of Clinical Oncology *31*, 1719-1725.
69. Gocke, C.B., and Yu, H. (2008). ZNF198 stabilizes the LSD1-CoREST-HDAC1 complex on chromatin through its MYM-type zinc fingers. PloS one *3*, e3255.
70. Gomez, A.V., Galleguillos, D., Maass, J.C., Battaglioli, E., Kukuljan, M., and Andres, M.E. (2008). CoREST represses the heat shock response mediated by HSF1. Molecular cell *31*, 222-231.
71. Gorski, J.J., James, C.R., Quinn, J.E., Stewart, G.E., Staunton, K.C., Buckley, N.E., McDyer, F.A., Kennedy, R.D., Wilson, R.H., Mullan, P.B.*, et al.* (2010). BRCA1 transcriptionally regulates genes associated with the basal-like phenotype in breast cancer. Breast cancer research and treatment *122*, 721-731.
72. Grivennikov, S.I., Greten, F.R., and Karin, M. (2010). Immunity, inflammation, and cancer. Cell *140*, 883-899.
73. Grivennikov, S.I., and Karin, M. (2010). Inflammation and oncogenesis: a vicious connection. Current opinion in genetics & development *20*, 65-71.

74. Guan, J.S., Haggarty, S.J., Giacometti, E., Dannenberg, J.H., Joseph, N., Gao, J., Nieland, T.J., Zhou, Y., Wang, X., Mazitschek, R.*, et al.* (2009). HDAC2 negatively regulates memory formation and synaptic plasticity. Nature *459*, 55-60.
75. Guo, W., Keckesova, Z., Donaher, J.L., Shibue, T., Tischler, V., Reinhardt, F., Itzkovitz, S., Noske, A., Zurrer-Hardi, U., Bell, G.*, et al.* (2012). Slug and Sox9 cooperatively determine the mammary stem cell state. Cell *148*, 1015-1028.
76. Gupta, P.B., Kuperwasser, C., Brunet, J.P., Ramaswamy, S., Kuo, W.L., Gray, J.W., Naber, S.P., and Weinberg, R.A. (2005). The melanocyte differentiation program predisposes to metastasis after neoplastic transformation. Nature genetics *37*, 1047-1054.
77. Gyorffy, B., Lanczky, A., Eklund, A.C., Denkert, C., Budczies, J., Li, Q., and Szallasi, Z. (2010). An online survival analysis tool to rapidly assess the effect of 22,277 genes on breast cancer prognosis using microarray data of 1,809 patients. Breast cancer research and treatment *123*, 725-731.
78. Hakimi, M.A., Bochar, D.A., Chenoweth, J., Lane, W.S., Mandel, G., and Shiekhattar, R. (2002). A core-BRAF35 complex containing histone deacetylase mediates repression of neuronal-specific genes. Proceedings of the National Academy of Sciences of the United States of America *99*, 7420-7425.
79. Hakimi, M.A., Dong, Y., Lane, W.S., Speicher, D.W., and Shiekhattar, R. (2003). A candidate X-linked mental retardation gene is a component of a new family of histone deacetylase-containing complexes. The Journal of biological chemistry *278*, 7234-7239.
80. Hanahan, D., and Folkman, J. (1996). Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell *86*, 353-364.
81. Hanahan, D., and Weinberg, R.A. (2011). Hallmarks of cancer: the next generation. Cell *144*, 646-674.
82. Harris, W.J., Huang, X., Lynch, J.T., Spencer, G.J., Hitchin, J.R., Li, Y., Ciceri, F., Blaser, J.G., Greystoke, B.F., Jordan, A.M.*, et al.* (2012). The histone demethylase KDM1A sustains the oncogenic potential of MLL-AF9 leukemia stem cells. Cancer cell *21*, 473-487.
83. Heichman, K.A., and Warren, J.D. (2012). DNA methylation biomarkers and their utility for solid cancer diagnostics. Clinical chemistry and laboratory medicine : CCLM / FESCC *50*, 1707-1721.
84. Hendriksen, E.M., Span, P.N., Schuuring, J., Peters, J.P., Sweep, F.C., van der Kogel, A.J., and Bussink, J. (2009). Angiogenesis, hypoxia and

VEGF expression during tumour growth in a human xenograft tumour model. Microvascular research *77*, 96-103.

85. Hopfl, G., Wenger, R.H., Ziegler, U., Stallmach, T., Gardelle, O., Achermann, R., Wergin, M., Kaser-Hotz, B., Saunders, H.M., Williams, K.J.*, et al.* (2002). Rescue of hypoxia-inducible factor-1alpha-deficient tumor growth by wild-type cells is independent of vascular endothelial growth factor. Cancer research *62*, 2962-2970.
86. Hu, X., Li, X., Valverde, K., Fu, X., Noguchi, C., Qiu, Y., and Huang, S. (2009). LSD1-mediated epigenetic modification is required for TAL1 function and hematopoiesis. Proceedings of the National Academy of Sciences of the United States of America *106*, 10141-10146.
87. Huang, J., Sengupta, R., Espejo, A.B., Lee, M.G., Dorsey, J.A., Richter, M., Opravil, S., Shiekhattar, R., Bedford, M.T., Jenuwein, T.*, et al.* (2007a). p53 is regulated by the lysine demethylase LSD1. Nature *449*, 105-108.
88. Huang, Y., Greene, E., Murray Stewart, T., Goodwin, A.C., Baylin, S.B., Woster, P.M., and Casero, R.A., Jr. (2007b). Inhibition of lysine-specific demethylase 1 by polyamine analogues results in reexpression of aberrantly silenced genes. Proceedings of the National Academy of Sciences of the United States of America *104*, 8023-8028.
89. Huang, Y., Marton, L.J., Woster, P.M., and Casero, R.A. (2009a). Polyamine analogues targeting epigenetic gene regulation. Essays in biochemistry *46*, 95-110.
90. Huang, Y., Stewart, T.M., Wu, Y., Baylin, S.B., Marton, L.J., Perkins, B., Jones, R.J., Woster, P.M., and Casero, R.A., Jr. (2009b). Novel oligoamine analogues inhibit lysine-specific demethylase 1 and induce reexpression of epigenetically silenced genes. Clinical cancer research : an official journal of the American Association for Cancer Research *15*, 7217-7228.
91. Huang, Y., Vasilatos, S.N., Boric, L., Shaw, P.G., and Davidson, N.E. (2012). Inhibitors of histone demethylation and histone deacetylation cooperate in regulating gene expression and inhibiting growth in human breast cancer cells. Breast cancer research and treatment *131*, 777-789.
92. Humphrey, G.W., Wang, Y., Russanova, V.R., Hirai, T., Qin, J., Nakatani, Y., and Howard, B.H. (2001). Stable histone deacetylase complexes distinguished by the presence of SANT domain proteins CoREST/kiaa0071 and Mta-L1. The Journal of biological chemistry *276*, 6817-6824.

93. Jensen, R.L., Ragel, B.T., Whang, K., and Gillespie, D. (2006). Inhibition of hypoxia inducible factor-1alpha (HIF-1alpha) decreases vascular endothelial growth factor (VEGF) secretion and tumor growth in malignant gliomas. Journal of neuro-oncology *78*, 233-247.
94. Jie, D., Zhongmin, Z., Guoqing, L., Sheng, L., Yi, Z., Jing, W., and Liang, Z. (2013). Positive Expression of LSD1 and Negative Expression of E-cadherin Correlate with Metastasis and Poor Prognosis of Colon Cancer. Digestive diseases and sciences.
95. Johannessen, T.C., Wagner, M., Straume, O., Bjerkvig, R., and Eikesdal, H.P. (2013). Tumor vasculature: the Achilles' heel of cancer? Expert opinion on therapeutic targets *17*, 7-20.
96. Kang, F.W., Que, L., Wu, M., Wang, Z.L., and Sun, J. (2012). Effects of trichostatin A on HIF-1alpha and VEGF expression in human tongue squamous cell carcinoma cells in vitro. Oncology reports *28*, 193-199.
97. Kashyap, V., Ahmad, S., Nilsson, E.M., Helczynski, L., Kenna, S., Persson, J.L., Gudas, L.J., and Mongan, N.P. (2013). The lysine specific demethylase-1 (LSD1/KDM1A) regulates VEGF-A expression in prostate cancer. Molecular oncology *7*, 555-566.
98. Kerenyi, M.A., Shao, Z., Hsu, Y.J., Guo, G., Luc, S., O'Brien, K., Fujiwara, Y., Peng, C., Nguyen, M., and Orkin, S.H. (2013). Histone demethylase Lsd1 represses hematopoietic stem and progenitor cell signatures during blood cell maturation. eLife *2*, e00633.
99. Kim, C.S., Choi, H.S., Hwang, C.K., Song, K.Y., Lee, B.K., Law, P.Y., Wei, L.N., and Loh, H.H. (2006). Evidence of the neuron-restrictive silencer factor (NRSF) interaction with Sp3 and its synergic repression to the mu opioid receptor (MOR) gene. Nucleic acids research *34*, 6392-6403.
100. Kim, J., Park, U.H., Moon, M., Um, S.J., and Kim, E.J. (2013). Negative regulation of ERalpha by a novel protein CAC1 through association with histone demethylase LSD1. FEBS letters *587*, 17-22.
101. Kouzarides, T. (2007). Chromatin modifications and their function. Cell *128*, 693-705.
102. Kuppuswamy, M., Vijayalingam, S., Zhao, L.J., Zhou, Y., Subramanian, T., Ryerse, J., and Chinnadurai, G. (2008). Role of the PLDLS-binding cleft region of CtBP1 in recruitment of core and auxiliary components of the corepressor complex. Molecular and cellular biology *28*, 269-281.
103. Kurdistani, S.K. (2011). Histone modifications in cancer biology and prognosis. Progress in drug research Fortschritte der

Arzneimittelforschung Progres des recherches pharmaceutiques *67*, 91-106.

104. Lakowski, B., Roelens, I., and Jacob, S. (2006). CoREST-like complexes regulate chromatin modification and neuronal gene expression. Journal of molecular neuroscience : MN *29*, 227-239.

105. Lan, F., Nottke, A.C., and Shi, Y. (2008). Mechanisms involved in the regulation of histone lysine demethylases. Current opinion in cell biology *20*, 316-325.

106. Lapidus, R.G., Nass, S.J., Butash, K.A., Parl, F.F., Weitzman, S.A., Graff, J.G., Herman, J.G., and Davidson, N.E. (1998). Mapping of ER gene CpG island methylation-specific polymerase chain reaction. Cancer research *58*, 2515-2519.

107. Laurent, B., Randrianarison-Huetz, V., Frisan, E., Andrieu-Soler, C., Soler, E., Fontenay, M., Dusanter-Fourt, I., and Dumenil, D. (2012). A short Gfi-1B isoform controls erythroid differentiation by recruiting the LSD1-CoREST complex through the dimethylation of its SNAG domain. Journal of cell science *125*, 993-1002.

108. Lee, M.G., Wynder, C., Cooch, N., and Shiekhattar, R. (2005). An essential role for CoREST in nucleosomal histone 3 lysine 4 demethylation. Nature *437*, 432-435.

109. Li, Q., Shi, L., Gui, B., Yu, W., Wang, J., Zhang, D., Han, X., Yao, Z., and Shang, Y. (2011). Binding of the JmjC demethylase JARID1B to LSD1/NuRD suppresses angiogenesis and metastasis in breast cancer cells by repressing chemokine CCL14. Cancer research *71*, 6899-6908.

110. Lim, S., Janzer, A., Becker, A., Zimmer, A., Schule, R., Buettner, R., and Kirfel, J. Lysine-specific demethylase 1 (LSD1) is highly expressed in ER-negative breast cancers and a biomarker predicting aggressive biology. Carcinogenesis *31*, 512-520.

111. Lim, S., Janzer, A., Becker, A., Zimmer, A., Schule, R., Buettner, R., and Kirfel, J. (2010). Lysine-specific demethylase 1 (LSD1) is highly expressed in ER-negative breast cancers and a biomarker predicting aggressive biology. Carcinogenesis *31*, 512-520.

112. Lin, T., Ponn, A., Hu, X., Law, B.K., and Lu, J. (2010a). Requirement of the histone demethylase LSD1 in Snai1-mediated transcriptional repression during epithelial-mesenchymal transition. Oncogene *29*, 4896-4904.

113. Lin, Y., Dong, C., and Zhou, B.P. (2013). Epigenetic Regulation of EMT: The Snail Story. Current pharmaceutical design.

114. Lin, Y., Wu, Y., Li, J., Dong, C., Ye, X., Chi, Y.I., Evers, B.M., and Zhou, B.P. (2010b). The SNAG domain of Snail1 functions as a molecular hook for recruiting lysine-specific demethylase 1. The EMBO journal *29*, 1803-1816.
115. Lindeman, G.J., and Visvader, J.E. (2011). Cell fate takes a slug in BRCA1-associated breast cancer. Breast cancer research : BCR *13*, 306.
116. Littlepage, L.E., Adler, A.S., Kouros-Mehr, H., Huang, G., Chou, J., Krig, S.R., Griffith, O.L., Korkola, J.E., Qu, K., Lawson, D.A.*, et al.* (2012). The transcription factor ZNF217 is a prognostic biomarker and therapeutic target during breast cancer progression. Cancer discovery *2*, 638-651.
117. Liu, H. (2012). MicroRNAs in breast cancer initiation and progression. Cellular and molecular life sciences : CMLS *69*, 3587-3599.
118. Lowe, S.W., and Lin, A.W. (2000). Apoptosis in cancer. Carcinogenesis *21*, 485-495.
119. Lv, T., Yuan, D., Miao, X., Lv, Y., Zhan, P., Shen, X., and Song, Y. (2012). Over-expression of LSD1 promotes proliferation, migration and invasion in non-small cell lung cancer. PloS one *7*, e35065.
120. Macfarlan, T.S., Gifford, W.D., Agarwal, S., Driscoll, S., Lettieri, K., Wang, J., Andrews, S.E., Franco, L., Rosenfeld, M.G., Ren, B.*, et al.* (2011). Endogenous retroviruses and neighboring genes are coordinately repressed by LSD1/KDM1A. Genes & development *25*, 594-607.
121. Mackey, J.R., Kerbel, R.S., Gelmon, K.A., McLeod, D.M., Chia, S.K., Rayson, D., Verma, S., Collins, L.L., Paterson, A.H., Robidoux, A.*, et al.* (2012). Controlling angiogenesis in breast cancer: a systematic review of anti-angiogenic trials. Cancer treatment reviews *38*, 673-688.
122. Mellacheruvu, D., Wright, Z., Couzens, A.L., Lambert, J.P., St-Denis, N.A., Li, T., Miteva, Y.V., Hauri, S., Sardiu, M.E., Low, T.Y.*, et al.* (2013). The CRAPome: a contaminant repository for affinity purification-mass spectrometry data. Nature methods *10*, 730-736.
123. Metzger, E., Wissmann, M., Yin, N., Muller, J.M., Schneider, R., Peters, A.H., Gunther, T., Buettner, R., and Schule, R. (2005). LSD1 demethylates repressive histone marks to promote androgen-receptor-dependent transcription. Nature *437*, 436-439.
124. Micalizzi, D.S., Farabaugh, S.M., and Ford, H.L. (2010). Epithelial-mesenchymal transition in cancer: parallels between normal development and tumor progression. Journal of mammary gland biology and neoplasia *15*, 117-134.
125. Miller, K., Wang, M., Gralow, J., Dickler, M., Cobleigh, M., Perez, E.A., Shenkier, T., Cella, D., and Davidson, N.E. (2007). Paclitaxel plus

bevacizumab versus paclitaxel alone for metastatic breast cancer. The New England journal of medicine *357*, 2666-2676.

126. Miller, K.D., Chap, L.I., Holmes, F.A., Cobleigh, M.A., Marcom, P.K., Fehrenbacher, L., Dickler, M., Overmoyer, B.A., Reimann, J.D., Sing, A.P.*, et al.* (2005). Randomized phase III trial of capecitabine compared with bevacizumab plus capecitabine in patients with previously treated metastatic breast cancer. Journal of clinical oncology : official journal of the American Society of Clinical Oncology *23*, 792-799.

127. Miyoshi, H., Blomer, U., Takahashi, M., Gage, F.H., and Verma, I.M. (1998). Development of a self-inactivating lentivirus vector. Journal of virology *72*, 8150-8157.

128. Muller, B.M., Jana, L., Kasajima, A., Lehmann, A., Prinzler, J., Budczies, J., Winzer, K.J., Dietel, M., Weichert, W., and Denkert, C. (2013). Differential expression of histone deacetylases HDAC1, 2 and 3 in human breast cancer--overexpression of HDAC2 and HDAC3 is associated with clinicopathological indicators of disease progression. BMC cancer *13*, 215.

129. Mulligan, P., Westbrook, T.F., Ottinger, M., Pavlova, N., Chang, B., Macia, E., Shi, Y.J., Barretina, J., Liu, J., Howley, P.M.*, et al.* (2008). CDYL bridges REST and histone methyltransferases for gene repression and suppression of cellular transformation. Molecular cell *32*, 718-726.

130. Mulligan, P., Yang, F., Di Stefano, L., Ji, J.Y., Ouyang, J., Nishikawa, J.L., Toiber, D., Kulkarni, M., Wang, Q., Najafi-Shoushtari, S.H.*, et al.* (2011). A SIRT1-LSD1 corepressor complex regulates Notch target gene expression and development. Molecular cell *42*, 689-699.

131. Munari, F., Soeroes, S., Zenn, H.M., Schomburg, A., Kost, N., Schroder, S., Klingberg, R., Rezaei-Ghaleh, N., Stutzer, A., Gelato, K.A.*, et al.* (2012). Methylation of lysine 9 in histone H3 directs alternative modes of highly dynamic interaction of heterochromatin protein hHP1beta with the nucleosome. The Journal of biological chemistry *287*, 33756-33765.

132. Munster, P.N., Thurn, K.T., Thomas, S., Raha, P., Lacevic, M., Miller, A., Melisko, M., Ismail-Khan, R., Rugo, H., Moasser, M.*, et al.* (2011). A phase II study of the histone deacetylase inhibitor vorinostat combined with tamoxifen for the treatment of patients with hormone therapy-resistant breast cancer. British journal of cancer *104*, 1828-1835.

133. Murdoch, C., Muthana, M., Coffelt, S.B., and Lewis, C.E. (2008). The role of myeloid cells in the promotion of tumour angiogenesis. Nature reviews Cancer *8*, 618-631.

134. Murray-Stewart, T., Woster, P.M., and Casero, R.A., Jr. (2013). The re-expression of the epigenetically silenced e-cadherin gene by a polyamine analogue lysine-specific demethylase-1 (LSD1) inhibitor in human acute myeloid leukemia cell lines. Amino acids.
135. Nagy, J.A., Chang, S.H., Shih, S.C., Dvorak, A.M., and Dvorak, H.F. (2010). Heterogeneity of the tumor vasculature. Seminars in thrombosis and hemostasis *36*, 321-331.
136. Nakamura, T., Mori, T., Tada, S., Krajewski, W., Rozovskaia, T., Wassell, R., Dubois, G., Mazo, A., Croce, C.M., and Canaani, E. (2002). ALL-1 is a histone methyltransferase that assembles a supercomplex of proteins involved in transcriptional regulation. Molecular cell *10*, 1119-1128.
137. Nakaya, Y., and Sheng, G. (2013). EMT in developmental morphogenesis. Cancer letters.
138. Naldini, A., Filippi, I., Cini, E., Rodriquez, M., Carraro, F., and Taddei, M. (2012). Downregulation of hypoxia-related responses by novel antitumor histone deacetylase inhibitors in MDAMB231 breast cancer cells. Anti-cancer agents in medicinal chemistry *12*, 407-413.
139. Nassour, M., Idoux-Gillet, Y., Selmi, A., Come, C., Faraldo, M.L., Deugnier, M.A., and Savagner, P. (2012). Slug controls stem/progenitor cell growth dynamics during mammary gland morphogenesis. PloS one *7*, e53498.
140. Neve, R.M., Chin, K., Fridlyand, J., Yeh, J., Baehner, F.L., Fevr, T., Clark, L., Bayani, N., Coppe, J.P., Tong, F.*, et al.* (2006). A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. Cancer cell *10*, 515-527.
141. Nishizawa, Y., Miki, T., Nakatsuka, K., Hagiwara, S., Iba, K., and Morii, H. (1992). [Clinical aspects of bone mineral content in osteoporosis]. Nihon Ronen Igakkai zasshi Japanese journal of geriatrics *29*, 257-261.
142. Nyberg, P., Salo, T., and Kalluri, R. (2008). Tumor microenvironment and angiogenesis. Frontiers in bioscience : a journal and virtual library *13*, 6537-6553.
143. Ombra, M.N., Di Santi, A., Abbondanza, C., Migliaccio, A., Avvedimento, E.V., and Perillo, B. (2013). Retinoic acid impairs estrogen signaling in breast cancer cells by interfering with activation of LSD1 via PKA. Biochimica et biophysica acta *1829*, 480-486.
144. Osawa, T., Tsuchida, R., Muramatsu, M., Shimamura, T., Wang, F., Suehiro, J., Kanki, Y., Wada, Y., Yuasa, Y., Aburatani, H.*, et al.* (2013). Inhibition of histone demethylase JMJD1A improves anti-angiogenic

therapy and reduces tumor-associated macrophages. Cancer research *73*, 3019-3028.

145. Ouyang, J., Shi, Y., Valin, A., Xuan, Y., and Gill, G. (2009). Direct binding of CoREST1 to SUMO-2/3 contributes to gene-specific repression by the LSD1/CoREST1/HDAC complex. Molecular cell *34*, 145-154.

146. Peinado, H., Olmeda, D., and Cano, A. (2007). Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype? Nat Rev Cancer *7*, 415-428.

147. Pereira, J.D., Sansom, S.N., Smith, J., Dobenecker, M.W., Tarakhovsky, A., and Livesey, F.J. (2010). Ezh2, the histone methyltransferase of PRC2, regulates the balance between self-renewal and differentiation in the cerebral cortex. Proceedings of the National Academy of Sciences of the United States of America *107*, 15957-15962.

148. Poggi, A., and Zocchi, M.R. (2006). Mechanisms of tumor escape: role of tumor microenvironment in inducing apoptosis of cytolytic effector cells. Archivum immunologiae et therapiae experimentalis *54*, 323-333.

149. Pollard, J.W. (2004). Tumour-educated macrophages promote tumour progression and metastasis. Nature reviews Cancer *4*, 71-78.

150. Pollock, J.A., Larrea, M.D., Jasper, J.S., McDonnell, D.P., and McCafferty, D.G. Lysine-Specific Histone Demethylase 1 Inhibitors Control Breast Cancer Proliferation in ERalpha-Dependent and -Independent Manners. ACS Chem Biol.

151. Porta, C., Subhra Kumar, B., Larghi, P., Rubino, L., Mancino, A., and Sica, A. (2007). Tumor promotion by tumor-associated macrophages. Advances in experimental medicine and biology *604*, 67-86.

152. Proia, T.A., Keller, P.J., Gupta, P.B., Klebba, I., Jones, A.D., Sedic, M., Gilmore, H., Tung, N., Naber, S.P., Schnitt, S.*, et al.* (2011). Genetic predisposition directs breast cancer phenotype by dictating progenitor cell fate. Cell stem cell *8*, 149-163.

153. Pugh, C.W., and Ratcliffe, P.J. (2003). Regulation of angiogenesis by hypoxia: role of the HIF system. Nature medicine *9*, 677-684.

154. Qureshi, I.A., Gokhan, S., and Mehler, M.F. (2010). REST and CoREST are transcriptional and epigenetic regulators of seminal neural fate decisions. Cell cycle *9*, 4477-4486.

155. Raica, M., Cimpean, A.M., and Ribatti, D. (2009). Angiogenesis in pre-malignant conditions. European journal of cancer *45*, 1924-1934.

156. Rankin, E.B., and Giaccia, A.J. (2008). The role of hypoxia-inducible factors in tumorigenesis. Cell death and differentiation *15*, 678-685.

157. Ray, A., Alalem, M., and Ray, B.K. (2013). Loss of Epigenetic Kruppel-like Factor 4 Histone Deacetylase (KLF-4-HDAC)-mediated Transcriptional Suppression is Crucial in Increasing Vascular Endothelial Growth Factor (VEGF) Expression in Breast Cancer. The Journal of biological chemistry.

158. Relf, M., LeJeune, S., Scott, P.A., Fox, S., Smith, K., Leek, R., Moghaddam, A., Whitehouse, R., Bicknell, R., and Harris, A.L. (1997). Expression of the angiogenic factors vascular endothelial cell growth factor, acidic and basic fibroblast growth factor, tumor growth factor beta-1, platelet-derived endothelial cell growth factor, placenta growth factor, and pleiotrophin in human primary breast cancer and its relation to angiogenesis. Cancer research *57*, 963-969.

159. Rinn, J.L., Kertesz, M., Wang, J.K., Squazzo, S.L., Xu, X., Brugmann, S.A., Goodnough, L.H., Helms, J.A., Farnham, P.J., Segal, E.*, et al.* (2007). Functional demarcation of active and silent chromatin domains in human HOX loci by noncoding RNAs. Cell *129*, 1311-1323.

160. Rudolph, T., Beuch, S., and Reuter, G. (2013). Lysine-specific histone demethylase LSD1 and the dynamic control of chromatin. Biological chemistry.

161. Ruthenburg, A.J., Allis, C.D., and Wysocka, J. (2007). Methylation of lysine 4 on histone H3: intricacy of writing and reading a single epigenetic mark. Molecular cell *25*, 15-30.

162. Saijo, K., Winner, B., Carson, C.T., Collier, J.G., Boyer, L., Rosenfeld, M.G., Gage, F.H., and Glass, C.K. (2009). A Nurr1/CoREST pathway in microglia and astrocytes protects dopaminergic neurons from inflammation-induced death. Cell *137*, 47-59.

163. Saleque, S., Kim, J., Rooke, H.M., and Orkin, S.H. (2007). Epigenetic regulation of hematopoietic differentiation by Gfi-1 and Gfi-1b is mediated by the cofactors CoREST and LSD1. Molecular cell *27*, 562-572.

164. Schneider, A.C., Heukamp, L.C., Rogenhofer, S., Fechner, G., Bastian, P.J., von Ruecker, A., Muller, S.C., and Ellinger, J. (2011). Global histone H4K20 trimethylation predicts cancer-specific survival in patients with muscle-invasive bladder cancer. BJU international *108*, E290-296.

165. Schneider, B.P., Wang, M., Radovich, M., Sledge, G.W., Badve, S., Thor, A., Flockhart, D.A., Hancock, B., Davidson, N., Gralow, J.*, et al.* (2008). Association of vascular endothelial growth factor and vascular endothelial growth factor receptor-2 genetic polymorphisms with outcome in a trial of paclitaxel compared with paclitaxel plus bevacizumab in

advanced breast cancer: ECOG 2100. Journal of clinical oncology : official journal of the American Society of Clinical Oncology *26*, 4672-4678.

166. Schulte, J.H., Lim, S., Schramm, A., Friedrichs, N., Koster, J., Versteeg, R., Ora, I., Pajtler, K., Klein-Hitpass, L., Kuhfittig-Kulle, S*., et al.* (2009). Lysine-specific demethylase 1 is strongly expressed in poorly differentiated neuroblastoma: implications for therapy. Cancer research *69*, 2065-2071.

167. Seligson, D.B., Horvath, S., McBrian, M.A., Mah, V., Yu, H., Tze, S., Wang, Q., Chia, D., Goodglick, L., and Kurdistani, S.K. (2009). Global levels of histone modifications predict prognosis in different cancers. The American journal of pathology *174*, 1619-1628.

168. Seligson, D.B., Horvath, S., Shi, T., Yu, H., Tze, S., Grunstein, M., and Kurdistani, S.K. (2005). Global histone modification patterns predict risk of prostate cancer recurrence. Nature *435*, 1262-1266.

169. Semenza, G.L. (2000). Hypoxia, clonal selection, and the role of HIF-1 in tumor progression. Critical reviews in biochemistry and molecular biology *35*, 71-103.

170. Serce, N., Gnatzy, A., Steiner, S., Lorenzen, H., Kirfel, J., and Buettner, R. (2012). Elevated expression of LSD1 (Lysine-specific demethylase 1) during tumour progression from pre-invasive to invasive ductal carcinoma of the breast. BMC clinical pathology *12*, 13.

171. Serrano, L., Vazquez, B.N., and Tischfield, J. (2013). Chromatin structure, pluripotency and differentiation. Experimental biology and medicine *238*, 259-270.

172. Shahbazian, M.D., and Grunstein, M. (2007). Functions of site-specific histone acetylation and deacetylation. Annual review of biochemistry *76*, 75-100.

173. Shi, Y., Lan, F., Matson, C., Mulligan, P., Whetstine, J.R., Cole, P.A., Casero, R.A., and Shi, Y. (2004). Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. Cell *119*, 941-953.

174. Shi, Y., Sawada, J., Sui, G., Affar el, B., Whetstine, J.R., Lan, F., Ogawa, H., Luke, M.P., Nakatani, Y., and Shi, Y. (2003). Coordinated histone modifications mediated by a CtBP co-repressor complex. Nature *422*, 735-738.

175. Shi, Y.J., Matson, C., Lan, F., Iwase, S., Baba, T., and Shi, Y. (2005). Regulation of LSD1 histone demethylase activity by its associated factors. Molecular cell *19*, 857-864.

176. Shipitsin, M., Campbell, L.L., Argani, P., Weremowicz, S., Bloushtain-Qimron, N., Yao, J., Nikolskaya, T., Serebryiskaya, T.,

Beroukhim, R., Hu, M.*, et al.* (2007). Molecular definition of breast tumor heterogeneity. Cancer cell *11*, 259-273.

177. Soares, J., Pinto, A.E., Cunha, C.V., Andre, S., Barao, I., Sousa, J.M., and Cravo, M. (1999). Global DNA hypomethylation in breast carcinoma: correlation with prognostic factors and tumor progression. Cancer *85*, 112-118.

178. Soon, P.S., and Kiaris, H. (2013). MicroRNAs in the tumor microenvironment: big role for small players. Endocrine-related cancer.

179. Sorlie, T. (2004). Molecular portraits of breast cancer: tumour subtypes as distinct disease entities. European journal of cancer *40*, 2667-2675.

180. Strahl, B.D., and Allis, C.D. (2000). The language of covalent histone modifications. Nature *403*, 41-45.

181. Sun, G., Alzayady, K., Stewart, R., Ye, P., Yang, S., Li, W., and Shi, Y. (2010). Histone demethylase LSD1 regulates neural stem cell proliferation. Molecular and cellular biology *30*, 1997-2005.

182. Swartz, M.A., Iida, N., Roberts, E.W., Sangaletti, S., Wong, M.H., Yull, F.E., Coussens, L.M., and DeClerck, Y.A. (2012). Tumor microenvironment complexity: emerging roles in cancer therapy. Cancer research *72*, 2473-2480.

183. Tang, N., Wang, L., Esko, J., Giordano, F.J., Huang, Y., Gerber, H.P., Ferrara, N., and Johnson, R.S. (2004). Loss of HIF-1alpha in endothelial cells disrupts a hypoxia-driven VEGF autocrine loop necessary for tumorigenesis. Cancer cell *6*, 485-495.

184. Thillainadesan, G., Isovic, M., Loney, E., Andrews, J., Tini, M., and Torchia, J. (2008). Genome analysis identifies the p15ink4b tumor suppressor as a direct target of the ZNF217/CoREST complex. Molecular and cellular biology *28*, 6066-6077.

185. Tontsch, S., Zach, O., and Bauer, H.C. (2001). Identification and localization of M-CoREST (1A13), a mouse homologue of the human transcriptional co-repressor CoREST, in the developing mouse CNS. Mechanisms of development *108*, 165-169.

186. Tsai, M.C., Manor, O., Wan, Y., Mosammaparast, N., Wang, J.K., Lan, F., Shi, Y., Segal, E., and Chang, H.Y. (2010). Long noncoding RNA as modular scaffold of histone modification complexes. Science *329*, 689-693.

187. Turner, B.M. (2000). Histone acetylation and an epigenetic code. BioEssays : news and reviews in molecular, cellular and developmental biology *22*, 836-845.

188. Vaissiere, T., Sawan, C., and Herceg, Z. (2008). Epigenetic interplay between histone modifications and DNA methylation in gene silencing. Mutation research *659*, 40-48.
189. Vakoc, C.R., Mandat, S.A., Olenchock, B.A., and Blobel, G.A. (2005). Histone H3 lysine 9 methylation and HP1gamma are associated with transcription elongation through mammalian chromatin. Molecular cell *19*, 381-391.
190. Vargo-Gogola, T., and Rosen, J.M. (2007). Modelling breast cancer: one size does not fit all. Nature reviews Cancer *7*, 659-672.
191. Vasilatos, S.N., Katz, T.A., Oesterreich, S., Wan, Y., Davidson, N.E., and Huang, Y. (2013). Crosstalk between lysine-specific demethylase 1 (LSD1) and histone deacetylases mediates antineoplastic efficacy of HDAC inhibitors in human breast cancer cells. Carcinogenesis.
192. Waldmann, T., and Schneider, R. (2013). Targeting histone modifications--epigenetics in cancer. Current opinion in cell biology *25*, 184-189.
193. Wang, J., Lu, F., Ren, Q., Sun, H., Xu, Z., Lan, R., Liu, Y., Ward, D., Quan, J., Ye, T.*, et al.* (2011). Novel histone demethylase LSD1 inhibitors selectively target cancer cells with pluripotent stem cell properties. Cancer research *71*, 7238-7249.
194. Wang, J., Scully, K., Zhu, X., Cai, L., Zhang, J., Prefontaine, G.G., Krones, A., Ohgi, K.A., Zhu, P., Garcia-Bassets, I.*, et al.* (2007). Opposing LSD1 complexes function in developmental gene activation and repression programmes. Nature *446*, 882-887.
195. Wang, Y., Guan, Y., Wang, F., Huang, A., Wang, S., and Zhang, Y.A. (2010). Bmi-1 regulates self-renewal, proliferation and senescence of human fetal neural stem cells in vitro. Neuroscience letters *476*, 74-78.
196. Weis, S.M., and Cheresh, D.A. (2011). Tumor angiogenesis: molecular pathways and therapeutic targets. Nature medicine *17*, 1359-1370.
197. Wen, B., Wu, H., Shinkai, Y., Irizarry, R.A., and Feinberg, A.P. (2009). Large histone H3 lysine 9 dimethylated chromatin blocks distinguish differentiated from embryonic stem cells. Nature genetics *41*, 246-250.
198. Widschwendter, M., and Jones, P.A. (2002). DNA methylation and breast carcinogenesis. Oncogene *21*, 5462-5482.
199. Wu, Z.Q., Li, X.Y., Hu, C.Y., Ford, M., Kleer, C.G., and Weiss, S.J. (2012). Canonical Wnt signaling regulates Slug activity and links epithelial-mesenchymal transition with epigenetic Breast Cancer 1, Early Onset

(BRCA1) repression. Proceedings of the National Academy of Sciences of the United States of America *109*, 16654-16659.

200. Xiao, J., Li, T., Wu, Z., Shi, Z., Chen, J., Lam, S.K., Zhao, Z., Yang, L., and Qiu, G. (2010). REST corepressor (CoREST) repression induces phenotypic gene regulation in advanced osteoarthritic chondrocytes. Journal of orthopaedic research : official publication of the Orthopaedic Research Society *28*, 1569-1575.

201. Xue, J.H., Zheng, M., Xu, X.W., Wu, S.S., Chen, Z., and Chen, F. (2011). Involvement of REST corepressor 3 in prognosis of human hepatitis B. Acta pharmacologica Sinica *32*, 1019-1024.

202. Yang, M., Gocke, C.B., Luo, X., Borek, D., Tomchick, D.R., Machius, M., Otwinowski, Z., and Yu, H. (2006). Structural basis for CoREST-dependent demethylation of nucleosomes by the human LSD1 histone demethylase. Molecular cell *23*, 377-387.

203. Yang, P., Wang, Y., Chen, J., Li, H., Kang, L., Zhang, Y., Chen, S., Zhu, B., and Gao, S. (2011). RCOR2 is a subunit of the LSD1 complex that regulates ESC property and substitutes for SOX2 in reprogramming somatic cells to pluripotency. Stem cells *29*, 791-801.

204. You, A., Tong, J.K., Grozinger, C.M., and Schreiber, S.L. (2001). CoREST is an integral component of the CoREST- human histone deacetylase complex. Proceedings of the National Academy of Sciences of the United States of America *98*, 1454-1458.

205. Zhang, J., Bonasio, R., Strino, F., Kluger, Y., Holloway, J.K., Modzelewski, A.J., Cohen, P.E., and Reinberg, D. (2013). SFMBT1 functions with LSD1 to regulate expression of canonical histone genes and chromatin-related factors. Genes & development *27*, 749-766.

206. Zhang, Z., Yamashita, H., Toyama, T., Sugiura, H., Omoto, Y., Ando, Y., Mita, K., Hamaguchi, M., Hayashi, S., and Iwase, H. (2004). HDAC6 expression is correlated with better survival in breast cancer. Clinical cancer research : an official journal of the American Association for Cancer Research *10*, 6962-6968.

207. Zhao, Z.K., Yu, H.F., Wang, D.R., Dong, P., Chen, L., Wu, W.G., Ding, W.J., and Liu, Y.B. (2012). Overexpression of lysine specific demethylase 1 predicts worse prognosis in primary hepatocellular carcinoma patients. World journal of gastroenterology : WJG *18*, 6651-6656.

208. Zhu, Q., Huang, Y., Marton, L.J., Woster, P.M., Davidson, N.E., and Casero, R.A., Jr. Polyamine analogs modulate gene expression by

inhibiting lysine-specific demethylase 1 (LSD1) and altering chromatin structure in human breast cancer cells. Amino acids.

209. Zhu, Q., Huang, Y., Marton, L.J., Woster, P.M., Davidson, N.E., and Casero, R.A., Jr. (2012). Polyamine analogs modulate gene expression by inhibiting lysine-specific demethylase 1 (LSD1) and altering chromatin structure in human breast cancer cells. Amino acids *42*, 887-898.