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UNIVERSITY OF CALIFORNIA, SAN DIEGO

**Sumoylation strategies in regulated repression by nuclear receptors and in  
function of tumor metastasis suppressor genes.**

A Dissertation submitted in partial satisfaction of the Requirements for the degree  
of Doctor of Philosophy

in

Biology

by

Ling Cai

Committee in Charge:

Professor Michael G. Rosenfeld, Chair  
Professor Cornelis Murre, Co-Chair  
Professor Christopher Glass  
Professor Randy Hampton  
Professor Mark Kamps

2008

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Co-Chair

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Chair

University of California, San Diego

2008

## **DEDICATION**

This thesis is dedicated to my parents who nurture me and offer me unconditional love and support throughout my life

Also, this thesis is dedicated to my husband who loves me, helps me and has been a great source of motivation and inspiration.

Finally, this thesis is dedicated to my lovely brother who cares me and supports me at my faraway hometown on the other side of the Pacific Ocean.

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## CURRICULUM VITAE

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- Characterized the molecular mechanisms for the selective down-regulation of a tumor metastasis-suppressor gene KAI1/CD82 in metastatic prostate cancer models.
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## **Publications**

1, Wang GG, **Cai L**, Pasillas MP, Kamps MP. NUP98-NSD1 links H3K36 methylation to Hox-A gene activation and leukaemogenesis. *Nat Cell Biol.* 2007 Jul;9(7):804-12.

2, Wang J, **Cai L\***, Scully K\*, Zhu X\*, Zhang J, Prefontaine GG, Krones A, Ohgi KA, Zhu P, Garcia-Bassets I, Liu F, Taylor H, Lozach J, Jayes FL, Korach KS, Glass CK, Fu XD, Rosenfeld MG. Opposing LSD1 complexes function in developmental gene activation and repression programmes. *Nature.* 2007 Apr 19;446(7138):882-7. \*These authors contributed equally to this paper.

3, **Cai L\***, Kim JH\*, Kim B\*, Choi HJ, Ohgi KA, Tran C, Chen C, Chung CH, Huber O, Rose DW, Sawyers CL, Rosenfeld MG, Baek SH. Transcriptional regulation of a metastasis suppressor gene by Tip60 and beta-catenin complexes. *Nature.* 2005 Apr 14; 434(7035): 921-6. \*These authors contributed equally to this paper.

4, **Cai L**, Zhu JD. The tumor-selective over-expression of the human Hsp70 gene is attributed to the aberrant controls at both initiation and elongation levels of transcription. *Cell Res.* 2003 Apr;13(2):93-109.

## **Conference Presentation**

2007 The 2007 research conference of the U.S. Department of Defense Prostate Cancer Research Program- “Innovative Minds in Prostate Cancer Today” (IMPACT) Meeting in Atlanta. Selected speech presentation, title “The Role of SUMOylation in Cancer Metastasis”.

## ABSTRACT OF THE DISSERTATION

**Sumoylation strategies in regulated repression by nuclear receptors and in function of tumor metastasis suppressor genes.**

By

Ling Cai

Doctor of Philosophy in Biology

University of California, San Diego, 2008

Professor Michael G. Rosenfeld, Chair

Professor Cornelis Murre, Co-Chair

Elucidating molecular mechanisms underlying transcriptional regulation of genes that are critical for normal and pathological development remains a central issue in biology and medicine.

Although down-regulation of tumor metastasis suppressor genes are commonly observed in high-risk tumors, the responsible mechanisms have rarely been identified. Here I report that the down-regulation of *KAI1*, a metastasis suppressor gene, in prostate cancer cells involved the inhibitory function of a  $\beta$ -catenin-Reptin complex, which required both induced  $\beta$ -catenin expression and recruitment of HDAC1 by Reptin. On the contrary, the transcriptional activation of

*KAI1* required a sufficiently high level of Tip60 coactivator, which itself was negatively regulated by  $\beta$ -catenin. The coordinated actions of  $\beta$ -catenin-Reptin repressive complex antagonize a Tip60 coactivator complex. The balance of these opposing complexes controlled the expression of *KAI1* and metastatic potential in prostate cancers.

To understand how Reptin confers its transcriptional repressive function, I identified Reptin-interacting cofactors including UBC9 and ASXL1 by utilizing yeast two-hybrid screening, and investigated their roles in the functional regulation of Reptin. I discovered that Reptin was subjected to sumo conjugation and sumoylation was essential for its repressive function. Furthermore, I found that, when working together with ASXL1 and LSD1, Reptin acted as a transcriptional coactivator on multiple *Hox* gene promoters in NTera2 cells. Thus, Reptin appears to play dual roles in transcriptional regulation in a context-dependent manner.

Lastly, I investigated how sumoylation mediated LSD1 functional switch from transcriptional repression to activation. I found that sumoylation enhanced LSD1 mediated-repression on Rest target promoters in nonneuronal cells; Knockdown of PIAS1, a LSD1 sumo E3 ligase, abrogated the recruitment of LSD1 on these target genes, concomitant with subsequent de-repression of these genes and increased H3K4me2 levels. On the other hand, activation of AR signaling by its ligand induced the recruitment of SENP1, a LSD1 sumo protease, and the de-sumoylation of LSD1 on the AR- target genes, accompanied by dramatically reduced H3K9me2 levels; Thus, sumoylated forms of LSD1

correlated with its gene repression function, while de-sumoylated forms of LSD1 correlated with its gene activation function together with AR. In summary, the changes in sumoylation-desumoylation status induced a switch of LSD1 functions in transcriptional regulation.

## **Chapter I**

### **Introduction**

**KAI1 is a tumor metastasis suppressor gene.**

Prostate cancer is one of the most common cancers affecting men in the United States, and its incidence has risen by 60~75% in the last 15 years in the developed countries (Siddiqui et al. 2004). Although surgery and radiotherapy is relatively effective for prostate cancer patients at early stage, one major difficulty to treat prostate cancer successfully is to suppress metastasis, which shows a positive correlation to high morbidity and high mortality (Siddiqui et al. 2004). Metastasis, by definition, is the process by which tumor cells disseminate from the primary tumor, migrate through the basement membrane, survive in the circulatory system, invade into a secondary site and start to proliferate (Stafford et al. 2008). The mechanism of metastasis remains one of the least understood aspects of prostate cancer and other cancer types. Specifically, mechanisms by which metastasis is promoted or inhibited in cancer cells are generally unclear, which is reflected by lack of effective treatments for metastatic cancers. The development for therapeutic methods to suppress metastasis remains to be one of most challenging issues in this field.

*KAI1* ('kang ai' [Chinese for anticancer], also designated as CD82) was initially identified as a metastasis suppressor gene that inhibits metastasis at any step of the metastatic cascade without blocking primary tumor growth (Stafford et al. 2008). *KAI1* gene, locates on human chromosome 11p11.2, encodes a transmembrane protein with 267 amino acids (Dong et al. 1995). Due to glycosylation, *KAI1* has a molecular weight of 46-60 KDa, rather than predicted 28KDa based on amino acid number (White et al. 1998). *KAI/CD82* protein has

cytoplasmic N- and C-terminals, and transverses the cell membrane four times, forming one small and one large extracellular loop with residues susceptible to post-translational modifications such as phosphorylation and glycosylation (Rubinstein et al. 1999). Initial evidence that defined *KAI1* as a metastasis suppressor gene came from rat prostate cancer models, where the expression of *KAI1* specifically inhibited tumor metastasis, but did not affect the incidence or growth rate of tumors (Dong et al. 1995). Consistently, the expression of KAI1 has been shown to be down regulated during the progression of a variety of human cancers, including prostate cancers (Dong et al. 1995), lung cancers (Adachi et al. 1996; Higashiyama et al. 1998; Miyake et al. 1999; Goncharuk et al. 2004), pancreas cancers (Guo et al. 1996), breast cancers (Yang et al. 1997; Huang et al. 1998), colorectal cancers (Takaoka et al. 1998b; Lombardi et al. 1999), ovarian cancers (Liu et al. 2000), esophageal cancers (Uchida et al. 1999), oral cancers (Farhadieh et al. 2004), cervical cancers (Liu et al. 2001) and melanomas (Takaoka et al. 1998a). *KAI1* down-regulation has been suggested to alter adhesion to specific components of the extracellular matrix such as fibronectin, reduce cell-cell interactions and increase cell motility, which leads to a more invasive and metastatic ability of tumor cells (Jackson et al. 2005). These observations indicate a general metastasis-inhibitory mechanism mediated by KAI1. In addition, KAI1 has also been reported to interact with integrin  $\alpha 4\beta 1$ , other transmembrane-4 superfamily (TM4SF) proteins and cell surface molecules such as CD4, CD8, CD19, CD21 and MHC class I and class II, forming what is now known as “ the tetraspanin web” (Hemler et al. 2001). How does KAI1/CD82

suppress cancer invasion and metastasis? One potential mechanism is that KAI1 inhibits cell motility by regulating its associated proteins such as integrin, EGF receptor (EGFR), PKC, KAI1-associated protein (KASP), KITENIN and etc. For example, it has been shown that interaction between KAI1 and EGFR attenuates EGFR-induced lamellipodia formation and migration signaling through affecting dimerization and internalization of EGFR (Odintsova et al. 2000; Odintsova et al. 2003). KAI1 also inhibits the formation of active p130<sup>CAS</sup>-CrkII complex, which positively regulates the organization of actin cytoskeleton and then suppresses cell motility (Zhang et al. 2003a). KASP, a member of the immunoglobulin superfamily, was shown to inhibit cell migration, and interaction with KAI1 enhances this inhibitory effect synergistically (Zhang et al. 2003b). KITENIN, another TM4SF protein, enhances invasion and metastatic behavior of tumor cells, and binding to KAI1 attenuates its function (Lee et al. 2004).

As mentioned earlier, *KAI1* expression is found frequently downregulated during prostate cancer progression (Dong et al. 1995). Such down-regulation does not appear to involve either an allelic loss or a mutation, implying that the reduction of *KAI1* gene expression is achieved at the transcriptional level (Dong et al. 1996). In Chapter II, I will report a potential mechanism that is responsible for regulating KAI1 down-expression in prostate cancer cells, which involves an antagonistic regulation of a  $\beta$ -catenin-Reptin transcriptional corepressor complex and a Tip60 transcriptional coactivator complex.

## **Reptin and Pontin**

Reptin and Pontin are two closely related ATP-dependent helicases. They are quite similar in primary protein sequence and exhibit a high evolutionary conservation among organisms from bacteria to mammals. Reptin is also called Reptin52, RUVBL2, Tip48, Tip49b, ECP51, TAP54 $\beta$ , RVB2 and TIH2. Pontin is also called Pontin52, RUVBL1, Tip49, Tip49a, ECP54, TAP54 $\alpha$ , RVB1 and TIH1. In bacteria, they are closely related to the bacterial DNA helicase RuvB, a member of the AAA+ family of helicases (Ogura et al. 2001). Bacterial RuvB helicase catalyzes the branch migration of Holliday junctions during homologous recombination or replication (Yamada et al. 2004; McGlynn et al. 2002). In yeast, Reptin and Pontin are essential components of INO80 chromatin remodeling complex, which either activates or represses a large number of genes by mobilizing nucleosomes and altering the accessibility of the underlying DNA to the transcriptional machinery (Shen et al. 2000; Jonsson et al. 2004). Reptin and Pontin share a subset of common target genes with the Ino80 complex (Jonsson et al. 2004). Reptin and Pontin regulate the transcription of over 5% of yeast genes, and it has also been suggested that they function in the same complex and have a role in both gene activation and gene repression in yeast (Jonsson et al. 2001). Reptin and Pontin are also present in the SWR1 complex (SRCAP complex in mammals). This complex deposits the variant histone Htz1 (also known as H2A.Z) in euchromatic sequences located next to the heterochromatin found at yeast telomeres, silent mating loci or in rDNA-encoding regions. In the absence of Htz1, heterochromatin spreads into the euchromatin and silences the expression of the genes in this region. Upon deletion of Swr1, many genes at the

same region are also repressed, indicating that Swr1 and Htz1 act in the same pathway (Krogan et al. 2003; Kobor et al. 2004; Mizuguchi et al. 2004). In *Drosophila*, Reptin and Pontin function antagonistically to regulate *Hox* gene expression. Reptin is incorporated into PRC1 complex and represses *Hox* gene expression, whereas Pontin interacts with the Brama complex and induces the activation of *Hox* genes (Diop et al. 2008). In mammals, Reptin and Pontin are integral subunits of Tip60 complex, a mammalian counterpart of the yeast Ino80 (or Swr1) complex and NuA4 complex combined. Tip60 complex has been shown to be involved in gene transcription and DNA repair (Ikura et al. 2000). Reptin and Pontin also share residency in other complexes including MLL1, Uri1 and Telomerase complexes. The presence of Reptin and Pontin in MLL1 complex suggested their involvement in *Hox* gene regulation (Dou et al. 2005). Uri1 complexes, which contain Reptin, Pontin, E3-ubiquitin ligase SCF<sup>Skp2</sup>, Rbp5, and several prefoldin-related proteins such as Uri1, play a critical role at the downstream of TOR pathway, mediating the repression of TOR-repressed genes in response to extracellular nutrient levels (Gstaiger et al. 2003). Recently, Reptin and Pontin have also been co-purified in the telomerase complex, and they have been shown to be essential for the assembly of telomerase holoenzyme (Venteicher et al. 2008). Furthermore, both Reptin and Pontin also interact with other transcription-associated proteins such as  $\beta$ -catenin (Bauer et al. 1998, 2000), TBP (Ohdate et al. 2003), and Myc (Wood et al. 2000). Pontin also associates with E2F1 (Dugan et al. 2002), and Reptin interacts with ATF2 (Cho et al. 2001). Despite their roles in chromatin remodeling and transcriptional

regulation, Reptin and Pontin also associate with microtubular structure and involved in mitosis (Gartner et al. 2003; Skop et al. 2004; Sigala et al. 2005), and play a critical role in the maturation of small nucleolar RNAs (Newman et al. 2000; King et al. 2001; Watkins et al. 2004). Combined together, the stable co-purification of Reptin and Pontin in multiple highly conserved protein complexes and their transient interaction with other critical regulators indicate their versatile function in many facets of cellular activities, which at least include gene transcription, DNA double-strand break repair, apoptosis, telomerase activities, and etc. In chapter II, I will elucidate the mechanisms by which Reptin and Pontin regulate the expression of *KAI1*, a tumor metastasis suppressor gene, in prostate cancer models.

### **Sumo pathway: sumoylation and desumoylation**

Post-translational modification, including phosphorylation, acetylation, ubiquitination, sumoylation, methylation and etc, is widely used to regulate protein behavior and protein-protein interaction. For example, ubiquitination plays an essential role in targeting proteins for proteasome-mediated degradation, although sometimes it rather regulates protein localization or activity instead. Besides ubiquitin, some ubiquitin like proteins (Ubls) have also been found to be conjugated to substrate proteins and affect their functions. SUMO (small ubiquitin related modifier) is one of the Ubls. Sumoylation has been reported to be important in many cellular processes including signal transduction, gene transcription, chromatin structure, cellular localization, DNA repair and

maintenance of the genome stability. A consensus sumo acceptor motif has been identified, i.e.,  $\Psi$ KXD/E where  $\Psi$  is a large hydrophobic amino acid (such as I, V or L), K is the site of sumo conjugation and X is any amino acid (Rodriguez et al. 2001). There is only a single sumo gene (Smt3) in yeast genome, and four sumo-encoding genes (sumo1, sumo2, sumo3 and sumo4) in mammals. The mature forms of sumo2 and sumo3 only differ from each other by three N-terminal residues and form a distinct subfamily known as sumo2/3. Sumo2/3 and sumo1 are identical in ~50% of their protein sequence. Sumo4 was recently identified and exhibits a more restricted expression pattern with high levels in the kidney cells (Bohren et al. 2004). Almost all of sumo1 proteins are conjugated to substrates, and a free pool of sumo2/3 is reserved for usage under stress condition such as heat shock or ethanol exposure (Saitoh et al. 2000). Sumo1 and sumo2/3 modify both common and distinct substrates. For example, YY1 was found simultaneously modified by sumo1 and sumo2/3, whereas RanGAP1 is preferentially modified by sumo1 and Topoisomerase II predominantly modified by sumo2/3 during mitosis (Deng et al. 2007; Saitoh et al. 2000; Azuma et al. 2003). Sumo1 modification of nuclear receptor PPAR $\gamma$  and sumo2/3 modification of LXR confer independent control of transrepression in macrophages (Ghisletti et al. 2007; Pascual et al. 2005). However, mechanisms underlying selective modification by specific sumo isoforms and their corresponding functional significance remain to be further elucidated.

It is known that ubiquitin can be conjugated to substrate either as monomer or polymer. The polymeric chains of ubiquitin have different biological

activities. For instance, while the K48-linked ubiquitin chain interacts with proteasome and mediates protein degradation, the K63-linked ubiquitin chain signals nonproteolytic outcomes such as regulating transient formation of functional macromolecular complexes or relocating modified proteins inside the cell (Hjerpe et al. 2008). In contrast, sumo is generally thought to function as a monomer, although sumo2/3 does possess sumo conjugation consensus motif that can be used to form polymeric chains *in vitro* (Tatham et al. 2001). Poly-sumo chains were detected in yeast, and yeast cells expressing a form of sumo that cannot form polymeric chains exhibit defects in spore formation, indicating that poly-sumoylation is essential for the formation of normal synaptonemal complex during meiosis (Bylebyl et al. 2003; Cheng et al. 2006). The activity and function of poly-sumoylation in mammals has not been reported yet.

In biochemistry, sumoylation pathway shares high similarity to ubiquitination pathway. First, ubiquitin and sumo are each covalently conjugated to target proteins by an isopeptide bond between a C-terminal glycine in the Ubl and a lysine residue in the substrate. Second, similar to ubiquitination, four categories of enzymes have been identified in sumo-conjugating process: sumo-specific proteases (SENPs), E1 activating enzyme, E2 conjugating enzyme and E3 ligases. Sumo precursor is cleaved by SENPs with their C-terminal hydrolase activity to expose a C-terminal glycine-glycine. Then, in an ATP-dependent reaction, mature sumo forms a thioester conjugate with the heterodimeric sumo E1 activating enzyme Aos1/Uba2. Sumo is then transferred to and forms a thioester intermediate with the only sumo E2 conjugating enzyme Ubc9. Finally,

sumo is transferred from Ubc9 to the lysine residue (K) in a substrate protein via an isopeptide bond. During the last step, several sumo E3 ligases have been identified to stimulate the transfer of sumo from Ubc9 to a specific substrate. So far, only three groups of sumo E3 ligase have been reported, RanBP2, the PIAS proteins, and the polycomb protein Pc2 (Pichler et al. 2002; Kahyo et al. 2001; Kagey et al. 2003). A common motif that is responsible for the E3 ligase activity has not been identified, as these proteins are quite distinct except that PIAS proteins share a RING finger, an essential domain for their SUMO E3 ligase activity (Kahyo et al. 2001). These E3 ligases also exhibit distinct subcellular localizations. RanBP2 is associated with the nuclear pore complex, PIAS proteins are localized to the nucleoplasm and nuclear bodies, and Pc2 is found in a subnuclear structure called “polycomb body” (Pichler et al. 2002; Sachdev et al. 2001; kotaja et al. 2002; Kagey et al. 2003). Specific cellular localization of these sumo E3 ligases may contribute to their functional specificity *in vivo*. For example, RanBP2 promotes sumo modification of RanGAP1 and Sp100 but not others such as p53 (pichler et al. 2002); Pc2 is co-localized with Ubc9, sumo and CtBP1 in polycomb bodies and promotes CtBP1 sumoylation (Kagey et al. 2003).

As a dynamic and reversible process, sumo can be removed from conjugated substrates by SENPs. Ulp1 and Ulp2, two only identified Smt3-specific proteases in yeast, deconjugate Smt3 (sumo) from substrates (or from Smt3 precursors, generating mature forms) (Li et al. 1999; Li et al. 2000; Takahashi et al. 2000). According to sequence homology to yeast Ulp1-2, seven human homologues (SEN1-3, 5-8) have been identified to possess activity of

sumo proteases (Yeh et al. 2000). Among them, SENP1, SENP2, SENP3 and SENP5 are related to Ulp1, and SENP6 and SENP7 are related to Ulp2 (Gong et al. 2000; Nishida et al. 2000; Hang et al. 2002; Best et al. 2002; Gong et al. 2006; Di Bacco et al. 2006; Kim et al. 2000). SENP8 is actually a NEDD8 specific protease (Mendoza et al. 2003). All SENPs share a conserved C-terminal catalytic domain, but the N-terminal regions are quite different. It was implicated that N-terminal regions are responsible for directing SENPs to distinct subcellular localizations and targeting their specific substrates (Melchior et al. 2003). SENP1 has been localized in the nucleoplasm and nuclear bodies, and SENP2 localized to the nuclear pore, SENP3 to the nucleolus, and SENP6 was originally reported to be cytoplasmic but recent evidence suggests that it is located in the nucleus (Gong et al. 2000; Nishida et al. 2000; Hang et al. 2002; Kim et al. 2000; Mukhopadhyay et al. 2006). SENP1 is an essential gene as SENP1 deficient embryos showed severe fetal anemia caused by deficient erythropoietin (Epo) production and died at midgestation (Cheng et al. 2007). SENP1-deficient cells accumulated both immature sumo1 and sumo1-conjugated substrates, but processing and deconjugation of sumo2/3 were found unchanged (Yamaguchi et al. 2005). SENP3 and SENP5 have a preference for deconjugating and processing sumo2/3 over sumo1 (Gong et al. 2006; Di Bacco et al. 2006). SENP6 seems to have a preference for cleavage of polymeric chains of sumo2/3.

Sumo modification of transcription factors is critical for their regulatory role in gene expression. In general, it is thought that sumo modification is linked to

transcriptional repression (Gill et al. 2005). While sumo conjugation enhances the repressive activity of transcription factors, the removal of sumo from transcription factors by specific SENPs reverses transcriptional repression (Hay et al. 2007). For instance, SENP2 negatively regulates sumo-dependent repression of Elk-1 and Sp3 (Yang et al. 2003; Ross et al. 2002); SENP1 relieved sumo-dependent repression of Reptin, Ets1, c-Jun and HDAC1 (Kim et al. 2006; Degerny et al. 2005; Cheng et al. 2005; Cheng et al. 2004).

In Chapter III and V, I will dissect the regulatory role of sumoylation-desumoylation cycling in the transcriptional regulation mediated by Reptin and LSD1, a histone lysine demethylase.

### ***Hox* gene regulation**

The *Hox* related homeobox genes encode homeodomain (HD)-containing transcription factors, which determine the anterior-to-posterior patterning and segmental identities during embryogenesis, *Hox* factors have also been found to be critical in regulating cellular proliferation and/or differentiation of lineage-specific progenitors during hematopoiesis (Pearson et al. 2005). A total of 39 mammalian class I *Hox* genes are located within four evolutionarily conserved chromosomal loci in forms of tandem arrays, designated as *Hox-A*, *Hox-B*, *Hox-C* and *Hox-D* gene clusters (Pearson et al. 2005). Precise regulation of spatial and temporal expression of *Hox* genes is critical as each of encoded *Hox* factors controls the formation of a diversified segmental structure along the anterior-to-posterior of animals (Pearson et al. 2005). In *Drosophila*, the phenotype induced

by inappropriate expression of *Hox* genes is termed as homeotic transformation, which is characterized by alteration of the morphology of one segment into that of another (Pearson et al. 2005). Earlier genetic screens based on homeotic transformation phenotype in fly led to identification of two antagonizing groups of the *Hox* locus regulators, the Trithorax group (TrxG) and Polycomb group (PcG) factors (Ringrose et al. 2004). Trithorax group proteins function as transcriptional activators and maintain the “ON” state of *Hox* loci, whereas polycomb group factors serve as transcriptional repressors and maintain the “OFF” state of *Hox* loci (Milne et al. 2002; Cao et al. 2004). Interestingly, Reptin and Pontin have recently been reported to act antagonistically to cooperate with polycomb and trithorax proteins, with Reptin as a corepressor of *Hox* gene transcription and Pontin as a coactivator (Diop et al. 2008). However, the function of Reptin and Pontin in mammalian *Hox* gene regulation remains unknown. In Chapter III, I identified ASXL1 as a novel Reptin-interacting protein by using yeast two-hybrid screening. Previous genetic studies have indicated that ASX, the *Drosophila* homologue of ASXL1, is unique in that it acts as an enhancer of both trithorax and polycomb genes and it has been shown required for both activation and repression of *Hox* gene clusters, as *Asx* mutation caused both anterior and posterior homeotic transformations (Milne et al. 1999). Consistently, mASXL1, murine homolog of ASX/ASXL1, exhibits a spatial expression pattern along the anterior-posterior axis, similar to that of *Hox* genes (Chen et al. 2004). The full-length ASXL1 protein contains 1541 amino acids, weighs about 170kDa and harbors three conserved regions named as the ASXN, ASXM, and PHD

domains. ASXL1 has recently been identified to interact with retinoic acid receptor (RAR) in retinoic acid (RA)-dependent fashion, acting as a coactivator of RAR through functional cooperation with SRC-1 (Cho et al. 2006). In Chapter III, I will examine how Reptin and its associated factor ASXL1 regulate mammalian *Hox* gene expression.

### **Histone lysine demethylase LSD1**

Epigenetic modification- is defined as heritable alteration that affects chromatin environment and gene expression without changing DNA sequence. Through epigenetic mechanism, an identical genome can be interpreted differently in a temporal and spatial-dependent manner (Lan et al. 2008). So far, two major ways have been identified to confer epigenetic regulation, DNA methylation and histone post-translational modifications. Covalent modifications discovered on histones include at least acetylation, phosphorylation, ubiquitination, sumoylation, ribosylation and methylation. Lysine methylation, in forms of either mono-, di-, or tri-methylation takes place on six major lysine residues, K4, K9, K27, K36 and K79 on histone H3 and K20 on histone H4. . Different degrees of histone lysine methylation at different residues impart distinct biological functions or consequences (Ruthenburg et al. 2007). Similar to histone acetylation, histone lysine methylation has recently been proven to be a dynamic process because of the discovery of histone lysine demethylases (Shi et al. 2004; Tsukada et al. 2006). Two kinds of histone lysine demethylases identified so far are distinct in their demethylating chemistry, coenzyme use, and

reaction product (Shi et al. 2004; Tsukada et al. 2006; Federico et al. 2008). The first class of histone lysine demethylase, LSD1(also called KDM1) belongs to the flavin-dependent amine oxidase family. Flavin-dependent histone demethylases react with oxygen and act only on mono- and dimethylated lysines and produce hydrogen peroxide ( $H_2O_2$ ) and formaldehyde through a classical amine oxidation reaction. The second class of histone demethylase, Jumonji domain containing histone demethylases, are iron-dependent enzymes that can act on mono-, di- and trimethylated lysine residues and even methylated arginine residues and specific Jumonji domain histone demethylases have been identified to specifically target mono-, di-, or trimethylated H3K4, H3K9, H3K27, H3K36, H3R2 or H4R3 (Shi et al. 2007).

Histone H3K4 methylation has been linked to gene activation (Sims et al. 2003), while H3K9 methylation correlates with gene repression (Nielsen et al. 2001; Shi et al. 2003). Interestingly, LSD1 can either demethylate mono- and dimethylated H3K4, acting as a repressor (Shi et al. 2004) or demethylate mono- and dimethylated H3K9, acting as an activator (Metzger et al. 2005). LSD1 was originally purified as a component of transcriptional repressor complex containing transcriptional corepressor CoREST and histone deacetylase HDAC1/2 (Ballas et al. 2001; Humphrey et al. 2001; You et al. 2001; Hakimi et al. 2002; Shi et al. 2003). The enzymatic activities of LSD1 are regulated at multiple layers. CoRest is essential for LSD1 to demethylate nucleosome substrates and prevents its proteasome-mediated degradation (Shi et al. 2005). BHC80 binds to unmethylated histone H3 K4, prevents its remethylation by MLL1 and promotes

the stable association of LSD1 with chromatin (Lan et al. 2007). It has also been shown that local chromatin environment regulates chromatin substrate association and enzymatic activity of LSD1. Typically, site-specific chromatin modifying enzymes require less than ten to fifteen amino acids for efficient substrate binding (Seet et al. 2006), whereas LSD1 requires all the first twenty N-terminal amino acids of the histone H3 for efficient binding (Forneris et al. 2005). Such unique recognition mechanism enables LSD1 to detect other epigenetic modifications on the histone tail (Shi et al. 2004; Forneris et al. 2005; Forneris et al. 2006; Forneris et al. 2007). LSD1 catalytic activity is affected by the presence of other histone epigenetic modifications on histone H3 such as lysine hyperacetylation (Shi et al. 2004; Shi et al. 2005) and Ser 10 phosphorylation (Forneris et al. 2005). These findings indicate that LSD1 catalytic activity occurs right after the operation of other chromatin modifying enzymes including histone deacetylases, serine phosphatases and arginine demethylases (Shi et al. 2005; Forneris et al. 2005; Lee et al. 2006; Forneris et al. 2007).

The co-crystal structure of LSD1, LSD1-CoREST complex and LSD1-CoREST-histone peptide ternary complex has recently been characterized and revealed three distinct structural identities in LSD1, the N-terminal SWIRM domain (named for its presence in the proteins Swi3, Rsc8, and Moira), the C-terminal FAD-binding amine oxidase domain, and the insertion tower domain. (Stavropoulos et al. 2006; Chen et al. 2006; Yang et al. 2006; Forneris et al. 2007). The SWIRM domain and the amine oxidase domain closely pack against

each other and form a globular core structure from which the tower domain protrudes as an elongated helix-turn-helix motif. LSD1-CoREST structure reveals an intensive intermolecular association with a long helical portion of CoREST running parallel to the tower helices of LSD1 (Yang et al. 2006). Co-crystal structure of the LSD1-CoREST-histone peptide ternary complex reveals that the peptide binds to the amine oxidase domain, forming a folded conformation to facilitate the binding site to accommodate the relatively long stretch of the N-terminal H3 tail (Forneris et al. 2007). Residues from CoREST are in close proximity to but do not interact directly with histone peptide, indicating that CoREST stabilizes an active conformation of LSD1 and promotes the docking of H3 tails into enzymatic active site (Forneris et al. 2007).

As a component co-purified with CoREST-HDAC complexes, LSD1 functions as a transcriptional repressor by its demethylase activity towards active epigenetic marks, methylated H3K4 (H3K4me). Transcription factor REST (repressor element 1 silencing transcription factor) mediates the long-term repression of neuronal genes in non-neuronal cells and in neuronal precursors by recruiting the CoREST-HDAC-LSD1 complex (Ballas et al. 2001; Battaglioli et al. 2002; Ballas et al. 2005; Shi et al. 2004). Knocking down LSD1 by siRNA caused the loss of REST-mediated repression and ectopic reactivation of neuronal genes (Shi et al. 2004), accompanied by increased levels of H3K4 methylation on the REST target promoters (Shi et al. 2004). LSD1-CoREST-HDAC core complex has also been shown to function as a transcriptional repressor during hematopoietic

differentiation. LSD1-CoREST-HDAC complexes physically associate with two zinc-finger transcriptional repressors, Gfi-1 and Gfi-1b (Saleque et al. 2007), and are recruited onto many Gfi-1/Gfi-1b target gene promoters. Knocking down LSD1 and/or CoREST induced abnormal hematopoietic differentiation, increased levels of H3K4 methylation marks on a large number of Gfi-1/Gfi-1b target genes, and reactivation of these Gfi-1/1b targets, indicating that the histone lysine demethylase activity of LSD1 is also critical for appropriate transcriptional silencing and cellular identities in both neuronal cells and in non-neuronal cells (Saleque et al. 2007; Shi et al. 2004). More recently, DNA methylase DNMT3L has been shown to be able to recognize histone tails that are unmethylated at H3K4, which provides evidence showing that unmethylated H3K4 acts as a novel epigenetic mark and induces *de novo* methylation of DNA. As LSD1 is known as the demethylase that completely converts methylated H3K4 (mono- or di-H3K4me) into unmodified H3K4, it has been suggested that LSD1-mediated H3K4 demethylation might be essential for *de novo* DNA methylation and stabilized gene repression (Ooi et al. 2007). LSD1 also interacts with an orphan nuclear receptor TLX directly through its SWIRM and amine oxidase domains, enhancing the transrepressive activity of TLX through its H3K4me demethylase activity (Yokoyama et al. 2008).

On the other hand, LSD1 also functions as a transcriptional activator under certain conditions. For instance, LSD1 interacts with androgen receptor (AR) and activates AR-responsive target genes by demethylating mono- and dimethylated H3K9 in a prostate cancer cell line LNCaP cells (Metzger et al.

2005). Consistently, LSD1 has been linked to an estrogen receptor $\alpha$  (ER $\alpha$ ) - mediated gene activation program in a ligand-dependent manner (Garcia-Bassets et al. 2007). In this case, a genome-wide ChIP-Chip analysis of LSD1 occupancy among promoters in MCF7 cells upon estrogen (E2) treatment reveals that LSD1 is recruited to nearly 20% of total gene promoters and a majority (84%) of these promoters are also associated with RNA polymerase II, an indication of an involvement of LSD1 in gene activation (Garcia-Bassets et al. 2007). Notably, LSD1 interacts with ER $\alpha$ , and about 58% of ER positive promoters also exhibit LSD1 co-occupancy. LSD1-ER $\alpha$  interaction functionally opposes transcriptional repression mediated by H3K9 methyltransferases (Garcia-Bassets et al. 2007). However, whether or not such activation function is due to LSD1-mediated demethylation of mono- and dimethylated H3K9 remains to be examined.

Taken together, LSD1 enzymatic activity is regulated at multiple levels. However, mechanism underlying its functional switch from transcriptional repression (demethylating H3K4me) to activation (demethylating H3K9me) is still unclear. In Chapter V, I will provide evidences supporting a potential mechanism of mediating a functional switch of LSD1 by sumoylation-desumoylation.

### **Scientific questions and general overviews**

Elucidating molecular mechanisms underlying the transcriptional regulation of genes that are critical for normal and pathological development remains a central issue in biology. Although down-regulation of tumor metastasis

suppressor genes are commonly observed in high-risk tumors, the responsible mechanisms have rarely been identified so far. When I joined my thesis lab five years ago, I was particularly interested in prostate cancer progression, the most common non-cutaneous malignant disease affecting men and the second leading cause of death from malignant tumors among men in the United States (Denmeade et al. 2002; Hsing et al. 2000). *KAI1*, one putative tumor metastasis suppressor, has been found down regulated in prostate cancer cells (Dong et al. 2005). *KAI1* is a NF- $\kappa$ B target gene and its expression is induced in response to IL-1 $\beta$  (Li et al. 2001). To our surprise, after IL-1 $\beta$  treatment, the message RNA level of *KAI1* was increased only among normal prostate cells and tumorigenic prostate cells, but not in highly metastatic prostate cancer cells. Does this imply that *KAI1* may play a tissue-selective role in inhibiting metastasis of human prostate cancers? And what is the mechanism underlying this inspiring phenomenon? What factors including transcriptional coactivators and corepressors are involved in the transcriptional regulation of *KAI1*? How do those cofactors confer their repression or activation roles in *KAI1* transcriptional regulation? With these questions in mind, I started my five-year-long research journey, which was full of joys and tears, pains and gains.

In Chapter II, I report that the down-regulation of *KAI1* in prostate cancer cells involves the inhibitory functions of a  $\beta$ -catenin-Reptin complex, which requires both induced  $\beta$ -catenin expression and recruitment of histone deacetylase 1 by Reptin. On the contrary, the transcriptional activation of *KAI1* requires a sufficiently high level of a histone acetyltransferase Tip60 coactivator,

which itself is negatively regulated by  $\beta$ -catenin. Indeed, we found that  $\beta$ -catenin expression was increased whereas Tip60 level was low in metastatic cancer cells. An increase in Tip60 expression is sufficient to restore the induction of *KAI1* by IL-1 $\beta$  in metastatic cancer cells. The coordinated actions of  $\beta$ -catenin-Reptin complex that mediate the repressive state serve to antagonize a Tip60-Pontin coactivator complex that is required for activation. The balance of these opposing complexes controls the expression of *KAI1* and metastatic potential.

In Chapter III, I describe a follow-up study on Reptin and investigate how Reptin confers the transcriptional repressive function as showed in Chapter II. Towards this end, I identified Reptin-interacting cofactors including UBC9 and ASXL1 by utilizing yeast two-hybrid screening, and investigated the roles of these Reptin-associated factors in the functional regulation of Reptin. After confirming *in vivo* interaction between Reptin and UBC9, a sole E2-conjugating enzyme essential for sumoylation, I showed that Reptin was a substrate of sumo conjugation and that sumoylation conferred the repressive function to Reptin. Furthermore, I examined the regulatory function of Reptin using *Hox* gene clusters as target in pluripotent embryonic cell line NTera2 as Reptin homologue in fly has been suggested to be involved in *Hox* gene regulation. Surprisingly, I found that, when working together with ASXL1 and LSD1, Reptin acted as transcriptional coactivator on multiple *Hox* gene promoters. Thus, Reptin appears to play dual roles in transcriptional regulation in a context-dependent manner.

In Chapter IV, I examined how post-translational modifications (such as sumoylation) mediate LSD1 functional switch from repression to activation. LSD1

was the first identified histone lysine demethylase. LSD1 possesses two opposite regulatory roles in transcription, either acting as a transcriptional repressor by demethylating mono- and dimethylated histone H3 Lys 4 (H3K4) (Shi et al. 2004), or acting as an activator when it associates with the androgen receptor (AR) and demethylates mono- and dimethylated H3 Lys9 (H3K9) (Metzger et al. 2005). I found that sumoylation enhanced LSD1 mediated-repression on some promoters such as CoREST target genes; Knockdown of PIAS1, a LSD1 sumo E3 ligase, abrogated the recruitment of LSD1 on these target genes, concomitant with subsequent de-repression of these genes. On the other hand, I also found that treatment with the AR ligand induced the desumoylation of LSD1 mediated by SENP1 on the AR target genes; Knocking down SENP1 decreased AR-mediated transcriptional activation. Thus, sumoylated forms of LSD1 correlated with its gene repression function in CoREST complexes, and de-sumoylated forms of LSD1 correlated with its gene activation function when associated with AR. In summary, the changes in sumoylation-desumoylation status induced a switch of LSD1 functions in transcriptional regulation.

Chapter V summarized the major findings and also pointed out the unsolved significant scientific questions that represent future directions of this field.

## **Chapter II**

### **Transcriptional regulation of a metastasis suppressor gene by Tip60 and $\beta$ -catenin complexes**

**Abstract**

Elucidating molecular mechanisms underlying transcriptional regulation of genes that are critical for normal and pathological development remains a central issue in biology and medicine. Although down-regulation of tumor metastasis suppressor genes are commonly observed in high-risk tumors, the responsible mechanisms have rarely been identified so far.

Here I report that the down-regulation of KAI1, a metastasis suppressor gene, in prostate cancer cells involved the inhibitory function of a  $\beta$ -catenin-Reptin complex, which required both induced  $\beta$ -catenin expression and recruitment of histone deacetylase 1 by Reptin. On the contrary, the transcriptional activation of KAI1 required a sufficiently high level of a histone acetyltransferase Tip60 coactivator. The coordinated actions of  $\beta$ -catenin-Reptin complex that mediated the repressive state serve to antagonize a Tip60-Pontin coactivator complex that was required for activation. The balance of these opposing complexes controlled the expression of KAI1 and metastatic potential in prostate cancers. This chapter is a revised version of our earlier publication (Kim and Cai et al. 2005). Here I would like to acknowledge again all the coauthors of this paper to let me present our collaborated work in my thesis.

KAI1 gene (also designated as CD82), locates on human chromosome 11p11.2, encodes a transmembrane protein with 267 amino acids (Dong et al. 1995). KAI/CD82 protein has cytoplasmic N- and C-terminal, and transverses the cell membrane four times forming one small and one large extracellular loop with residues susceptible to post-translational modifications such as phosphorylation and glycosylation (Rubinstein et al. 1999).

KAI1 is among a list of molecules identified as genes inhibiting metastasis, or metastasis suppressor genes. KAI1 was initially defined as a metastasis suppressor gene with evidence in rat prostate cancer models, showing that KAI1 expression specifically inhibited tumor metastasis, but did not affect the incidence or growth rate of tumors (Dong et al. 1995). The expression of KAI1 is largely reduced during the progression of a variety of different human cancers including prostate (Dong et al. 1995). These observations indicate a general metastasis-inhibitory mechanism mediated by KAI1. Since KAI1 is a NF- $\kappa$ B target gene, its message levels can be induced in response to interleukin-1 $\beta$  (IL-1 $\beta$ ) (Li et al. 2001). RT-PCR analysis of KAI1 mRNA revealed that its level was increased by treatment with IL-1 $\beta$  in normal prostate cells (RWPE1) and tumorigenic prostate cells (RWPE2), but not in prostate metastatic cells (LNCaP and PC3) (Figure 2.1). These data suggest a potential tissue-selective role for KAI1 in suppressing human prostate cancer metastasis. Interestingly, this downregulation of KAI1 gene expression seems to be regulated at the transcriptional level, because neither an allelic loss nor a mutation of KAI1 appears to be involved (Dong et al.

1996). The underlying molecular mechanism for the reduction of KAI1 remains to be furthered identified (Kim and Cai et al. 2005).

With the help of Dr. Charles Sawyers's lab, we initially performed in vivo metastasis assay to address the potential functional role of KAI1 as a metastasis suppressor gene (Figure 2.2). We restored KAI1 expression to LNCaP prostate cancer cells, which did not effectively express KAI1, by stably expressing an exogenous KAI1 expression vector. After injection of this KAI1 expressing LNCaP cells together with luciferase reporter into mouse prostate, the level of prostate-specific antigen (PSA) in the mouse was monitored. When serum PSA level reached 15ng/ml, luciferin solution was injected intravenously and the lung was later dissected from each mouse and luciferase activity was recorded. As summarized in Figure 2.2, there was a dramatic decrease in lung metastases in the mouse injected with KAI1-expressing LNCaP cells, although the primary tumor weights were comparable both in control and in KAI1-expressing cell tumors (data not shown). These data strongly suggest that expression of KAI1 significantly inhibits the in vivo incidence of lung metastases in prostate cancer cells (Kim and Cai et al. 2005).

To elucidate the underlying molecular mechanisms involved in the differential transcriptional regulation of KAI1 expression in non-metastatic and metastatic cancer cells, we performed chromatin immunoprecipitation (ChIP) assay to detect the dynamic recruitment of various transcription cofactors

(including coactivators and corepressors) and histone code modifications on KAI1 promoter in response to IL-1 $\beta$  signaling. From here, non-metastatic prostate cells refer to RWPE1 cells, while metastatic prostate cancer cells refer to LNCaP cells. According to Dr. Sung Hee Baek's earlier work, IL-1 $\beta$  induces the dismissal of the N-CoR/TAB2 corepressor complex on KAI1 promoter (Baek et al. 2002). As shown in Figure 2.3, we observed the similar IL-1 $\beta$  dependent dismissal of the N-CoR/TAB2 corepressor complex on KAI1 promoter in both non-metastatic and metastatic prostate cancer cells. By a candidate screening approach, surprisingly, we found that recruitment of one important transcription coactivator Tip60 represents a major difference on KAI1 promoter in response to IL-1 $\beta$  comparing non-metastatic with metastatic cancer cells. In non-metastatic cells, coincident with IL-1 $\beta$ -dependent dismissal of the N-CoR/TAB2 corepressor complex, the Tip60 coactivator was recruited onto KAI1 promoter along with gene activation mark such as acetylated histones H3 and H4; however, in metastatic cells, the Tip60 coactivator was not recruited to the KAI1 promoter upon IL-1 $\beta$  treatment even after release of the N-CoR corepressor complex, and the KAI1 promoter remained silenced. This result suggests Tip60 might play a role in regulating KAI1 expression in those two different prostate cells (Kim and Cai et al. 2005).

In addition to Tip60, we tried to identify more cofactors potentially involved in the differential regulation of KAI1 expression.  $\beta$ -catenin, a transcriptional activator in the canonical Wnt signaling pathway, has become to our another

candidate, because  $\beta$ -catenin has been demonstrated to form a complex with NF- $\kappa$ B and repressed NF- $\kappa$ B activity in human colon and breast cancer cells (Deng et al. 2002). Since KAI1 has been shown to be an NF- $\kappa$ B target gene (Li et al. 2001), we were wondering whether  $\beta$ -catenin could play a similar role in prostate cancers. More interestingly, two cofactors, called reptin and pontin, are common components of both Tip60 and  $\beta$ -catenin complexes. The Tip60 coactivator has been purified as a multi-component complex including histone acetyltransferase Tip60, TRRAP, BAF53, reptin and pontin etc. (Ikura et al. 2000). It has been shown that the ATPase activity of Tip60 complex is intrinsic to pontin and reptin (Ikura et al. 2000). Both pontin and reptin possess intrinsic ATPase and DNA helicase activities, implying a function in DNA unwinding and promoter opening (Bauer et al. 1998; 2000; Ikura et al 2000; Wood et al. 2000; Rottbauer et al. 2002; Feng et al. 2003). They have also been characterized to be components of chromatin remodeling complexes such as INO80 and SWR1 complexes (Shen et al. 2000; Mizuguchi et al. 2004). Pontin and reptin have been reported to interact with  $\beta$ -catenin and function as antagonistic regulators of  $\beta$ -catenin signaling using transient transfection and reporter gene assays (Bauer et al. 2000). However, the exact functions of pontin and reptin in the Tip60 or  $\beta$ -catenin complexes have not been studied thoroughly. So we decided to study how these four cofactors Tip60,  $\beta$ -catenin, reptin and pontin involved in regulating KAI1 expression in prostate cancer cells.

We examined  $\beta$ -catenin and Tip60 levels in various metastatic and non-metastatic cell lines and found that  $\beta$ -catenin expression was high in metastatic cancer cells and low in non-metastatic cells, while Tip60 exhibited a low level of expression in metastatic cancer cells and high in non-metastatic cells (Figure 2.4a). To determine whether down-regulation of KAI1 in metastatic cancer cells correlated with low level of Tip60, we overexpressed an exogenous Tip60 in metastatic cancer cells which resulted in re-activation of KAI1 in the presence of IL-1 $\beta$ , indicating that the difference in the level of Tip60 expression was sufficient to account for the failure of transcriptional activation of KAI1 in these metastatic cancer cells (Figure 2.4b). On the other hand, overexpression of  $\beta$ -catenin (a constitutive active form) in non-metastatic cells inhibited KAI1 gene expression under IL-1 $\beta$  treatment, supporting that high levels of nuclear  $\beta$ -catenin were competent to mediate down-regulation of KAI1 (Figure 2.4c) (Kim and Cai et al. 2005).

Through extensive ChIP analysis by using antibodies against components of Tip60 and  $\beta$ -catenin complexes, as shown in Figure 2.5a, we observed that in non-metastatic cells, Tip60 and pontin, but not reptin, were recruited; in contrast, in metastatic cancer cells,  $\beta$ -catenin and reptin, but not pontin, were recruited. Remarkably, with overexpression of Tip60 in metastatic cancer cells in the presence of IL-1 $\beta$ , Tip60 and pontin actively displaced  $\beta$ -catenin and reptin, and were recruited on the KAI1 promoter. On the other hand, in non-metastatic cells,

overexpression of a constitutive active  $\beta$ -catenin restored its occupancy on KAI1 promoter together with repressin by dismissing the recruitment of Tip60 and pontin (Figure 2.5b). This pattern indicated a competitive recruitment mechanism of Tip60/Pontin as coactivators while  $\beta$ -catenin/repressin as corepressors on KAI1 promoter upon IL-1 $\beta$  treatment based on non-metastatic and metastatic cell line models. The occupancy of the KAI1 promoter by either a Tip60/pontin coactivator complex or a  $\beta$ -catenin/repressin repressor complex appeared to be mutually exclusive (Kim and Cai et al. 2005).

To further prove Tip60/pontin coactivator complex and  $\beta$ -catenin/repressin corepressor complex are crucial for the differential expression of KAI1 in non-metastatic and metastatic prostate cancer cells, we designed specific shRNAs targeting Tip60, pontin,  $\beta$ -catenin or repressin. As shown in Figure 2.6a, an shRNA against Tip60 in non-metastatic cells resulted in loss of induction of KAI1 by IL-1 $\beta$ , whereas knockdown of pontin had no dramatic effect. This indicates Tip60 is required for mediating the transcriptional activation of KAI1 in response to IL-1 $\beta$  in non-metastatic cells. On the other hand, when we silenced  $\beta$ -catenin or repressin in metastatic cells by using specific shRNAs, we observed that knockdown of either  $\beta$ -catenin or repressin restored the induction of KAI1 in the presence of IL-1 $\beta$  in metastatic cells, although knockdown of  $\beta$ -catenin alone caused much higher level of induction of the KAI1 transcript than knockdown of repressin alone (Figure

2.6b). These results provide evidence that both  $\beta$ -catenin and reptin play significant roles in regulating KAI1 expression (Kim and Cai et al. 2005).

KAI1 is an NF- $\kappa$ B target gene (Li et al. 2001). NF- $\kappa$ B p50 is the DNA binding protein tethering  $\beta$ -catenin/reptin complex to the KAI1 promoter as well as Tip60/pontin coactivators (data not shown).  $\beta$ -catenin plays a crucial role in development and homeostasis, and deregulated expression of  $\beta$ -catenin is involved in oncogenesis and tumor progression (Moon et al. 2002; Cheshire et al. 2003). As  $\beta$ -catenin exerts a dual role in TCF/LEF-mediated activation and NF- $\kappa$ B-mediated selective repression, the biological activity of  $\beta$ -catenin is likely to be modified by the cellular context and/or effects of other signaling pathways (Deng et al. 2002). The interaction between  $\beta$ -catenin and NF- $\kappa$ B is indirect and additional cellular proteins are required which implies the interaction between  $\beta$ -catenin and NF- $\kappa$ B may be subject to another level of regulation (Deng et al. 2002). Thus, identification of the intermediate proteins for this interaction would be important for understanding the detailed mechanism of the crossregulation by  $\beta$ -catenin on the NF- $\kappa$ B pathway.

Since reptin was found to harbor intrinsic corepressor properties (a Gal4-Reptin fusion protein caused repression of a UAS/TK luciferase reporter, data not shown) and knockdown of reptin alone restored KAI1 induction in metastatic cells (Figure 2.6b), along with weak histone acetylation signals (data not shown), we wanted to test whether the repressive function of  $\beta$ -catenin was conferred by

reptin possibly through histone deacetylases (HDACs). Indeed, treatment of cells with a known HDAC inhibitor TSA significantly decreased the repressive activity of Gal4-Reptin, indicating that HDACs were involved in reptin-mediated repression (Figure 2.7a). Among single cell microinjections of specific IgGs against HDAC1 to HDAC6, only HDAC1 IgG relieved the repression by Gal4-Reptin, suggesting selective HDAC requirement for reptin-mediated repression (Figure 2.7b). GST pull-down experiments and in vivo coimmunoprecipitation assays confirmed the interaction between reptin and HDAC1 (data not shown). In addition, siRNA against HDAC1 further enhanced the activation of KAI1 promoter driven reporter about 2.5 fold in LNCaP cells (Figure 2.7c). Therefore, HDAC1 activity at least partially contributes to the repressive function of  $\beta$ -catenin/reptin (Kim and Cai et al. 2005).

So far, our data suggest the down-regulation of KAI1 in metastatic prostate cancer cells involves the actions of  $\beta$ -catenin/reptin corepressor complex, serving to antagonize a Tip60 coactivator complex, with the balance of these opposing complexes controlling the expression of KAI1. To test whether altering the ratio of Tip60 and  $\beta$ -catenin will actually modulate the metastatic potential of invasive prostate cancer cells and normal prostate cells, we performed in vitro Matrigel invasion assay (Figure 2.8). We measured the ability of cells to traverse a Matrigel-coated membrane with 8- $\mu$ m pores, a correlate of metastatic potential in vivo (Albini et al. 1987; Kobayashi et al. 1992). As shown in Figure 2.8, overexpression of either Tip60 or shRNA against  $\beta$ -catenin in IL-1 $\beta$ -

treated LNCaP cells reduced cell invasion compared to the IL-1 $\beta$ -treated control cells, while RWPE1 cells expressing  $\beta$ -catenin expression vector or vector-based shRNA against Tip60 increased cell invasion. We obtained similar results in another metastatic prostate cancer cells PC3 cells as in LNCaP cells (data not shown). These experiments suggest that the changes in the level of Tip60 and  $\beta$ -catenin expression can indeed affect the metastatic potential of prostate cancer cells (Kim and Cai et al. 2005).

In summary, our data revealed that the down-regulation of KAI1, a metastasis suppressor gene, in prostate cancer cells involved the inhibitory function of a  $\beta$ -catenin-reptin complex, which required both induced  $\beta$ -catenin expression and recruitment of histone deacetylase 1 by Reptin. On the contrary, the transcriptional activation of KAI1 required a sufficiently high level of a histone acetyltransferase Tip60 coactivator, which itself was negatively regulated by  $\beta$ -catenin (data not shown). The coordinated actions of  $\beta$ -catenin-reptin complex that mediated the repressive state antagonized a Tip60 coactivator complex that was required for activation (Figure 2.9). The balance of these opposing complexes controlled the expression of KAI1 and metastatic potential in prostate cancers. Our findings indicate that the crosstalk between Wnt/ $\beta$ -catenin and the NF- $\kappa$ B pathway might be critical for regulating tumorigenesis and metastasis in prostate cancers (Madrid et al. 2003; Kim and Cai et al. 2005).

**Methods** (Kim and Cai et al. 2005)**In vivo metastasis assay**

The prostate of each mouse (one million cells/mouse) was injected with a luciferase reporter and LNCaP cells stably expressing either empty vector or KAI1 expression vector. After injection, the level of prostate specific antigen (PSA) in these xenograft mice was monitored every week and the luciferin (5 mg/kg of mice) was injected intravenously into a mouse when serum PSA level reached 15 ng/ml. The lung was dissected from each mouse and luciferase activity, representative of lung metastasis, was recorded. Each tumor in prostate was weighed as control.

**ChIP assay**

ChIP assay was performed as previously described (Shang et al. 2000) to test cofactor recruitments on endogenous KAI1 promoter by using specific antibodies, with average size of sonicated fragments about 300 bp-1kb. The primer sequences for PCR are as follows, KAI1 promoter sense strand 5'-ACCGTTAGGCAGCGCCGTGAG-3' and antisense strand 5'-CTTGGGAAGGCGGTGCGCTC-3'.

**GST-pulldown and CoIP**

GST-pulldown and CoIP experiments were performed as described previously (Dasen et al. 1999).

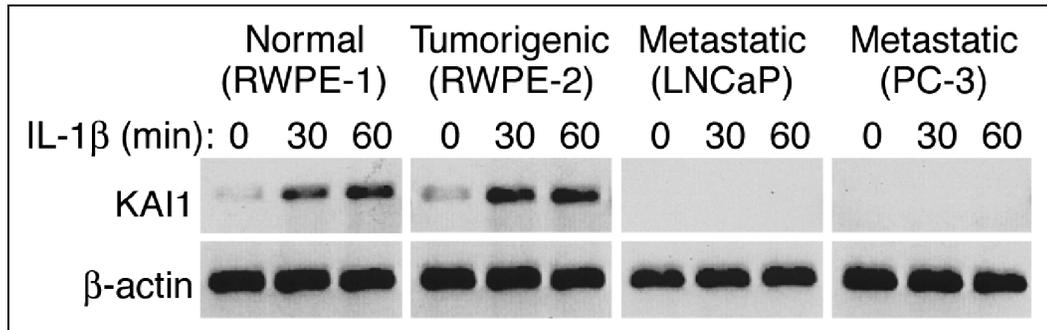
For the interaction assays, 0.1–0.5  $\mu$ g of GST proteins will be combined with  $^{35}$ S-labeled proteins in a binding/washing buffer containing 150 mM NaCl, 20 mM Tris (pH 7.8), 10% glycerol, 0.02% NP-40, and 0.5 mM DTT. GST-coupled

and  $^{35}\text{S}$ -labeled proteins will be bound for 2 hr at 4°C, washed four times, and interactions visualized after SDS-PAGE and autoradiography.

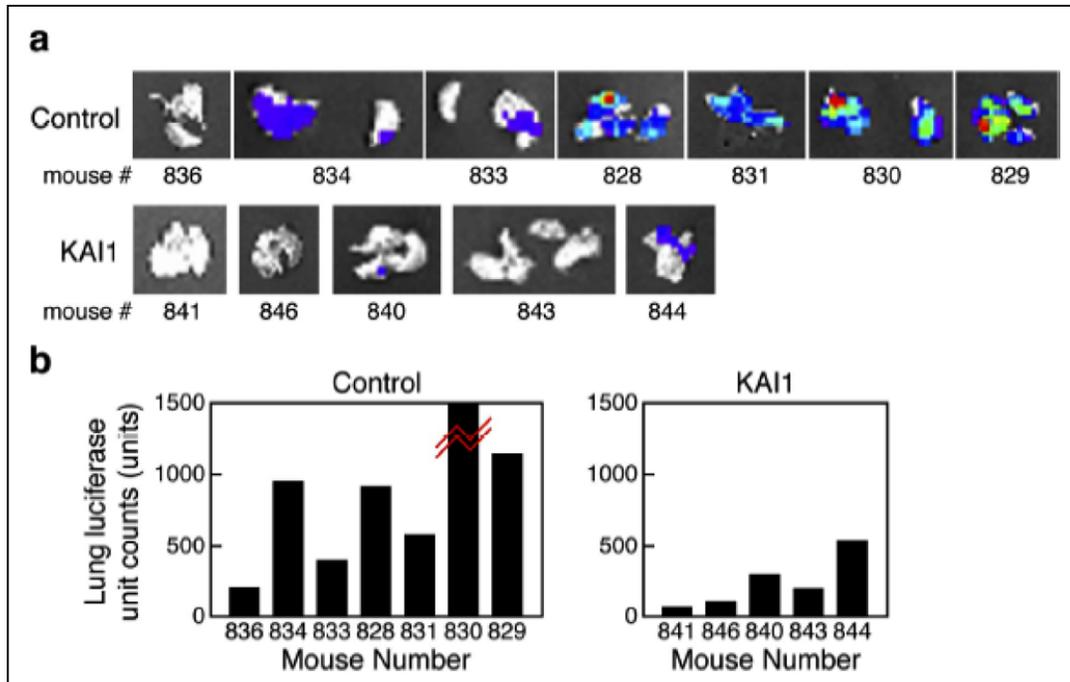
For coimmunoprecipitation studies, after 48 hr transfection, cells were harvested and lysed in binding buffer containing 20 mM Tris (pH 7.8), 150 mM NaCl, 0.1% NP-40, 10% glycerol, 0.5 mM EDTA, and 1× Complete Protease Inhibitor Cocktail (Boehringer Mannheim). Then, lysates were incubated with specific antibody overnight at 4°C, precipitated with protein A/G plus agarose, and washed four times in binding buffer. Complexes were resolved by SDS-PAGE and transferred to nitrocellulose membranes, followed by primary and secondary antibodies, and developed by ECL (Amersham).

### **Single-cell nuclear microinjection**

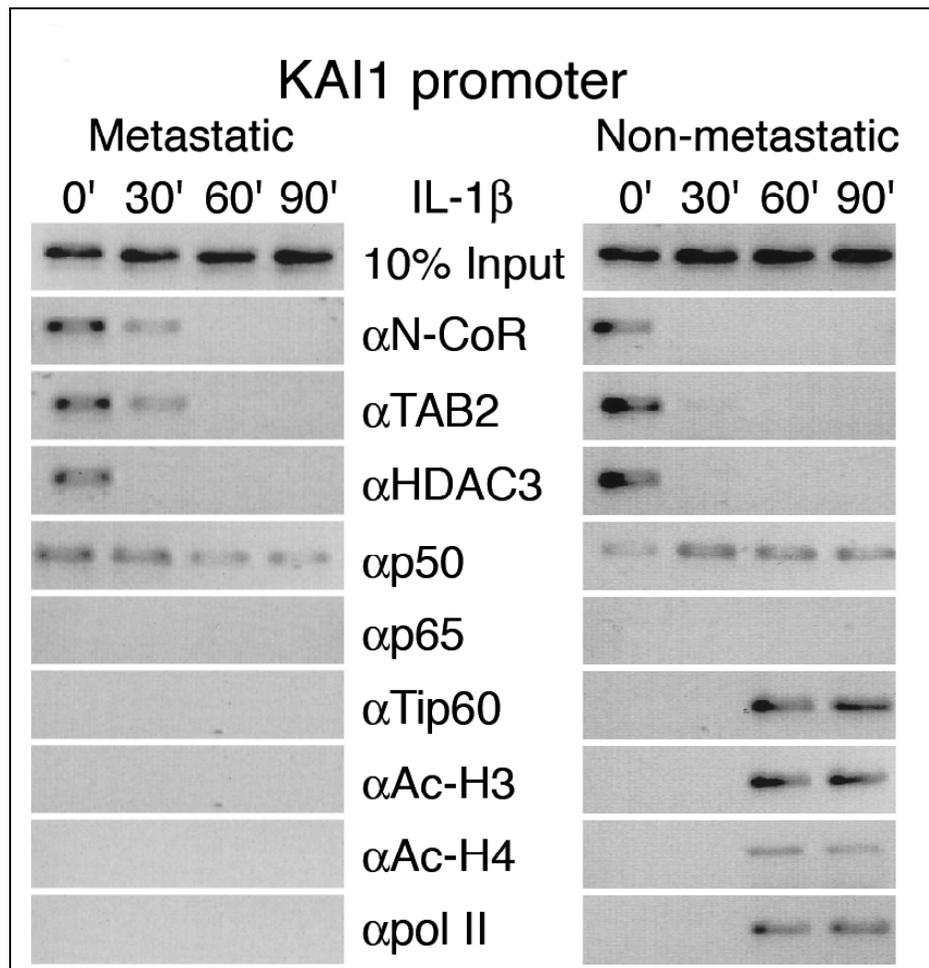
Single-cell nuclear microinjection experiments were carried out as previously described (McInerney et al. 1998). Each experiment was performed on three independent cover slips consisting of 1,000 cells, with more than 300 cells injected, and rhodamine-conjugated dextran was used as a negative control in each experiment. Before injection, cells were rendered quiescent by incubation in serum-free medium for 24-36 hours. The LacZ reporters were previously described (McInerney et al. 1998).



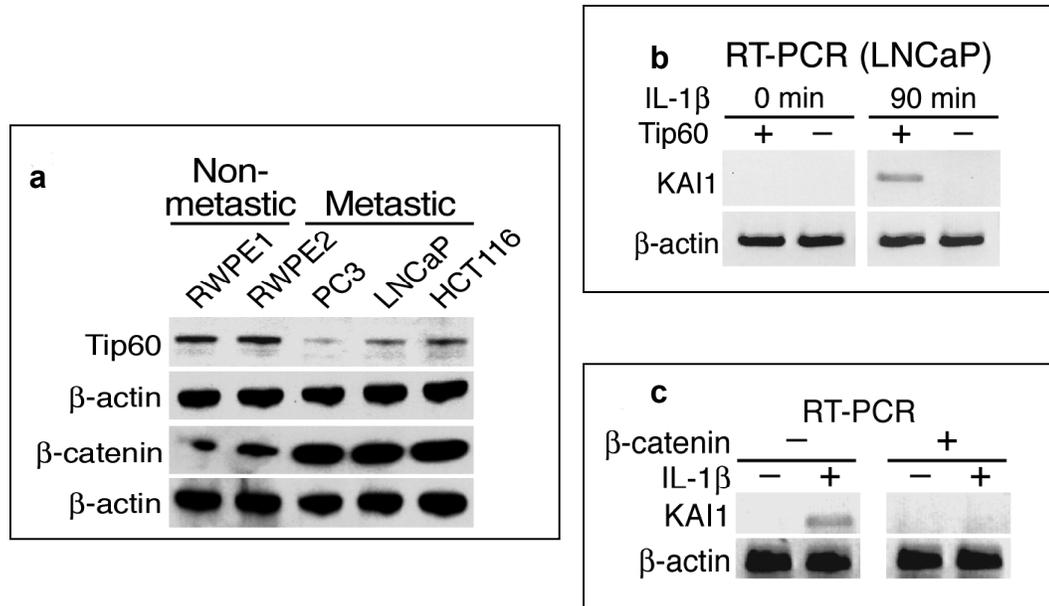
**Figure 2.1. KAI1 mRNA level in the presence of IL-1 $\beta$  is down-regulated in metastatic prostate cancer cells.** (adapted from Kim and Cai et al. 2005). Various prostate cell lines were treated with IL-1 $\beta$  0, 30, 60 minutes (min). Total RNA was isolated and amplified by RT-PCR using transcript-specific primers.  $\beta$ -actin was an internal control.



**Figure 2.2 In vivo metastasis assay.** (adapted from Kim and Cai et al. 2005) a, Lung images representing lung metastasis from different mice were shown. LNCaP cells expressing luciferase reporter with either empty vector or KAI1 expression vector were injected into mouse prostate and luciferase activity in the lung was recorded for each mouse. b, Quantification results of luciferase activities from either each control mouse or each KAI1-expressing mouse.

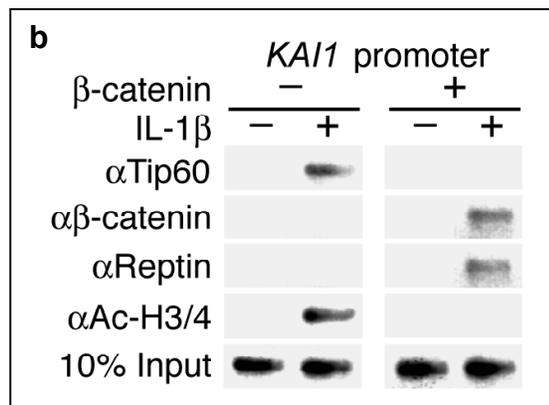
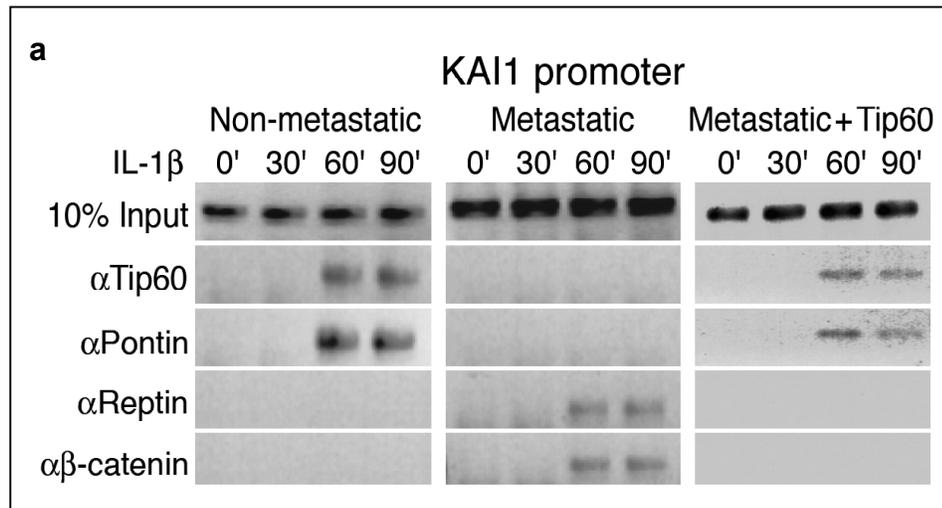


**Figure 2.3. ChIP assay on KAI1 promoter.** (adapted from Kim and Cai et al. 2005) ChIP assay was performed to compare cofactor dynamics in response to IL-1 $\beta$  (at time 0', 30', 60', 90') on KAI1 promoter in LNCaP (metastatic) and RWPE1 (non-metastatic) cells.

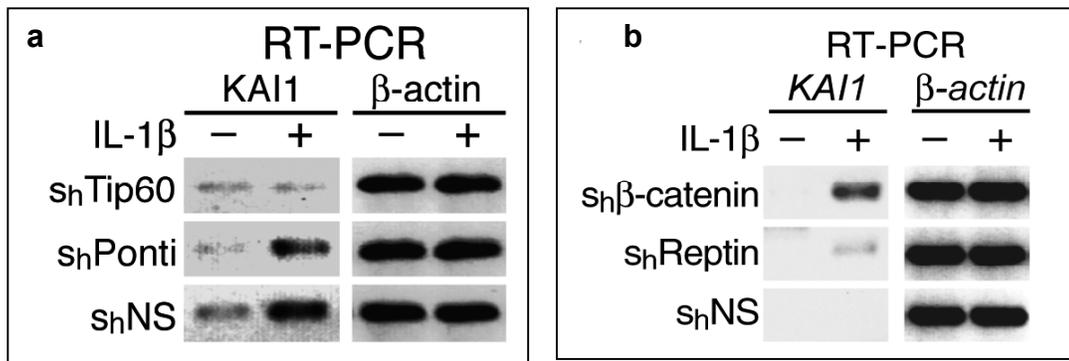


**Figure 2.4. Tip60 and  $\beta$ -catenin are crucial for regulation of KAI1.**

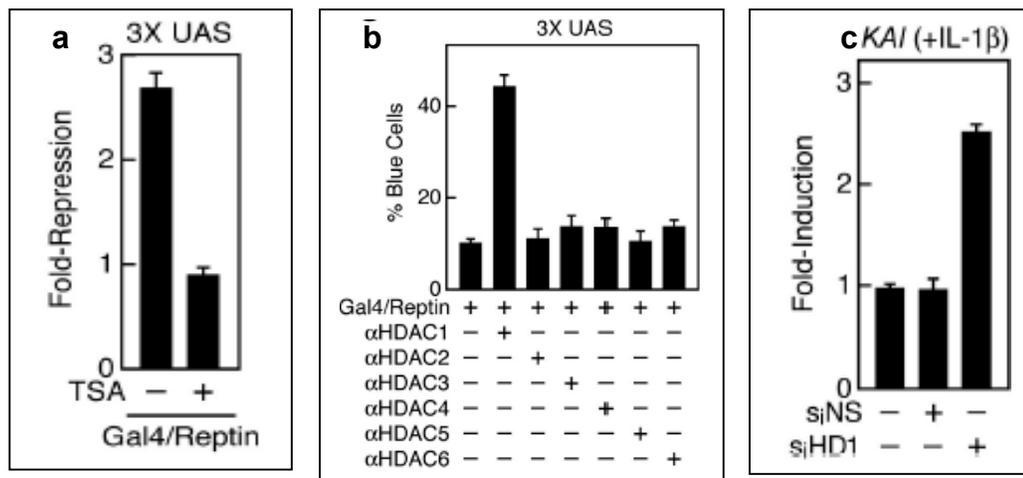
(adapted from Kim and Cai et al. 2005) a, Immunoblot analysis of Tip60 and  $\beta$ -catenin levels in various prostate cell lines. b, Overexpression of an exogenous Tip60 restored KAI1 induction in response to IL-1 $\beta$  in LNCaP cells. c, Overexpression of  $\beta$ -catenin inhibited KAI1 induction in response to IL-1 $\beta$  in RWPE1 cells.



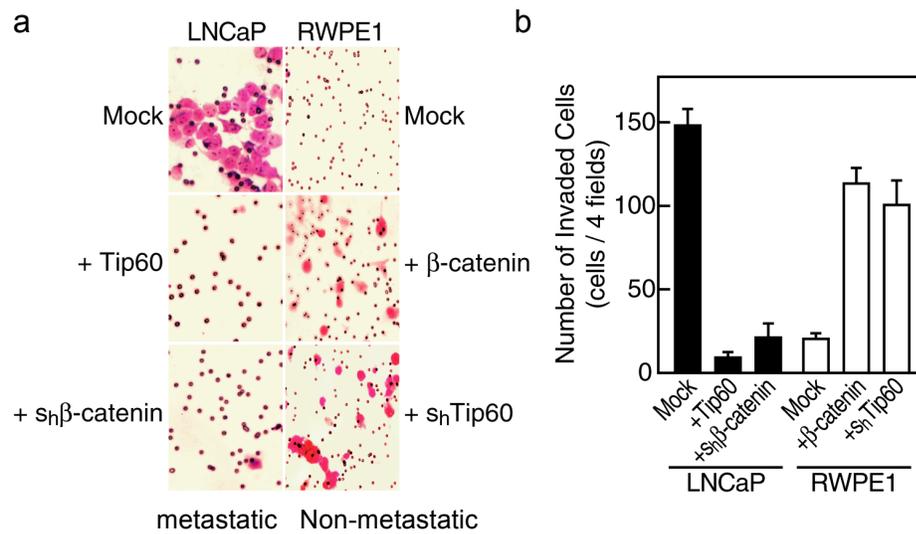
**Figure 2.5. ChIP analysis of Tip60,  $\beta$ -catenin and associated factors on KAI1 promoter in various prostate cells.** (adapted from Kim and Cai et al. 2005) a, ChIP analysis of cofactors recruited on KAI1 promoter was performed in RWPE1, LNCaP, LNCaP cells stably overexpressing exogenous Tip60; b, ChIP analysis of cofactors recruited on KAI1 promoter was performed in RWPE1 and RWPE1 cells stably overexpressing  $\beta$ -catenin.



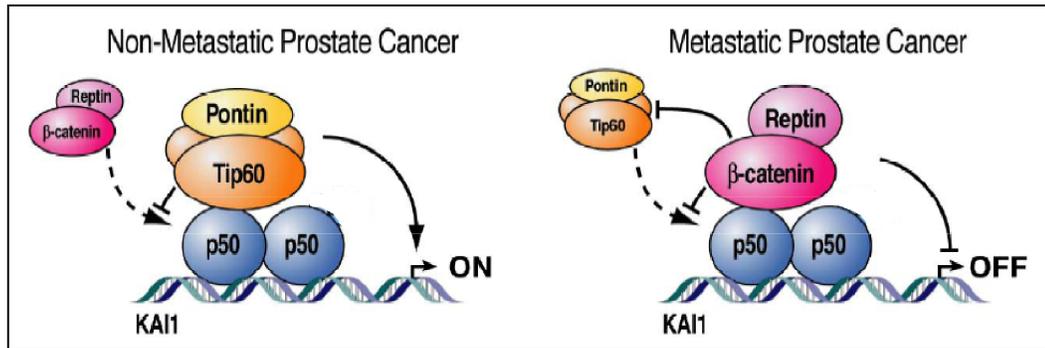
**Figure 2.6. Tip60, β-catenin and associated factors are important for the regulation of KAI1 expression.** (adapted from Kim and Cai et al. 2005) a, The effects of knockdown of either Tip60 or pontin on KAI1 induction in response to IL-1β in RWPE1 cells. b, The effects of knockdown of either β-catenin or reptin on KAI1 induction in response to IL-1β in LNCaP cells.



**Figure 2.7. Histone deacetylase activity is crucial for the repressive function of reptin.** (adapted from Kim and Cai et al. 2005) a. The effect of Gal4-reptin on a UAS/TK luciferase reporter in the presence or absence of an HDAC inhibitor TSA. Luciferase activity was measured and normalized by  $\beta$ -galactosidase assay. Values are expressed as mean  $\pm$  sd (standard deviation) for three independent experiments. b. Single cell microinjection assay. Specific IgGs against HDAC1 to HDAC6 were injected together with a UAS/TK LacZ reporter and Gal4-reptin into cells.  $\beta$ -galactosidase assay was performed later and blue cells were counted under microscope. Values are expressed as mean  $\pm$  sd (standard deviation) for three independent experiments. c. The effect of knockdown HDAC1 by siRNA transfection on KAI1 promoter driven reporter in LNCaP cells. Values are expressed as mean  $\pm$  sd (standard deviation) for three independent experiments.



**Figure 2.8. In vitro Matrigel Invasion Assay.** (adapted from Kim and Cai et al. 2005) a. The invasive activities of LNCaP and RWPE1 cells were measured in Matrigel chambers. The cells that traverse the Matrigel membrane were fixed and stained with phenol red and counted under microscope. b. The quantification result of the left panel. Values are expressed as mean  $\pm$  sd (standard deviation) for three independent experiments.



**Figure 2.9. Schematic model of alternative recruitment of Tip60/pontin coactivators and  $\beta$ -catenin/reptin repressors as a key regulatory switch for KAI1 expression in prostate cancer cells. (adapted from Kim and Cai et al. 2005)**

## **Chapter III**

### **The dual roles of Reptin in transcriptional regulation**

**Abstract**

Reptin, a member of the AAA+ helicase family, is evolutionarily conserved in structure and function. It has been identified as component in many multi-subunit protein complexes that are actively involved in the regulation of various cellular activities including transcription, cell cycle control, DNA replication, DNA repair, apoptosis and etc. We have recently reported that a Reptin/ $\beta$ -catenin complex selectively repressed a subset of NF- $\kappa$ B target genes, including a metastasis suppressor gene KAI1. Here, we identified its additional interacting cofactors such as UBC9 and ASXL1 by utilizing a yeast two-hybrid screening and investigated their roles in the functional regulation of Reptin. After confirming *in vivo* interaction between Reptin and UBC9, a sole E2-conjugating enzyme essential for sumoylation, we further showed that Reptin was indeed a sumo-conjugating target and sumoylation conferred the repressive function to Reptin. Furthermore, we examined the function of Reptin in Hox gene regulation in NTera2 cells, and surprisingly, we found that, when working together with ASXL1 and LSD1, Reptin acted as transcriptional coactivator on *Hox* gene promoter.. To our knowledge, this is the first report showing that Reptin acts as a coactivator instead of corepressor in gene regulation. Taken together, these observations suggest that Reptin plays dual roles in transcriptional regulation in a context-dependent manner.

## Introduction

Reptin (synonyms: Reptin52, RUVBL2, Rvb2, Tip49b, Tip48, ECP51, TAP54 $\beta$ , Tih2) and Pontin (synonyms: Pontin52, RUVBL1, Rvb1, Tip49a, Tip49, ECP54 $\alpha$ , Tih1) are paralogous ATP-dependent DNA helicases that are evolutionarily conserved from yeast to human. They are closely related to bacterial RuvB helicase, a member of the AAA+ family of helicases (Ogura et al. 2001). Bacterial RuvB helicase catalyzes the branch migration of Holliday junctions during homologous recombination or DNA replication (Yamada et al. 2004; McGlynn et al. 2002). Mammalian Reptin and Pontin possess intrinsic ATPase activities, which are stimulated by single-stranded DNA, and ATP-dependent helicase activities of opposite polarity (Kanemaki et al. 1999; Makino et al. 1999). Gene disruption of either Reptin or Pontin was lethal in all species examined so far, indicating their essential and nonredundant functions in cell growth and early development (Bauer et al 1998; Bauer et al 2000; Lim et al. 2000).

Reptin, together with Pontin, has been identified from numerous multimeric protein complexes functioning in chromatin remodeling and gene transcription.

Reptin is identified as a component of the yeast chromatin remodeling INO80 complex and required for the correct assembly of the INO80 complex (Shen et al. 2000; Jonsson et al. 2004). It is involved in transcription of about 5% of yeast genes (Jonsson et al. 2001). It is also co-purified in the yeast Swr1

complex that can exchange histone H2A with the variant histone H2A.Z (Krogan et al. 2003; Kobor et al. 2004; Mizuguchi et al. 2004). In *Drosophila*, it is present in the PRC1 complex that maintains an inherited repressive state of genes by repressing ectopic expression (Saurin et al. 2001). In mammals, it is part of the Tip60 complex, which plays a crucial role in regulating DNA repair and apoptosis (Ikura et al. 2000). It is also a component of URI1 complex that functions downstream of TOR pathway to mediate the repression of TOR-repressed genes in response to extracellular nutrient levels (Gstaiger et al. 2003). Very recently, it is found in the telomerase complex and essential for the holoenzyme assembly (Venteicher et al. 2008). Furthermore, Reptin and Pontin are also involved in the regulation of various cellular activities including transcription, growth control, DNA repair and apoptosis through interacting with many transcriptional factors such as  $\beta$ -catenin (Bauer et al. 2000), c-Myc (Wood et al. 2000), ATF2 (Reptin only, Cho et al. 2001), E2F1 (Pontin only, Dugan et al. 2002) and etc. We reported earlier that a  $\beta$ -catenin-Reptin complex repressed a subset of NF- $\kappa$ B target gene, including a tumor metastasis suppressor gene KAI1, in metastatic prostate cancer cells (Kim et al. 2005). The repressive function of the  $\beta$ -catenin-Reptin complex was at least partially conferred by Reptin. Collectively, Reptin is a versatile factor that is involved in the regulation of various cellular activities including transcription, cell cycle control, DNA replication, DNA repair, apoptosis and etc.

In this chapter, we investigated the roles of two additional interacting cofactors, i.e., UBC9, the sole E2-conjugating enzyme essential for sumoylation, and ASXL1 as identified by a yeast two-hybrid screening, in the functional regulation of Reptin. Sumoylation is a very similar process of protein modification to ubiquitination. Sumo (small ubiquitin-like modifier) is covalently conjugated to substrate proteins by an isopeptide bond between a C-terminal glycine in the sumo and a lysine residue in the substrate. There are four kinds of enzymes involved in this process. They are sumo-specific proteases (SENPs), E1 activating enzyme, E2 conjugating enzyme and E3 ligases. The sumo precursor is cleaved by SENPs with C-terminal hydrolase activity to expose a C-terminal glycine-glycine. In an ATP-dependent reaction, mature sumo forms a thioester conjugate with the heterodimeric sumo E1 activating enzyme Aos1/Uba2. Sumo is then transferred to and forms a thioester intermediate with the only sumo E2 conjugating enzyme UBC9. After that, sumo is transferred from UBC9 to the lysine residue (K) in a substrate protein via an isopeptide bond. During the last step, several sumo E3 ligases have been identified to stimulate the transfer of sumo from UBC9 to a specific substrate. The identification of UBC9 as Reptin interacting partner by the yeast two-hybrid screening indicated Reptin could be a potential sumo modification substrate. Indeed, we confirmed the interaction between Reptin and UBC9, and further proved that Reptin was able to be sumoylated and that sumoylation was essential for its repressive function.

ASXL1 (additional sex comb-like 1) was another potential Reptin interacting protein identified from the initial screening. It was a mammalian homolog of *Drosophila* ASX. ASXL1 was previously identified as a protein that interacted with retinoic acid receptor (RAR) in the presence of retinoic acid (RA) by yeast two-hybrid screening, and was shown to act as a coactivator of RAR through the functional cooperation with SRC-1 (Cho et al. 2006). One prominent subset of RA-regulated genes is the *Hox* gene family, which plays an essential role in embryogenesis and hematopoiesis (Pearson et al. 2005). RAR binds to retinoic acid response elements (RAREs) within *Hox* regulatory regions and activates *Hox* gene transcription in response to RA (Marshall et al. 1994; Dupe et al. 1997; Langston et al. 1997). Despite the crucial role of RAR in *Hox* gene activation, little is known about how RAR affects *Hox* gene expression and what other cofactors are involved. In *Drosophila*, the *Asx* mutant phenotype belongs to phenotypes termed as homeotic transformation that are induced by inappropriate expression of *Hox* genes and are characterized by the alteration of the morphology of one segment into that of another (Pearson et al. 2005). Previous investigation by using fly genetic screening based on homeotic transformation phenotype, led to the identification of two antagonizing groups of the *Hox* locus regulators: *Trithorax* group (TrxG) and *Polycomb* group (PcG) (Ringrose et al. 2004). *Trithorax* group functions as transcriptional activators that maintain the “ON” state of the *Hox* locus, whereas *Polycomb* group serves as transcriptional repressors that maintain the “OFF” state of the *Hox* locus. Like their *drosophila*

counterparts, the mammalian PcG and TrxG proteins maintain the correct expression patterns of Hox genes.

Interestingly, Reptin and Pontin were reported recently to act as antagonistic mediators of *Drosophila Hox* gene transcription through cooperation with polycomb and trithorax proteins, and during this process, Reptin was a corepressor and Pontin was a coactivator (Diop et al. 2008). However, the role of Reptin and Pontin in mammalian *Hox* gene regulation remains unknown. The identification a polycomb protein ASXL1 from our initial yeast two-hybrid screening promoted us to further investigate how Reptin would affect *Hox* gene expression. Surprisingly, we found Reptin functioned as a coactivator in *Hox* gene regulation in NTera2 cells together with its cofactors ASXL1 and LSD1. It is the first observation showing that Reptin acts as a coactivator instead of a corepressor in transcriptional regulation. Collectively, these data suggest that Reptin could function in both activation and repression in transcriptional regulation in a highly context-dependent manner.

## **Results**

### **Identification of UBC9 as a Reptin-interacting protein**

To further identify how Reptin and its putative interacting cofactors confers their regulatory role in gene transcription, we first performed yeast two-hybrid screening by using a full-length Reptin as bait and using human fetal brain cDNA library as prey. From over 1 million colonies screened, 41 positive clones growing up under the most stringent condition were isolated, and their identities were

obtained by sequencing as summarized in Table 3.1. One of them encoded the N-terminal region (amino acids 1-62) of UBC9, the sole E2 SUMO-conjugating enzyme. The interaction between Reptin and UBC9 was further validated by co-immunoprecipitation (CoIP) assay. Total cell lysates from 293T cells transiently transfected with FLAG-Reptin and V5-UBC9 were immunoprecipitated with either anti-FLAG or nonspecific IgG antibodies, and the resultant precipitates were subjected to immunoblotting with anti-V5 antibody. As shown in Figure 3.1, Reptin specifically bound to UBC9, which is consistent with direct interaction identified in the yeast two-hybrid experiment. The interaction between Reptin and some other identified potential interaction partners such as FHL2 and Id2 were also confirmed by co-immunoprecipitation analysis (data not shown).

#### **K456 of Reptin is critical for SUMO conjugation.**

The direct interaction between Reptin and UBC9, the only E2 conjugating enzyme of sumoylation pathway, provided an early indication that Reptin may be a target of sumo modification. We first utilized an *in vitro* sumo conjugation system to determine whether or not Reptin may be a substrate for sumo modification. *In vitro* translated Reptin proteins were incubated with a sumoylation reaction mixture containing recombinant E1 enzyme (a heterodimer of Aos1/Uba2), E2 enzyme (UBC9), SUMO and ATP. In the presence of SUMO, an additional more slowly migrating band with higher molecular weight (about 10kDa more) appeared, indicating Reptin underwent sumo conjugation (Figure 3.2a). To map the sumo acceptor site, we searched the Reptin amino acid

sequence for potential consensus sumo modification motifs  $\psi$ KXD/E (where  $\psi$  represents a large hydrophobic amino acid and X represents any amino acid). Each of the potential sumo conjugating lysine residues of Reptin was mutated individually to arginine and the resultant mutants were tested for the potentials to be sumoylated by *in vivo* sumoylation assay. Mutation of K115R, K132R, K269R, K279R and K288R revealed little or no impact on sumo conjugation of Reptin, whereas the K456R mutant abrogated Reptin from SUMO modification (Figure 3.2b). Thus, it appears that K456 is the sole lysine residue in Reptin subjected to SUMO modification.

Protein posttranslational modifications play an important role in the functional regulation of transcriptional cofactors. SUMO modification affects many cellular processes including transcriptional regulation, sub-cellular localization, signal transduction, DNA repair, and etc. In most cases, sumo modification of transcriptional factors correlates with inhibition of gene transcription. As Reptin has been reported to function as a transcriptional co-repressor, we then test whether or not sumo modification of Reptin modulates its repression activities. We first performed luciferase reporter assays using a UAS-TK-promoter reporter and Gal4- Reptin fusion. Gal4-fused wild-type Reptin consistently repressed the expression of a UAS-TK-luciferase reporter, whereas the Gal4-fused sumo null mutant (K456R) relieved the transcriptional repression (Figure 3.3, panel 2 versus panel 3). Furthermore, increased sumoylation as result of overexpressing wild-type UBC9 enhanced the Gal4-Reptin mediated repression, whereas a dominant negative form of UBC9 (C93S mutant) released

the Gal4-Reptin mediated repression (Figure 3.3, panel 2 versus panels 4 and 5). These data suggest that sumo modification is important for the repression activities mediated by Reptin.

### **PIAS1, PIAS3-isoform1 and Pc2 are Reptin E3 sumo ligases.**

So far, there have been at least three distinct families of sumo E3 ligases identified, including PIAS family, RanBP2 and Pc2. *In vivo* sumoylation assays showed that PIAS1, PIAS3 isoform1 and Pc2 strongly enhanced Reptin sumoylation (Figure 3.4a). Consistently, overexpression of PIAS1 and Pc2 also enhanced Reptin-mediated repression on the artificial luciferase reporter (Figure 3.4b). Future experiments using chromatin immunoprecipitation and knock down analysis will delineate whether or not these sumo ligases affect the expression of KAI1 in tumor metastatic models.

### **SEN1 and SEN3 are Reptin sumo proteases.**

As a dynamic process, sumoylation can be removed by specific sumo proteases (Li et al. 1999; Kim et al. 2000; Best et al. 2002; Bachant et al. 2002). Notably, Reptin has recently been reported to form a complex together with two sumo-deconjugating enzymes SEN1 and SUSP1 in 293T cells (Kim et al. 2006). Consistent with that, we found that over-expression of SEN1 released the transcriptional repression mediated by Reptin (Figure 3.3, panel 8; Kim et al. 2006). Interestingly, we also found that another sumo protease, SEN3, could relieve Reptin-mediated gene repression to the similar extents as SEN1 (Figure

3.3, panel 7). Indeed, co-immunoprecipitation analysis showed that Reptin interacted with both SENP1 and SENP3 in 293T cells (Figure 3.5a and 3.5b). To further determine whether or not the increased transcriptional activation induced by these sumo proteases was due to their enzymatic activity towards Reptin, we performed *in vivo* desumoylation assays. Overexpression of either wild-type SENP1 or wild-type SENP3 led to the removal of SUMO from Reptin-SUMO conjugates efficiently, whereas enzymatic dead mutant of SENP1 (C603A) and SENP3 (C532A) failed to deconjugate SUMO off SUMO-modified Reptin (Figure 3.5c). Notably, SENP3 enzymatic dead mutant actually enhanced the sumo conjugation of Reptin by possibly acting as a dominant negative form (Figure 3.5c). Compared to SENP1 and SENP3, SENP6 possessed much weaker SUMO protease activities towards Reptin (Figure 3.5c and Kim et al. 2006). The above observations clearly demonstrated that Reptin was an *in vivo* substrate for SENP1- and SENP3-mediated desumoylation.

### **Identification of ASXL1 as a Reptin interacting protein**

In addition to UBC9, another interesting positive clone from the initial yeast two-hybrid screening called our attention, that is, ASXL1 (additional sex comb-like 1), a mammalian homolog of *Drosophila* ASX. Two partially overlapping ASXL1 C-terminal fragments that shared a minimal interacting region, human ASXL1 amino acids 1313-1412, came out from the yeast two-hybrid screening (Table 3.1). The full-length ASXL1 protein contains 1541 amino acids, weighs about 170kDa and harbors three conserved regions named as the

ASXN, ASXM, and PHD domains. Interestingly, the identified Reptin-interacting region covers the very C-terminal PHD domain of ASXL1. To further confirm the interaction between ASXL1 and Reptin, we performed Co-IP assays in an embryonic carcinoma cell line Ntera2 using antibodies against endogenous ASXL1, Reptin and LSD1. As shown in Figure 3.6, Reptin interacted specifically with ASXL1 and LSD1. ASXL1, previously identified as a protein that interacted with retinoic acid receptor (RAR) in the presence of retinoic acid (RA), was shown to act as a coactivator of RAR through the functional cooperation with SRC-1 (Cho et al. 2006). The interaction between Reptin and ASXL1 promoted us to test whether or not Reptin could also interact with RAR and regulate the expression of RAR target genes. We first examined the interaction between Reptin and RAR by using Co-IP assays in 293T cells transfected with FLAG-tagged Reptin in the absence or presence of RA. Cell lysates were immunoprecipitated with anti-FLAG antibody and precipitated elutes were detected with anti-RAR antibodies. As shown in Figure 3.7A, Reptin interacted with RAR strongly whereas, as a negative control, pcDNA and LSD1 did not. The interaction between Pontin and RAR was very weak. Then, we investigated the effect after knocking down Reptin in Ntera2 cells on the gene expression of canonical RAR target genes such as RAR $\beta$ 2. Surprisingly, the induced mRNA level of RAR $\beta$ 2 under RA treatment was dramatically decreased after transfection of specific siRNA against Reptin or LSD1, indicating that Reptin and LSD1 act as an unexpected transcriptional coactivator in this case (Figure 3.7b). To further determine whether or not Reptin is directly involved in RAR-mediated

gene activation, we performed CHIP assays. As expected, the recruitments of RAR on RAR $\beta$ 2 promoter were induced in response to RA (Figure 3.7c). At the same time, Reptin bound to RAR $\beta$ 2 promoter both in the absence and presence of RA treatment (Figure 3.7c). The above observations demonstrated that Reptin played an important role in transcriptional activation in contrast to its previously described repressive functions.

We also investigated whether or not Reptin could regulate *Hox* gene expression in response to RA treatment. First, knocking down of Reptin by specific siRNAs caused a significant decrease in the induction of message levels of a subset of *Hox* genes (*Hoxa1*, *Hoxa2* and *Hoxa4*) following RA treatment (Figure 3.8a). Using CHIP assays, we then showed that Reptin directly bound to *Hoxa2* promoter region both before and after RA treatment in NTERA2 cells, with relatively higher levels of recruitment three days after RA treatment (Figure 3.8b).

To further examine how Reptin plays a role in activation instead of repression, we test whether it is through the help of its binding partner ASXL1. As shown in Figure 3.9A, ASXL1 depletion by siRNA transfection caused a dramatic decrease in RA-induced activation of *Hoxa1* and *Hoxa4* genes, similar to the effects of knocking down Reptin. CHIP assay also detected the occupancy of ASXL1 on both *Hoxa1* and *Hoxa4* gene promoters only after RA treatment (Figure 3.9b). This indicates that Reptin confers an activation role on *Hox* gene clusters at least partially through its collaboration with ASXL1. To our surprise, we found that LSD1 could also interact with Reptin and ASXL1 in NTERA2 cells.

SiRNA against LSD1 also dramatically decreased RA-induced *Hox* gene activation. Consistently, the occupancy of LSD1 on *Hox* gene promoters was detected by ChIP assays. These data indicated that Reptin, ASXL1 and LSD1, possibly acting in forms of complexes, are equal contributors of transcriptional activation of *Hox* gene clusters.

## **Discussion**

In this chapter, we identified several additional interacting cofactors of Reptin by using a yeast two-hybrid screening, and investigated their roles in Reptin-mediated transcriptional regulation. Identification of the sole sumoylation E2 conjugating enzyme, UBC9, as Reptin-interacting partner led us to probe whether or not Reptin was a potential sumo targeting substrate. Indeed, by using *in vitro* and *in vivo* sumoylation assays, we showed that Reptin was sumoylated. Like most previously reported cases, in which sumoylation is linked to transcriptional repression, we also found that it stands true for Reptin. Sumo conjugation enhanced Reptin-mediated repressive activities, while the Reptin mutant defective in sumo modification lost its repression potentials in an artificial target reporter assay. In addition, we showed that a subset of PIAS family members (PIAS1, PIAS3 isoform1) and Pc2 act as Reptin sumo E3 ligases. SiRNA knockdown of these E3 ligases relieved Reptin-mediated gene repression. These data further proved that sumoylation is essential for Reptin to function as a repressor. As sumoylation is a very dynamic process, we also identified the sumo protease responsible for the removal of sumo off Reptin. We

found SENP1 and SENP3, but not SENP6, desumoylate Reptin. During the time of our work, similar results on the effect of SENP1 on Reptin were published (Kim et al. 2006). Yet, we found that, compared to SENP1, SENP3 was an even more robust sumo protease for Reptin. Reptin, Pontin and SENP3 were all present in MLL1 transcriptional complex in HeLa cells (Dou et al. 2005), however their functional significance in this complex has not been studied. One intriguing possibility is that these factors and their covalent modification may finely modulate *Hox* gene regulation during embryonic development.

In *Drosophila*, Reptin was previously co-purified with PRC1 complex (Saurin et al. 2001), and yet, neither Pontin nor Tip60 was present in this specific complex. Later on, Reptin was shown to interact genetically with polycomb group genes in the PRC1 complex and Reptin mutants share similar properties with PRC1 mutants (Qi et al. 2006). However, because *Reptin* mutants suppressed PEV and failed to derepress endogenous Hox gene expression, Reptin was not considered a bona fide PcG gene, and it was found unlikely that Reptin protein played an essential role in the PRC1 complex (Qi et al. 2006). Very recently, Reptin and Pontin were reported to function antagonistically with polycomb and trithorax complexes to mediate Hox gene control in *Drosophila* (Diop et al. 2008). The exact role that Reptin plays in Hox gene regulation in mammals is still unknown.

Identification of a polycomb protein ASXL1 as Reptin-interacting factor further promoted us to investigate the function of Reptin on Hox gene regulation. Previously, ASXL1 was reported to function as a coactivator of RAR. Genetic

studies indicated that ASX, the *Drosophila* homologue of ASXL1, is an enhancer of both trithorax and polycomb (ETP) genes and is required for both the activation and repression of *Hox* gene clusters, as *Asx* mutation caused both anterior and posterior homeotic transformations (Milne et al. 1999). mASXL1, the mouse homolog of ASX/ASXL1, exhibits a spatially expression pattern along the anterior-posterior axis, similar to that of *Hox* genes (Chen et al. 2004). In our study, we found that ASXL1 functions as a transcriptional coactivator for *Hox* genes in NTera2 cells. NTera2/NT2 cells are embryonic carcinoma cell lines and serve as a good system to study *Hox* gene regulation in response to RA treatment. siRNA transfection against ASXL1 caused a decrease in activation of at least a subset of *Hox* genes including *Hoxa1* and *Hoxa4*. Our initial yeast two-hybrid experiments indicated that this C-terminal PHD domain of ASXL1 is responsible for interaction with Reptin, and consistently, this PHD domain has been previously reported critical for RAR-mediated transcriptional activation *in vivo* (Cho et al. 2006). To our surprise, knockdown of Reptin caused an impaired activation of these *Hox* genes, suggesting Reptin as transcriptional co-activator instead of co-repressor. Besides ASXL1, we found that LSD1 acted as another potential Reptin coactivator in this case. Knockdown of LSD1 dramatically decreased *Hox* gene activation in response to RA treatment. ChIP experiments showed co-recruitment of all these cofactors on *Hox* gene promoter regions, indicating they may regulate *Hox* gene expression in forms of transcriptional complexes in development.

It has been reported that the yeast homologue of Reptin regulated the expression of about 5% yeast genes, exhibiting both repression and activation potentials. In mammals, although the transcriptional repressive role of Reptin has been well characterized, its putative activation function has not been reported. Our observations in Reptin-mediated *Hox* gene activation in NT2 cells represent a first novel gene activation activities of Reptin in mammalian cells, -that is, Reptin acts as a coactivator potentially through interacting with ASXL1 and a histone lysine demethylase LSD1. It is likely that additional cofactors may participate in Reptin-mediated activation. For example, trithorax group protein MLL5 was also identified in initial screening, and, similar to prominent *Hox* activator MLL1, MLL5 may also be involved in the regulation of *Hox* gene activation.

Taken together, our data suggest Reptin, similar to its yeast homologue, plays an important role in both gene activation and repression, as exemplified in Figure 3.10. Since Reptin is involved in many multi-component transcriptional complexes, it seems that Reptin may contribute different activities in a highly cellular context dependent fashion. Furthermore, post-translational covalent modifications of Reptin (such as sumoylation and phosphorylation, data not shown) add additional fine regulatory mechanisms for its role in gene transcription and maybe other cellular activities. Further studies such as investigating a context-dependent transcription will dissect exact roles of Reptin in *Hox* gene regulation, tumor metastasis (KAI1), organismal development, and etc.

## **Materials and Methods**

### **Chemicals, Antibodies, and other Reagents**

The following commercially available antibodies were used: anti-Reptin (Abcam); anti-ASXL1 (Abcam); anti-LSD1 (Bethyl Lab.); anti-Flag (Sigma); anti-HA (Covance); anti-diMeH3K4 (UP07-030); anti-diMeH3K9 (UP05-768); anti-panH3Ac (UP06-599). DHT was from Sigma. Lipofectamine 2000 reagent was purchased from Invitrogen.

### **Yeast two-hybrid screening**

A fetal brain yeast two-hybrid library was purchased from BD Bioscience. The full-length Reptin was constructed into plasmid pGBKT7 as the “bait” protein. It was transformed into yeast strain AH109. The Matchmaker cDNA library was pretransformed into yeast strain Y187. The strain AH109 with Reptin was mated with strain Y187 that carries prey protein(s). Colonies were picked 4–6 days post mating. PCR inserts were amplified and sequenced. Yeast plasmids were purified from individual clones and were transformed back to an Escherichia coli strain for isolating the plasmid. Finally, interactions were verified by  $\alpha$ -galactosidase activity assays.

### **Co-immunoprecipitation (Co-IP)**

Cell lysates were harvested and lysed in binding buffer containing 20 mM Tris (pH 7.8), 150 mM NaCl, 0.1% NP-40, 10% glycerol, 0.5 mM EDTA, and 1 $\times$  Complete Protease Inhibitor Cocktail (Boehringer Mannheim). Then, lysates were incubated with specific antibody overnight at 4°C, precipitated with protein A/G plus agarose, and washed four times in binding buffer. The resultant precipitates

were resolved by SDS-PAGE gel, transferred to nitrocellulose membranes, detected with a specific primary antibody, followed by HRP-coupled secondary antibodies, and developed by ECL (Amersham).

### **In vitro sumoylation assay**

In vitro sumoylation assay was carried out according to the instructions of the commercial sumoylation control kit (LAE Biotech.). [<sup>35</sup>S] methionine-labeled in vitro-translated Reptin protein was incubated with a sumoylation mixture containing purified E1 (SAE1/SAE2) and E2 (Ubc9) in the presence or absence of the purified sumo in a final volume of 20 ul in reaction buffer containing 20 mM Hepes pH 7.5, 5mM MgCl<sub>2</sub>, 2mM ATP for 30~60 minutes at 37°C. The reaction products were analyzed by autoradiography.

### **In vivo sumoylation assay**

For in vivo sumoylation experiments, 293T cells were cotransfected with Flag- tagged Reptin wildtype or sumo point mutants and HA-tagged SUMO3. 36hrs after transfection, the cells were lysed in lysis buffer containing 0.1% SDS, 0.5% deoxycholate, 0.5% TritonX-100, 1 mM EDTA, 20 mM Tris-HCl pH 7.8 and 150 mM NaCl, supplemented with complex protease inhibitor cocktail (Roche Bochem.) and sumo protease inhibitor NEM (Sigma). The clarified extracts were subjected to immunoblot with anti-Flag antibody.

### **Cell transfection and Luciferase Reporter assays.**

Cells were transfected with Lipofectamine 2000. 48h after transfection, luciferase activity was measured in a luminometer and normalized by β-

galactosidase expression. The results were shown from at least three independent experiments.

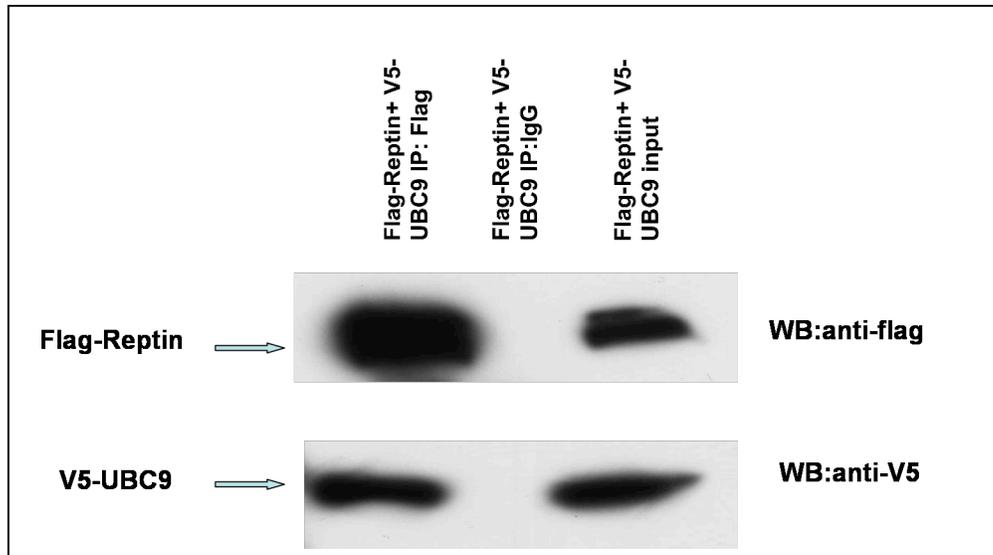
### **Chromatin immunoprecipitation assay (ChIP)**

ChIP assay was performed according to the protocol of ChIP assay kit from Upstate Biotechnology (NY) with minor modifications.  $1 \times 10^6$  cells were subjected to DNA-protein cross-linking with 1% formaldehyde for 10 to 15 min at room temperature, followed by a 5-minute treatment in 5 ml of 0.125 M glycine to stop the cross-linking reaction. After washing with cold phosphate-buffered saline (PBS), the cell pellet was suspended in 300-400  $\mu$ l of sodium dodecyl sulfate lysis buffer (1% SDS, 10mM EDTA, 50 mM Tris-HCl pH8.0, protease inhibitors), incubated on ice for 15 min, and then subjected to sonication. The size of sheared DNA fragments was about 400 bp to 1kb. The sonicated sample was centrifuged at 14,000 rpm for 10 min at 4°C, and the supernatants were diluted 1:10 with ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2mM EDTA, 16.7mM Tris-HCl, pH 8.1, 167mM NaCl ). The chromatin was precleared with 45-50  $\mu$ l protein A/G-agarose, followed by a 45 min rotation (4°C) and removal of the beads by brief centrifugation. The precleared sample was subjected to immunoprecipitation with control IgG antibody or specific antibody with rotation at 4°C overnight. 45-50ul of protein A/G-agarose was then added to each tube followed by rotation for 2 h and sequential washing with low-salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH8.0, 150 mM NaCl), high-salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH8.0, 500 mM NaCl), LiCl wash buffer (0.25 M LiCl, 1% NP-40, 1%

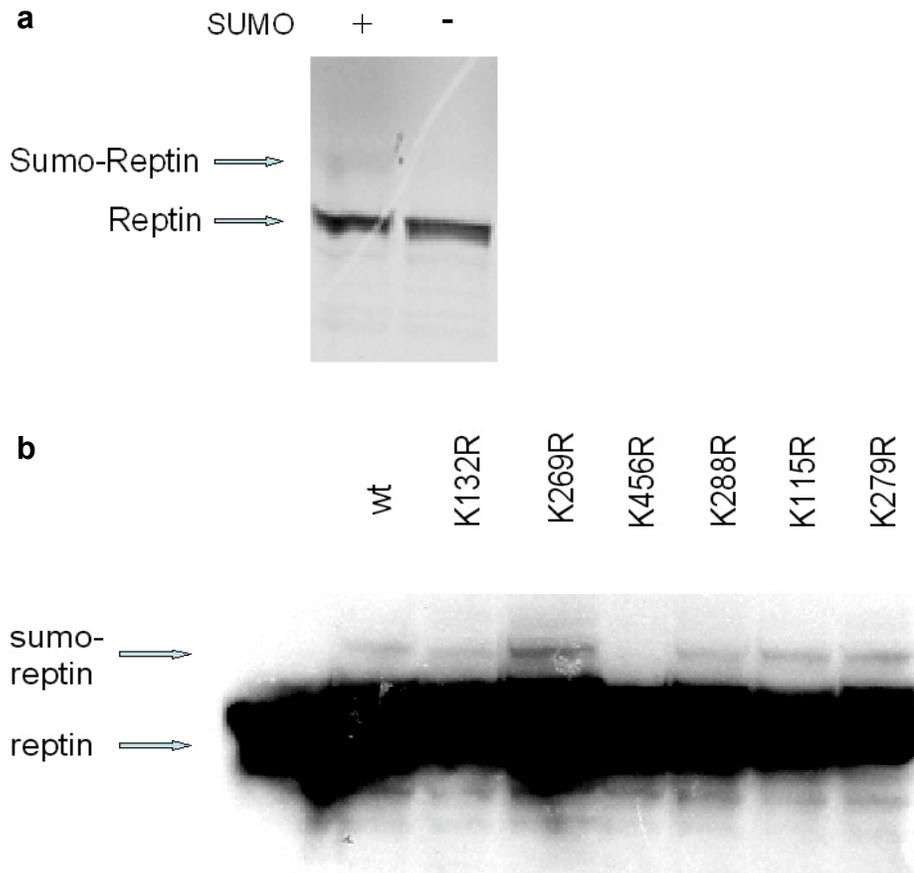
SDC, 1 mM EDTA, 10 mM Tris-HCl pH8.0); TE buffer (20 mM Tris-HCl pH8.0, 1 mM EDTA pH8.0). Chromatin was eluted twice with 100  $\mu$ l of freshly made elution buffer (1% SDS, 0.1 M NaHCO<sub>3</sub>) with 30 min of vigorous shaking on a vortex mixer. The eluted chromatin-protein was subjected to reverse cross-linking by incubation at 65°C for 6 to 16 h. DNA was recovered using a QIAGEN Quick Spin Column and suspended in 100  $\mu$ l of elution buffer (QIAGEN). PCR amplification was performed using SYBR green real-time PCR master mix and 2  $\mu$ l of eluted DNA.

**Table 3.1. Identification of Reptin-interacting proteins by using yeast two-hybrid screening.**

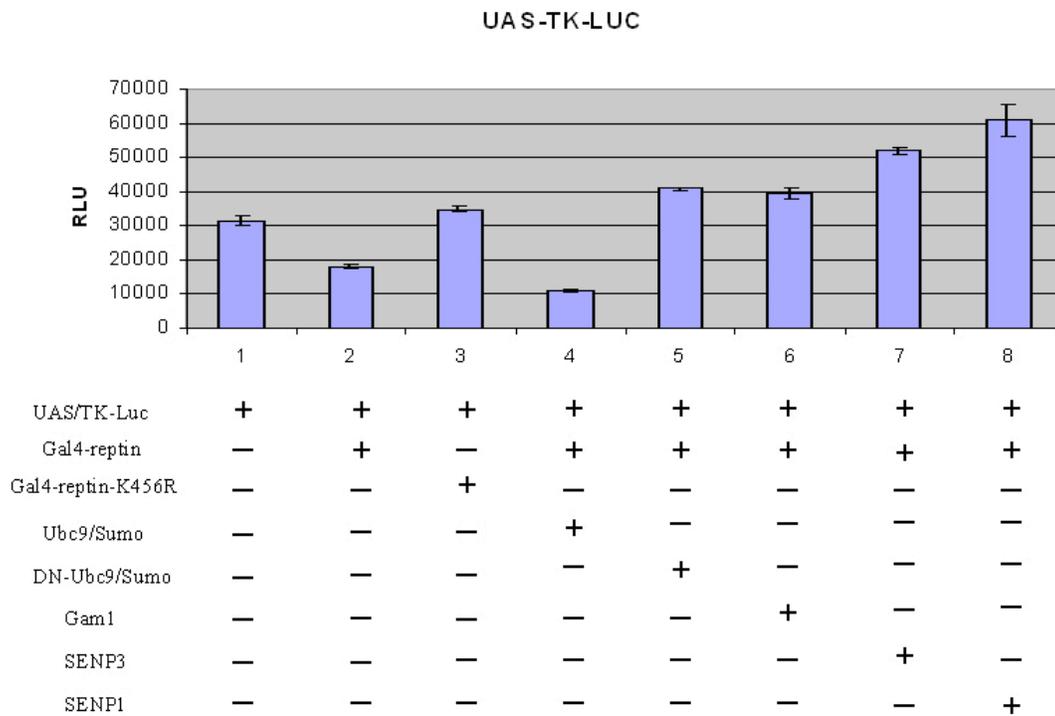
Clone #	Gene name	In Frame	interaction region
#6	fibronectin type 3	Yes	AA 1-285
#7	ASXL1	Yes	AA 1313-1536
#9	MLL5	Yes	AA 116-323
#13	RPS20	Yes	AA 301-375
#19	FHL2	Yes	
#20	ASXL1	Yes	AA 1287-1412
#24	TRIP3	Yes	AA 4-155
#25	RPS20	Yes	AA 315-375
#26	UBC9	Yes	AA 1-62
#27(L)	KIAA1503	Yes	
#27(S)	ALU subfamily	Yes	
#31	Collagen	Yes	
#36	Id2	Yes	AA 1-100
#40	Id2	Yes	AA 1-17



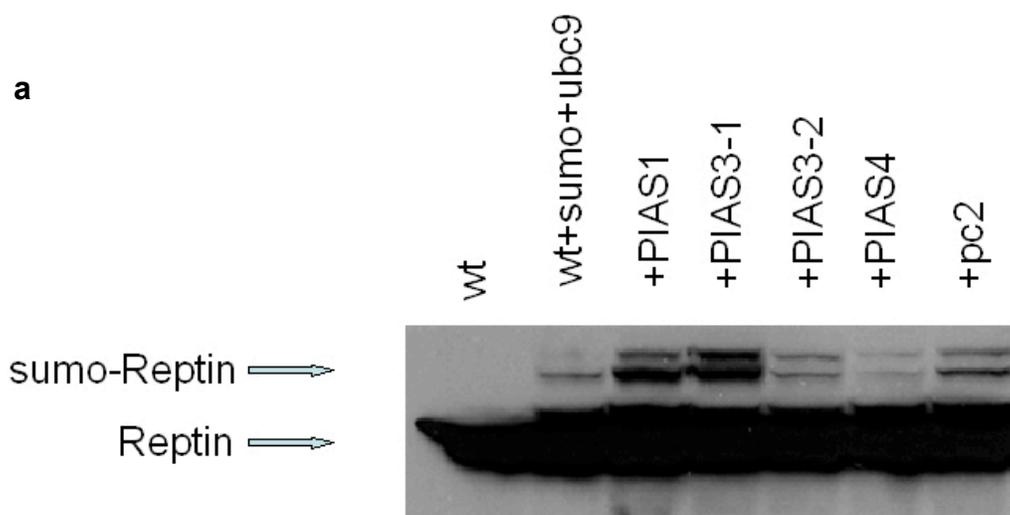
**Figure 3.1. Reptin interacts with UBC9 in 293T cells.** Flag-Reptin and V5-Ubc9 plasmids were co-transfected into 293T cells. After 36hrs, whole cell lysates were immunoprecipitated with antibody against either anti-Flag or control IgG, and the resultant precipitates were immunoblotted with either anti-Flag (upper panel) or anti-V5 antibodies (lower panel).



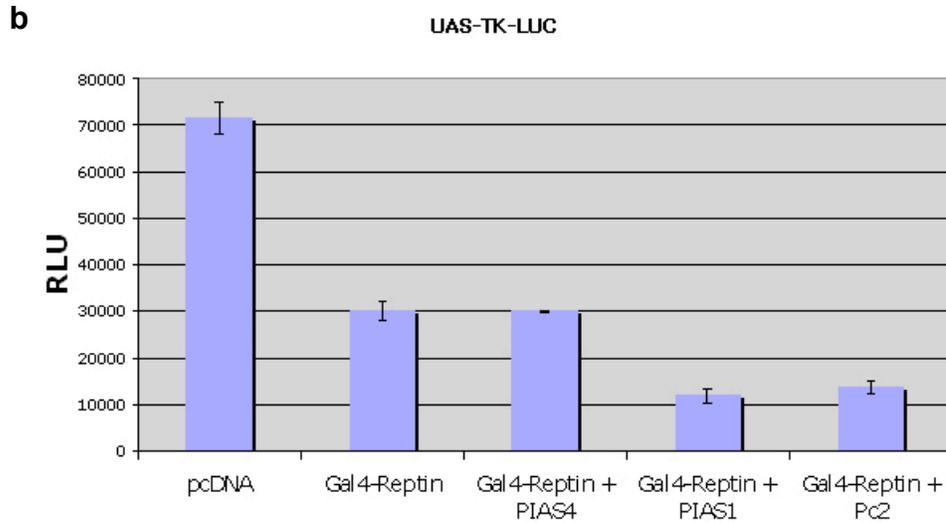
**Figure 3.2. Lysine 456 of Reptin is crucial for SUMO modification.** a. In vitro modification of Reptin by SUMO. S35-labelled in vitro translated Reptin was incubated with a sumoylation assay kit (LAE Biotech) containing purified SAE1-SAE2, Ubc9, and ATP in the presence or absence of SUMO. b. Lysine 456 of reptin is a major SUMO conjugation site. 293T cells were cotransfected with plasmids expressing Flag-tagged either wild-type (wt) Reptin or mutant Reptin with each potential SUMO conjugating lysine individually mutated to arginine in the presence of SUMO and Ubc9 followed by western blot analysis using anti-Flag antibody.



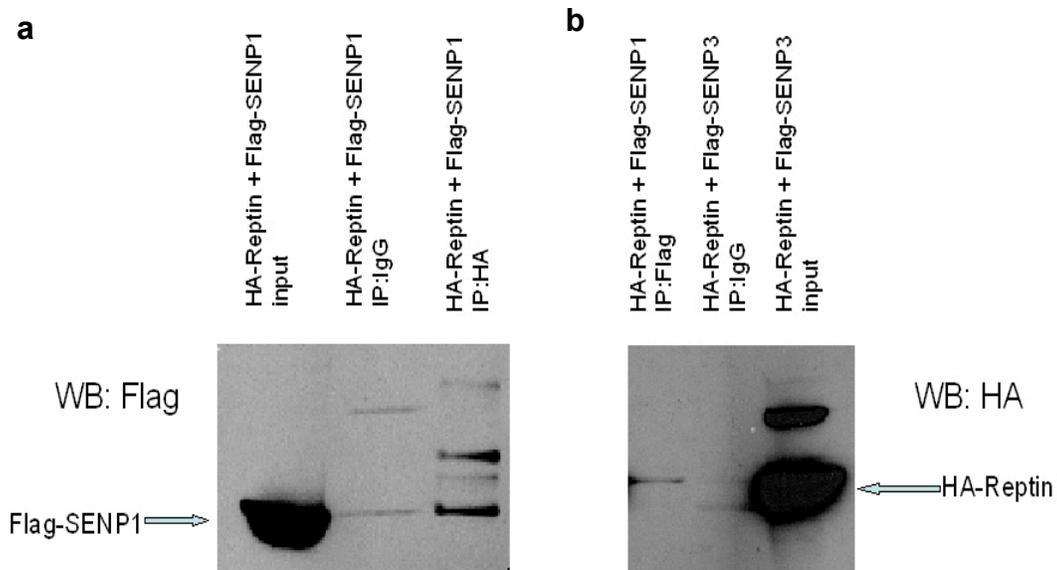
**Figure 3.3. Sumoylation pathway is important for the repression function of Reptin.** 293T cells were cotransfected with a UAS-TK-luciferase reporter, Gal4-Reptin, Gal4-Reptin-K456R, Ubc9, DN-Ubc9, SENP1, SENP3, Gam1. DN-Ubc9 represents dominant negative Ubc9. Gam1 is a SUMO pathway inhibitor. Extracts of transfected cells were subjected to luciferase assays. Luciferase activity was measured and normalized by  $\beta$ -galactosidase assay. Values are expressed as mean  $\pm$  sd (standard deviation) for three independent experiments.



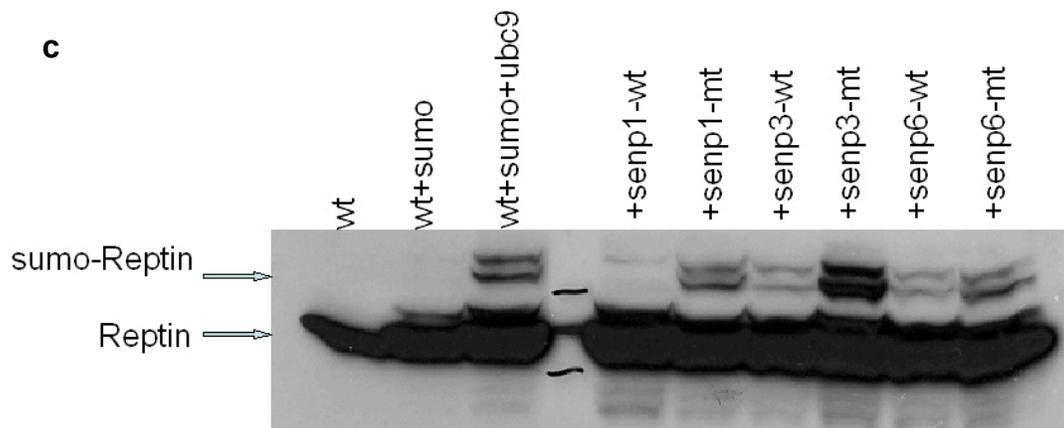
**Figure 3.4. PIAS family members and Pc2 are Reptin SUMO E3 ligases. a.** Reptin E3 SUMO ligases. 293T cells were cotransfected with wildtype (wt) HA-Reptin, Ubc9 and SUMO together with each indicated plasmids PIAS1, PIAS3-isoform1, PIAS3-isoform2, PIAS4 or Pc2. Whole cell extracts were assessed by western blotting analysis against anti-HA antibody. PIAS1, PIAS3-isoform1 and Pc2 increase Reptin sumoylation.



**Figure 3.4, continued.** b. Reptin sumo E3 ligases enhance Reptin repression function on a UAS-TK promoter reporter. 293T cells were cotransfected with a UAS-TK-luciferase reporter, Gal4-Reptin, and different sumo E3 ligases such as PIAS1, PIAS4, or Pc2. Extracts of transfected cells were subjected to luciferase assays. Luciferase activity was measured and normalized by b-galatosidase assay. Values are expressed as mean  $\pm$  sd (standard deviation) for three independent experiments.

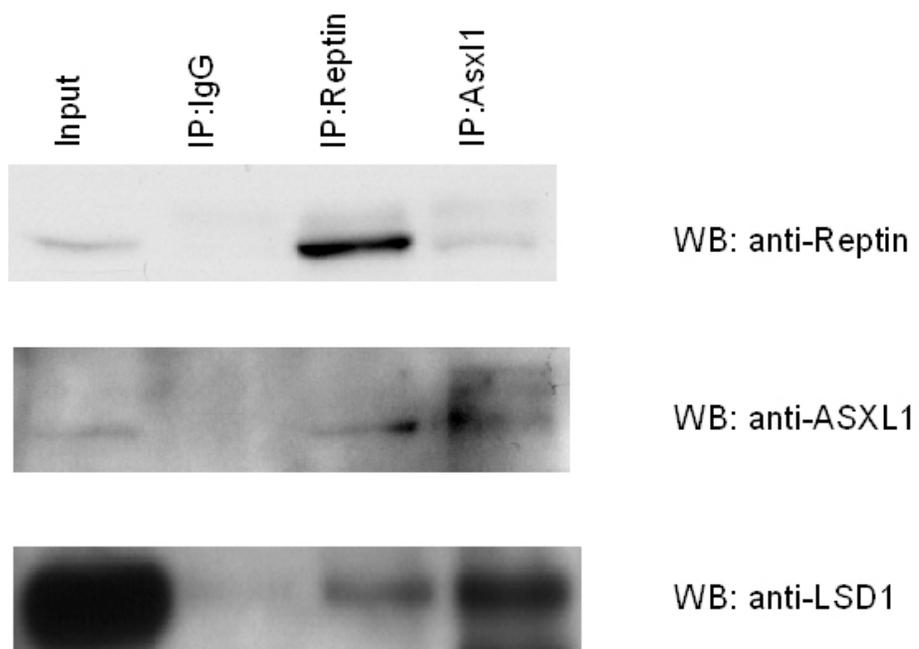


**Figure 3.5. Reptin interacts with both SENP1 and SENP3.** a. Reptin interacts with SENP1. HA-Reptin and Flag-SENP1 plasmids were cotransfected into 293T cells by using lipofectamine 2000. Whole cell lysates of transfected cells were immunoprecipitated with antibody against either HA or control IgG, and the resultant precipitates were immunoblotted with anti-Flag antibody. b. Reptin interacts with SENP3. HA-Reptin and Flag-SENP3 plasmids were cotransfected into 293T cells by using lipofectamine 2000. Whole cell lysates of transfected cells were immunoprecipitated with antibody against either Flag or control IgG, and the resultant precipitates were immunoblotted with anti-HA antibody.

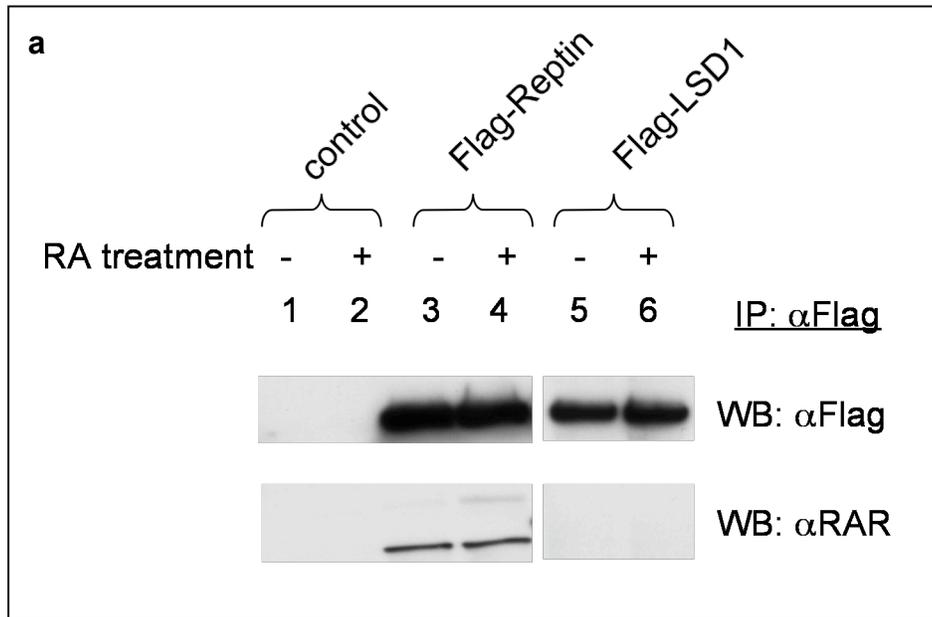


**Figure 3.5. continued.**

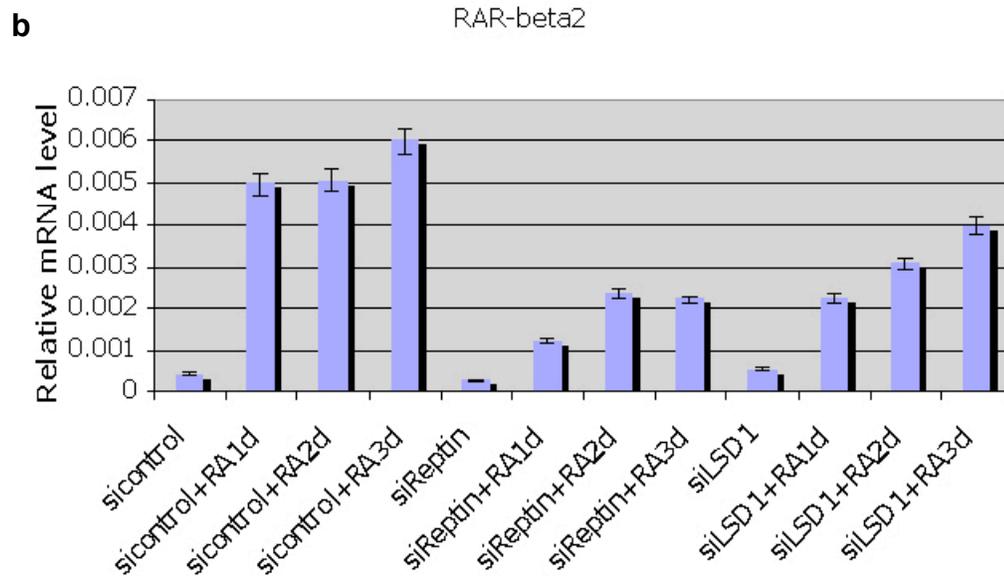
c. 293T cells were cotransfected with wild-type(wt) HA-Reptin, Ubc9 and SUMO together with each indicated plasmids SENP1-wt, SENP1-mutant (mt), SENP3-wt, SENP3-mt, SENP6-wt, or SENP6-mt. SENP-mt indicates the active site mutant that has impaired enzymatic activity. Whole cell extracts of transfected cells were subjected to western blotting analysis using anti-HA antibody.



**Figure 3.6. Reptin interacts with ASXL1 and LSD1 in NT2 cells.** NT2 cell lysates were subjected to immunoprecipitation with anti-Reptin, anti-ASXL1 or control IgG individually, and the resultant precipitates were subjected to immunoblotting against anti-Reptin (upper panel), anti-ASXL1 (middle panel) or anti-LSD1 (bottom panel).

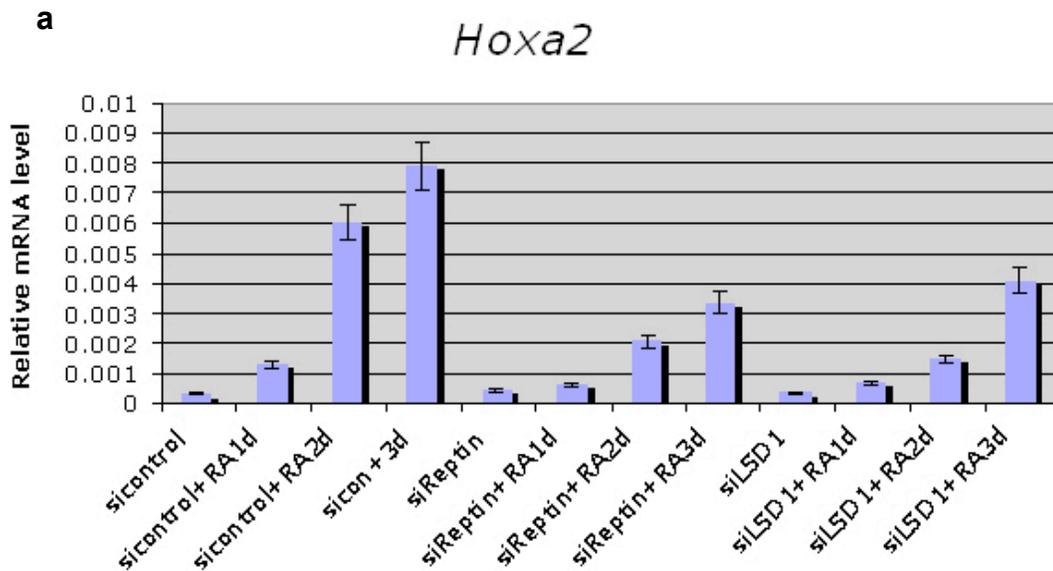


**Figure 3.7. Reptin is a coactivator for RAR target gene in NT2 cells.** a. Reptin interacts with RAR. 293 cells were transfected with pcDNA, Flag-Reptin, or Flag-LSD1 individually. After transfection, cells were treated with or without retinoic acid (RA) and whole cell lysates were immunoprecipitated with anti-FLAG antibody, and the resultant precipitates were immunoblotted with either anti-Flag (upper panel) or anti-RAR antibodies (lower panel).

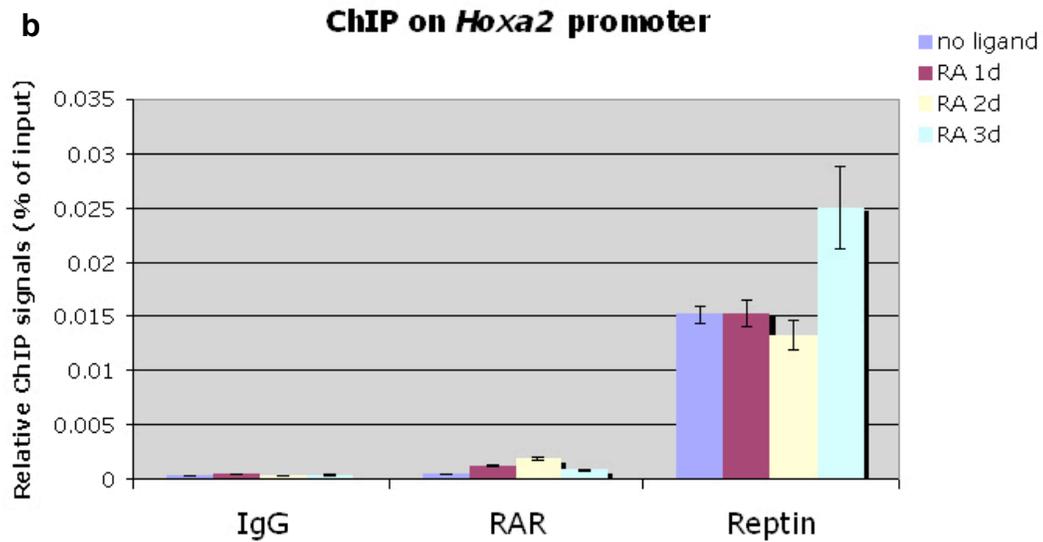


**Figure 3.7. continued.** b. The knockdown of endogenous Reptin or LSD1 in Ntera2 cells affected the expression level of RAR $\beta$ 2 gene. Ntera2 cells were transfected with siRNA targeting Reptin, LSD1 or a control siRNA individually. After transfection, cells were treated with or without retinoic acid (RA) for 1 to 3 days (1d to 3d). Total RNA was isolated and amplified by real-time RT-PCR using transcript-specific primers. Values are normalized with GAPDH and expressed as mean  $\pm$  sd (standard deviation) for three independent experiments.

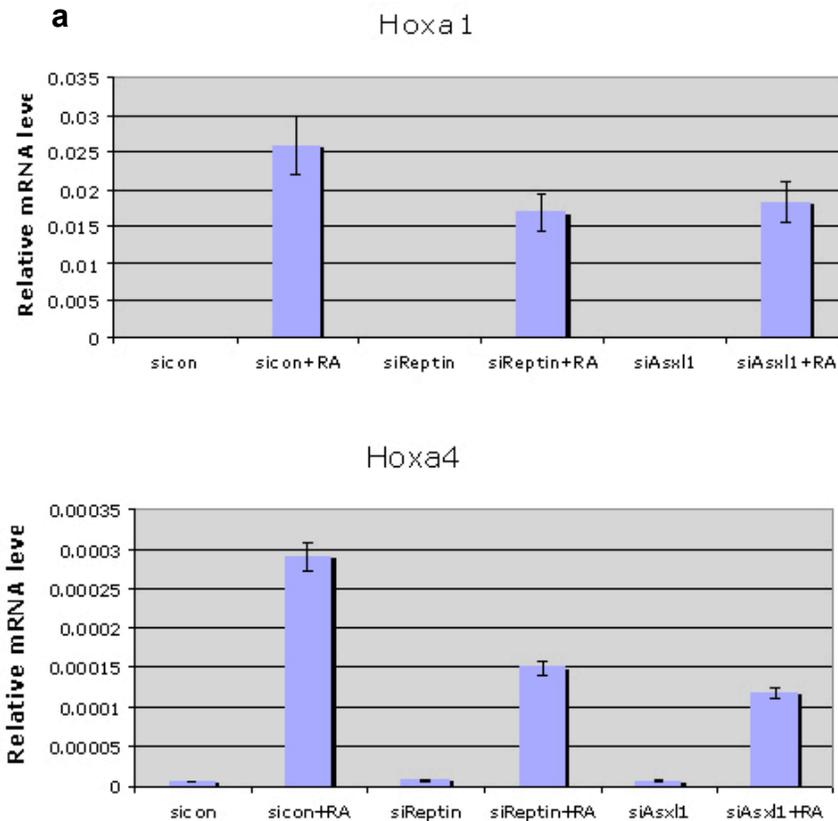




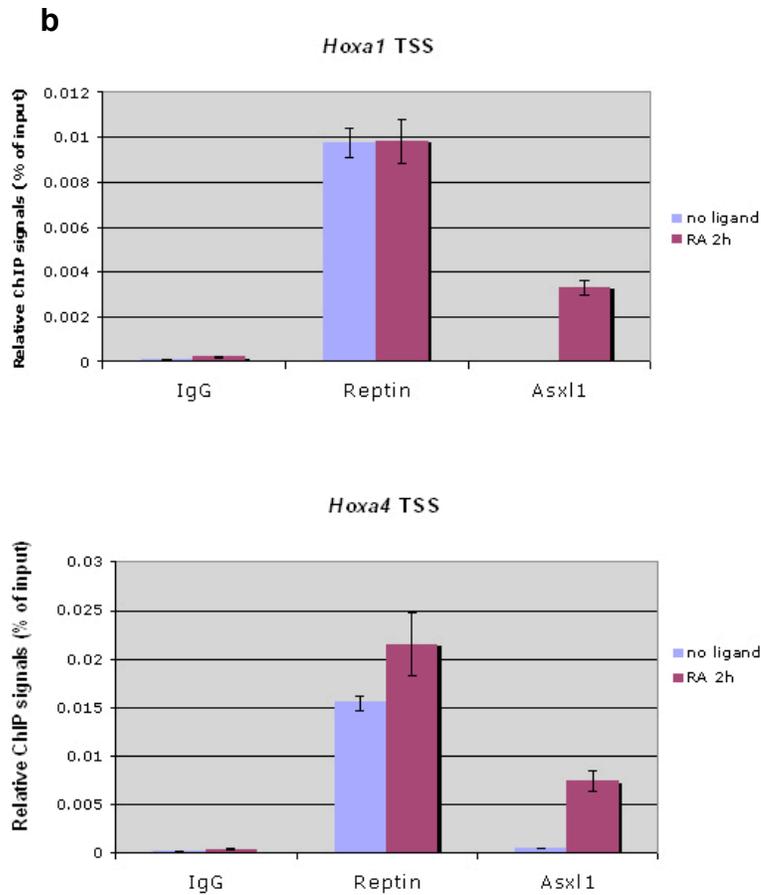
**Figure 3.8. Reptin is a coactivator for Hox genes in NTERA2 cells.** a. The knockdown of endogenous Reptin or LSD1 in NTERA2 cells affected the expression level of specific Hox genes. NTERA2 cells were transfected with siRNA targeting Reptin, LSD1 or a control siRNA individually. After transfection, cells were treated with or without retinoic acid (RA) for 1 to 3 days (1d to 3d). Total RNA was isolated and amplified by real-time RT-PCR using transcript-specific primers. Values are normalized with GAPDH and expressed as mean  $\pm$  s.d. (standard deviation) for three independent experiments.



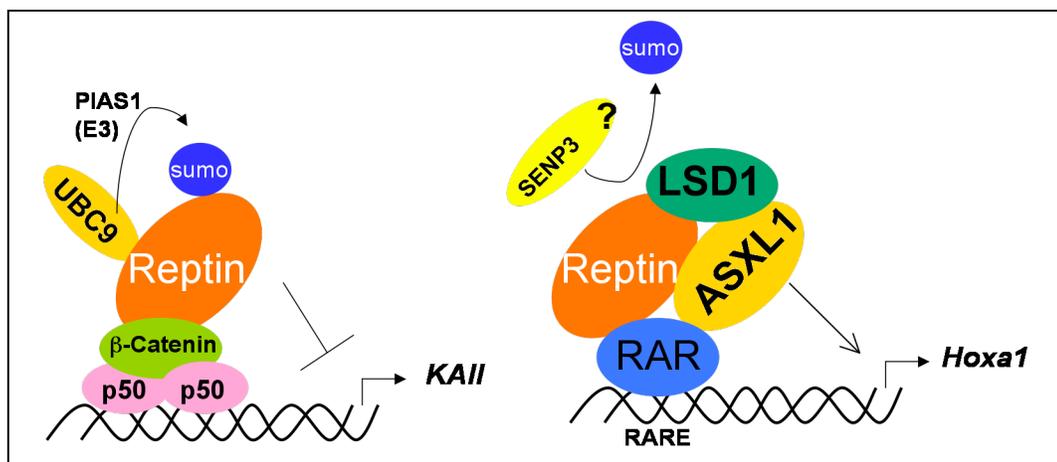
**Figure 3.8. continued.** b. Reptin was directly recruited to *Hoxa2* promoter in NTERA2 cells. NTERA2 cells treated with or without RA for 1 to 3d were subjected to ChIP assay with the indicated antibodies. The precipitated chromatin was amplified by PCR using primers flanking the *Hoxa2* promoter region. Values are expressed as mean  $\pm$  s.d. (standard deviation) for three independent experiments.



**Figure 3.9. Reptin and ASXL1 function together on Hox gene expression in NT2 cells.** a. The knockdown of endogenous Reptin or ASXL1 in NTera2 cells affected the expression level of specific Hox genes. NTera2 cells were transfected with siRNA targeting Reptin (siReptin), ASXL1 (siAsxl1) or a control siRNA (sicon) individually. After transfection, cells were treated with or without retinoic acid (RA) for 2 days. Total RNA was isolated and amplified by real-time RT-PCR using transcript-specific primers. Values are normalized with GAPDH and expressed as mean  $\pm$  s.d. (standard deviation) for three independent experiments.

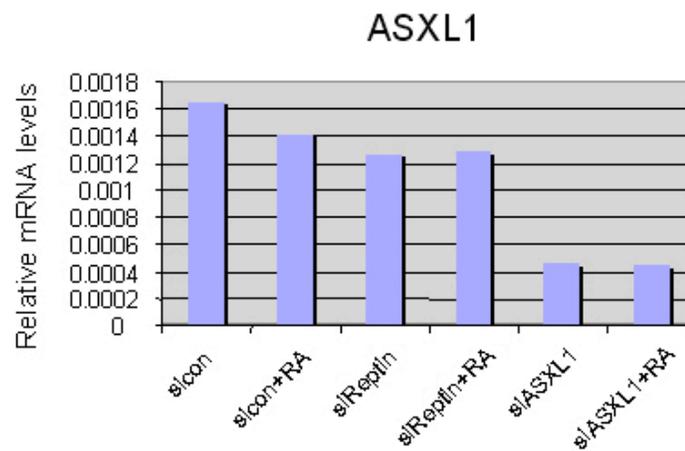
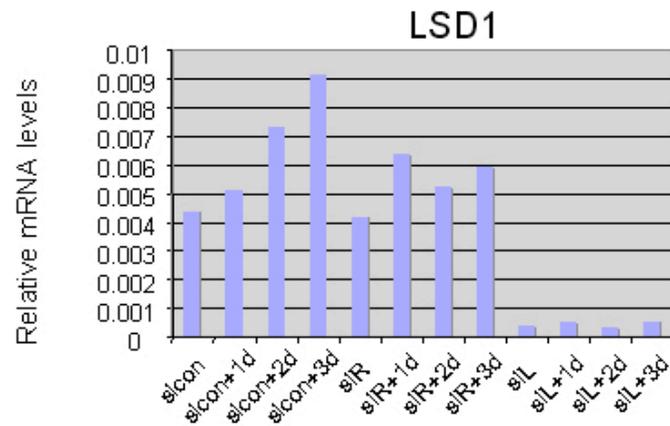
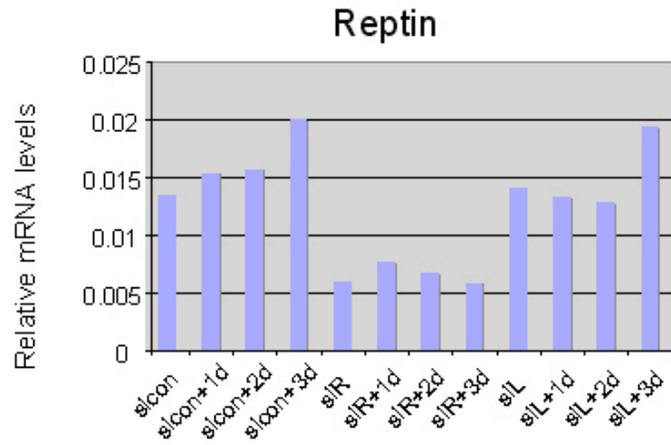


**Figure 3.9. continued.** b. Reptin and ASXL1 were directly recruited to specific Hox gene promoters in Ntera2 cells. Ntera2 cells treated with or without RA for 2hrs were subjected to ChIP assay with the indicated antibodies. The precipitated chromatin was amplified by PCR using primers flanking the transcriptional start sites (TSS) of *Hoxa1* and *Hoxa4*. Values are expressed as mean  $\pm$  s.d. (standard deviation) for three independent experiments.



**Figure 3.10.** Reptin plays dual roles in gene transcription in a context dependent manner.

**Supplementary Figure 3.1. The knockdown efficiency of siRNA that specifically target Reptin, LSD1 or ASXL1 in NTera2 cells.** NTera2 cells were transfected with siRNA targeting Reptin, LSD1, ASXL1 or a control siRNA individually. After transfection, cells were treated with or without retinoic acid (RA). Total RNA was isolated and amplified by real-time RT-PCR using transcript-specific primers. siR stands for siReptin; siL stands for siLSD1; sicon stands for sicontrol. Values from signals in real-time PCR are normalized against signals of GAPDH and expressed as mean  $\pm$  s.d. (standard deviation) for three independent experiments.



## **Chapter IV**

**Sumoylation regulates multiple aspects of LSD1 function.**

**Abstract**

Most DNA-templated processes, such as transcriptional regulation and DNA replication, are actively regulated by dynamic histone modifications at tail regions (such as acetylation, phosphorylation, ubiquitination, sumoylation and methylation) and their specific chromatin-modifying enzymes. LSD1 was the first identified histone lysine demethylase. LSD1 possesses two opposite regulatory roles in transcription, either acting as a transcriptional repressor by demethylating mono- and dimethylated histone H3 Lys 4 (H3K4) (Shi et al. 2004), or acting as an activator when it associates with the androgen receptor (AR) and demethylates mono- and dimethylated H3 Lys9 (H3K9) (Metzger et al. 2005). Here, we reported that LSD1 was modulated by sumo modification and that changes in sumoylation-desumoylation status induced a switch of LSD1 functions in transcriptional regulation. Specifically, sumoylation enhanced LSD1 mediated-repression on some promoters such as CoREST target genes; Knockdown of PIAS1, a LSD1 sumo E3 ligase, abrogated the recruitment of LSD1 on these target genes, concomitant with subsequent de-repression of these genes. On the other hand, we also found that treatment with the AR ligand induced the desumoylation of LSD1 mediated by SENP1 on the AR target genes; Knocking down SENP1 decreased AR-mediated transcriptional activation. Thus, sumoylated forms of LSD1 correlated with its gene repression function in CoREST complexes, and de-sumoylated forms of LSD1 correlated with its gene activation function when associated with AR. Our observations suggest that

sumoylation-desumoylation cycling plays an important role in -regulating LSD1 function.

## Introduction

The fundamental unit of chromatin, the nucleosome core particle, consists of approximately 146 base pairs of DNA wrapped around a histone octamer consisting of two copies each of the core histone proteins – H2A, H2B, H3 and H4. The N-terminal tails of these histones are subject to post-translational covalent modifications, which at least include phosphorylation, acetylation, ubiquitination, sumoylation, methylation and etc. (Strahl et al. 2000). It has been suggested that these histone modifications are critical in the regulation of chromatin structure, gene transcription, and cellular identities (Bannister et al. 2002; Lachner et al. 2002; Zhang et al. 2001). Unlike histone acetylation that occurs only on lysines (K), histone methylation takes place on both lysine (K) and arginine (R) residues. While histone acetylation is usually linked with transcriptional activation, histone methylation is correlated with both activation and repression (Roth et al. 2001; Zhang et al. 2001). The position and extent of lysine methylation differentially regulate diverse physiological responses. For example, methylation on histone H3 Lys9 (H3K9) plays a significant role in heterochromatin formation and in euchromatin gene repression (Nakayama et al. 2001; Nielsen et al. 2001; Rice et al. 2001; Zhang et al. 2001); In contrast, methylation of histone H3 Lys4 (H3K4) is associated with active transcription (Liang et al. 2004; Schneider et al. 2004;). Recent identification of the first histone lysine demethylase, LSD1, and the Jmj domain-containing families of hydroxylases demonstrated that histone lysine methylation is clearly reversible and dynamically regulated.

LSD1, also called KIAA0601, BHC110, NPAO, KDM1, was initially identified as an amine oxidase, which demethylates mono and dimethylated H3K4 via a FAD-dependent oxidative reaction and functions as a transcriptional corepressor (Shi et al. 2004). LSD1 is unable to demethylate trimethylated H3K4 due to the inherent chemistry of flavin-containing amine oxidases, which requires a protonated nitrogen in the substrates (Bannister et al. 2002). Later on, LSD1 was also shown to play an important role in the activation of androgen receptor (AR) target genes (Metzger et al. 2005). LSD1 interacted with AR in vitro and in vivo (Metzger et al. 2005). In response to AR ligand treatment, LSD1 and AR co-occupied on their target gene promoters and stimulated H3K9 demethylation without affecting H3K4 methylation status (Metzger et al. 2005). Furthermore, siRNA knockdown of LSD1 resulted in decreased activation of AR target genes (Metzger et al. 2005). However, the mechanism by which the LSD1 function is changed from transcriptional repression (H3K4 demethylation) to activation (H3K9 demethylation) in the context of AR-dependent gene regulation remains largely unknown. Hypothetically, either a conformational change induced by protein-protein interactions or a post-translational modification on LSD1 may result in this alteration. It is known that LSD1 enzymatic activity is regulated by its associated cofactors, CoREST, HDAC1/2 and BHC80 (Shi et al. 2005). For example, CoREST was reported to be essential for LSD1 to demethylate nucleosomal substrates, and it also protects LSD1 from proteasomal degradation in vivo (Shi et al. 2005). HDAC1 and HDAC2 are implicated to generate hypo-acetylated nucleosomes that serve as better substrate for LSD1-mediated H3K4

demethylation (Shi et al. 2005). In addition, BHC80 binds to unmethylated H3K4 and is required for stable association of the LSD1-containing complexes to their target promoters (Lan et al. 2007). These findings clearly suggest that LSD1 histone demethylase activity can be regulated dynamically *in vivo*.

In this chapter, we demonstrated that covalent post-translational modification such as sumoylation was involved in the regulation of LSD1 function. Both LSD1 sumoylation and desumoylation were important for its demethylase activity. First, we proved that LSD1 was subject to sumo modification *in vivo* and sumoylation is essential for LSD1-mediated gene repression. We identified PIAS family members as LSD1 sumo E3 ligases. Knockdown of PIAS1, one of LSD1 sumo E3 ligases, dramatically abrogated the recruitment of LSD1 on LSD1/CoREST target genes, which was concomitant with increased levels of dimethylated H3K4 marks and resultant gene de-repression. On the other hand, LSD1 sumoylation and desumoylation were also important for its gene activation activity when associated with AR. In LNCaP cells, we found that treatment with an androgen receptor ligand (DHT) induced dramatic desumoylation of LSD1 by sumo protease SENP1. Then, both SENP1 and desumoylated form of LSD1 were recruited to AR target genes in response to DHT treatment, and knocking down either of them decreased AR-mediated transcriptional activation, which was concomitant with increased occupancy of dimethylated H3-K9 marks. Taken together, our data suggest that cycling between sumoylation and desumoylation of LSD1 plays a significant role in regulating its regulatory roles in gene transcription.

## Results and Discussion

Previously, LSD1 has been copurified within a number of transcriptional repressive complexes including NRD-, CoREST-, CtBP-, and HDAC1/2-containing complexes. (Tong et al. 1998; You et al. 2001; Shi et al. 2003; Hakimi et al. 2002, 2003; Humphrey et al. 2001). CtBP-CoREST corepressor complexes contain LSD1, CoREST, CtBP1/2, ZNF217, Zeb1, Pc2 and et al. (Shi et al. 2003). Notably, one component Pc2 is a sumo E3 ligase. The presence of a sumo E3 ligase and LSD1 in the same complex promoted us to test whether or not LSD1 is a sumo modification substrate. First, we carried out an *in vitro* sumo conjugation assay to examine this possibility. *In vitro* translated LSD1 was incubated with a sumoylation reaction mixture containing recombinant E1 (a heterodimer of Aos1/Uba2), E2 (UBC9), SUMO and ATP. In the presence of SUMO, an additional more slowly migrating band appeared, which is corresponding to the size of sumo conjugated LSD1 (Figure 4.1a). To map the sumo acceptor site, we searched LSD1 amino acid sequence for the presence of potential consensus sumo modification motif(s),  $\psi$ KXD/E (where  $\psi$  represents a large hydrophobic amino acid and X represents any amino acid), and each of these potentially sumo-modification lysine residues were mutated individually to arginine. These mutants were then tested for the potentials to be sumoylated by *in vivo* sumoylation assay. Mutation of K117R, K144R, K469R and K503R exhibited little or no alterations of LSD1 sumo conjugation, whereas the K424R mutant almost completely abrogated LSD1 from SUMO modification (Figure 4.1b

and data not shown). This suggests that K424 is a major sumoylation site within LSD1.

To investigate whether or not sumoylation regulates LSD1 function in transcriptional regulation, we first performed luciferase reporter analyses. We examined the promoter activities of Cdc2 and growth hormone, two known LSD1 target genes (Wang et al. 2007). As shown in Fig.2A, overexpression of wild-type LSD1 repressed the expression of cdc2-promoter driven luciferase reporter, whereas the sumo null mutant of LSD1 (K424R) failed to repress it. Similar results were observed using a luciferase reporter driven by the growth hormone promoter (Figure 4.2b).

To further examine whether or not LSD1 sumoylation plays a significant role in LSD1 mediated repression *in vivo*, we next performed a rescue experiment by using LSD1 knockout MEF cells (Wang et al. 2007). In non-neuronal cells, LSD1/CoREST complexes induced the silencing of neuronal specific genes such as genes encoding the sodium channels (SCN1A, SCN2A, and SCN3A) and acetylcholine receptors (AchR) (Ballas et al. 2001). These genes were shown to be derepressed in LSD1 knockout MEFs. We asked whether or not the repression of these target genes can be restored after transfection of LSD1 plasmids, either wildtype or sumo-defective mutant. As shown by real-time RT-PCR in Figure 4.2c, expression of wild-type LSD1 induced the repression of these CoREST target genes, whereas expression of either a sumo null LSD1 mutant or an enzymatic dead mutant of LSD1 did not.

**PIAS family functions as LSD1 E3 sumo ligases.**

At least three distinct families of sumo E3 ligases have been identified so far, i.e., PIAS, RanBP2 and Pc2. *In vivo* sumoylation assays showed that each of PIAS family members tested (PIAS1, PIAS3 isoform1 and 2 and PIAS4) enhanced the sumoylation of LSD1 (Figure 4.3). Surprisingly, although LSD1 interacted with another sumo E3 ligase Pc2, overexpression of Pc2 did not induce significant increase in LSD1 sumo conjugation (Figure 4.3). Next, we examined how LSD1 sumo E3 ligases regulated LSD1 functions in gene expression. As shown in Figure 4.4a, knocking down PIAS1 by specific siRNA relieved LSD1-mediated repression of CoREST target gene, sodium channel 1A (SCN1A), which was accompanied with dramatic loss of LSD1 recruitment and increased levels of H3K4 dimethylation marks, as detected by chromatin immunoprecipitation (ChIP) assays (Figure 4.4b). This indicated that sumoylation of LSD1 played an important role in its gene repression function when associated with CoREST complexes.

Since sumoylation may regulate protein-protein interactions, we tested whether or not sumoylation would affect the interaction between LSD1 and CoREST. As shown in Figure 4.5, interaction between sumo null mutant (K424R) of LSD1 and CoREST was relatively weaker than interaction between wildtype LSD1 and CoREST. As control, both the wildtype and the sumo null mutant of LSD interacted with HDAC1 at the same levels. This suggests that sumoylation decreased LSD1 repressive activities at least partially through affecting its interaction with CoREST.

**SENP1 is a LSD1 sumo protease.**

Sumoylation is a dynamic process and the removal of sumo is accomplished by specific sumo proteases SENPs (Li et al. 1999; Kim et al. 2000; Best et al. 2002; Bachant et al. 2002). Six members of SENPs (SENP1, SENP2, SENP3, SENP5, SENP6, SENP7) have been identified in mammals (Yeh et al. 2000; Best et al. 2002; Hang et al. 2002; Kim et al. 2000; Nishida et al. 2000, 2001; Gong et al. 2000). All these SENPs share a conserved C-terminal catalytic domain, but the N-terminal regions are quite divergent. The N-terminal regions direct different SENP proteins to distinct subcellular localizations, targeting their specific substrates (Melchior et al. 2003). For example, SENP1 has been shown localized in the nucleoplasm and nuclear bodies, Senp2 localized to the nuclear pore, Senp3 localized to the nucleolus, and Senp6 was reported to be cytoplasmic and also in the nucleus (Gong et al. 2000; Nishida et al. 2000; Hang et al. 2002; Kim et al. 2000; Mukhopadhyay et al. 2006). SENP1 is an essential gene during development as SENP1-null embryos exhibited severe fetal anemia caused by deficient erythropoietin (Epo) production and died at mid-gestation (Cheng et al. 2007). Cells deficient in SENP1 accumulated both immature sumo1 and sumo1 conjugated substrates, whereas procession and deconjugation of sumo2/3 were unchanged (Yamaguchi et al. 2005). Senp3 and Senp5 had a preference for deconjugating and processing sumo2/3 over sumo1 (Gong et al. 2006; Di Bacco et al. 2006). Senp6 seemed to have a preference for the cleavage of polymeric chains of sumo2/3.

To identify the sumo protease that mediates the removal of sumo off LSD1, we performed an *in vivo* desumoylation assay (Figure 4.6). Overexpression of wild-type SENP1 led to the complete removal of sumo off LSD1-sumo conjugates, and a SENP1 enzymatic dead mutant (C603A) dramatically enhanced the sumo conjugation of LSD1 presumably through functioning as a dominant negative form (Figure 4.6). Compared to SENP1, SENP3 and SENP6 had much weaker sumo protease activity towards LSD1 (Figure 4.6). Co-immunoprecipitation assays showed that LSD1 indeed bound specifically to SENP1 after transient transfection in 293T cells (Figure 4.7). The above results demonstrated that LSD1 is an *in vivo* substrate for SENP1-mediated desumoylation.

Interestingly, SENP1 has been shown to promote the transcriptional activation of AR target genes by deconjugating sumoylated HDAC1 and thus reducing HDAC1 deacetylase activities in LNCaP cells (Gong et al. 2004). As discussed earlier, mechanisms underlying LSD1-mediated transcriptional activation of AR target genes (by demethylating H3K9) remained unclear. Hypothetically, the observed increase in AR target gene transcription induced by SENP1 may also be attributed to its enzymatic activity towards additional substrates such as LSD1. Towards this end, we first examined whether or not SENP1 could desumoylate LSD1 in response to treatment with an AR ligand, DHT. Since HeLa cells do not express endogenous AR, we transiently transfected a Flag-tagged AR in them. As shown in Figure 4.8, in these cells expressing AR, treatment with DHT, but not control vehicle, dramatically induced the

desumoylation of LSD1. DHT-induced desumoylation was totally dependent on signaling through its receptor AR, because levels of LSD1 sumoylation stayed at the same levels after treatment with DHT in the cells that did not express AR, when compared to treatment with control vehicle. This result demonstrated that treatment with the AR agonist DHT induced efficient removal of sumo off LSD1 presumably through recruitment of SENP1. To further investigate how SENP1 and LSD1 affect AR target gene expression, we examined the message RNA level of PSA by performing RT-PCR analysis after knocking down either SENP1 or LSD1 by transfection of specific siRNAs into LNCaP cells. We found that knocking down SENP1 dramatically decreased the activation of PSA after treatment with DHT (Figure 4.9). As previously reported, knocking down LSD1 induced the similar effect. This suggested that like LSD1, SENP1, a LSD1 de-sumoylating enzyme, was also required for transactivation of AR target genes.

To further determine whether SENP1 and LSD1 associate with chromatin *in vivo*, we performed ChIP analysis in LNCaP cells with or without DHT treatment. As shown in Figure 4.10, LSD1 was specifically bound to the enhancer region of PSA both in the absence and presence of DHT, with a slightly stronger binding after ligand treatment. Interestingly, the recruitment of SENP1 on the PSA enhancer exhibited an AR ligand-dependent manner: SENP1 was absent from PSA enhancer before DHT treatment, and efficiently bound there after DHT treatment (Figure 4.10). This observation was consistent with our earlier experiments showing that SENP1-induced desumoylation of LSD1 only occurred after AR ligand treatment.

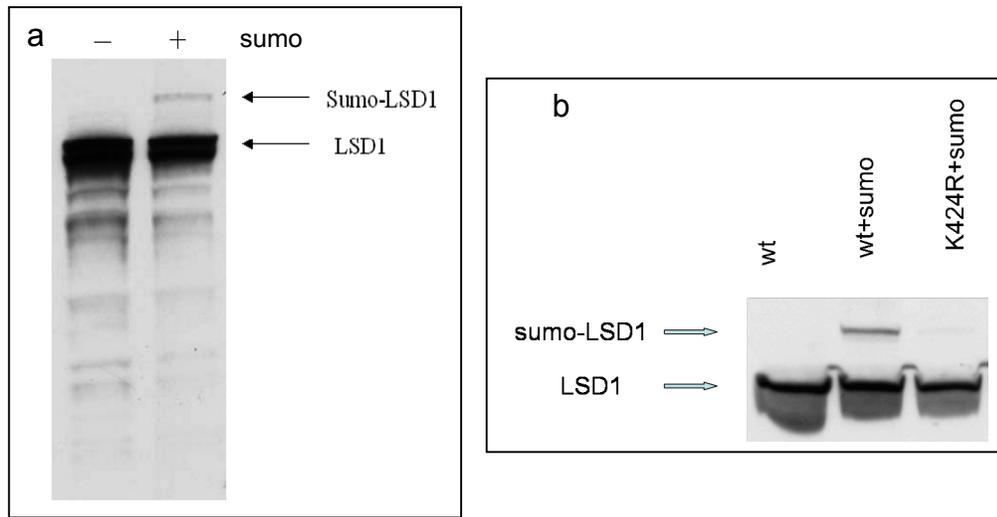
Because DHT treatment induced the expression of PSA in LNCaP cells, we next examined the status of several histone marks representing either gene repression or activation on PSA enhancer regions. As expected, the occupancy of AR and acetylated H3 were dramatically increased in response to DHT treatment. We also examined two potential histone methylation substrates of LSD1: dimethylated H3K4 levels did not show a dramatic change before and after treatment with DHT, whereas we detected a robust decrease in dimethylated H3K9 levels on the PSA enhancer after DHT treatment (Figure 4.10). This indicated specific effect of LSD1 on H3K9 demethylation and resultant transcriptional activation on AR target genes, an observation similar to previous reports.

In summary, we showed that LSD1 was a sumo conjugation substrate *in vivo*. Sumoylation of LSD1 modulated multiple aspects of LSD1-mediated transcriptional regulation. As exemplified in Figure 4.11, LSD1 sumoylation enhanced LSD1-mediated gene repression on LSD1/CoRest complex target promoters. On the other hand, in LNCaP cells, LSD1 associated with AR, and in response to treatment of the androgen receptor ligand DHT, SENP1 removed sumo off LSD1. SENP1-mediated desumoylation of LSD1 correlated with transcriptional activation of AR target promoters. Mechanisms underlying that LSD1 acts as a coactivator remain unclear as LSD1 demethylase activities towards methylated H3K9 have not been detected so far in any *in vitro* enzymatic assays by using purified LSD1 protein alone. Several hypothetical possibilities as follows may explain this issue. First, LSD1 may directly remove the repressive

marks, mono or dimethylated H3K9, and induce gene transcription, which may be accomplished by a conformational change of LSD1 induced by protein-protein interactions or post-translational modifications such as sumoylation reported here. Recently, the crystal structure of LSD1 has been characterized, revealing at least three distinct structural identities within LSD1: the N-terminal SWIRM domain (named for its presence in the proteins Swi3, Rsc8, and Moira), the C-terminal FAD-binding amine oxidase domain, and the insertion tower domain (Stavropoulos et al. 2006; Chen et al. 2006; Yang et al. 2006; Forneris et al. 2007). The SWIRM domain and the amine oxidase domain closely pack against each other and form a globular core structure from which the tower domain protrudes as an elongated helix-turn-helix motif. The major sumo-conjugating site that we identified in LSD1 is located in the tower domain, which gave an indication that sumo may be able to change LSD1 substrate specificity (Stavropoulos et al. 2006; Chen et al. 2006; Yang et al. 2006; Forneris et al. 2007). Alternatively, the observed H3K9 demethylation is catalyzed by an unknown H3K9 demethylase present in the LSD1 complexes. Indeed, a recent report showing that LSD1 cooperates with the H3K9 demethylase JMJD2C to activate AR target genes supported this hypothesis (Wissmann et al. 2007). Lastly, LSD1 may induce H3K9 demethylation indirectly through modifying non-histone substrates, which then may functionally oppose H3K9 methyltransferases.

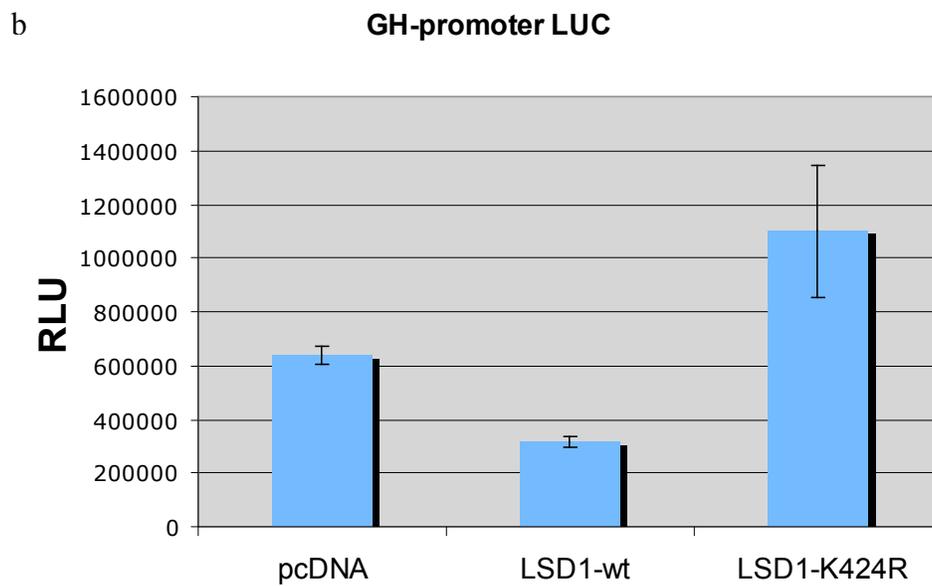
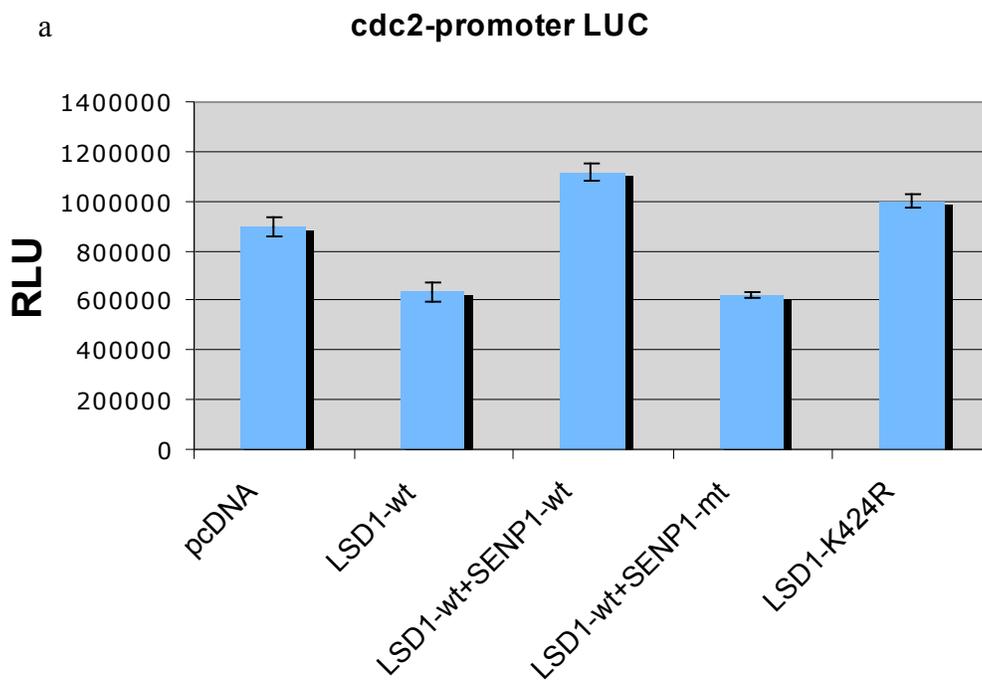
Loss of H3K4 methylation and increased levels of H3K9 methylation have been reported to be associated with several types of solid tumors (Wang et al.

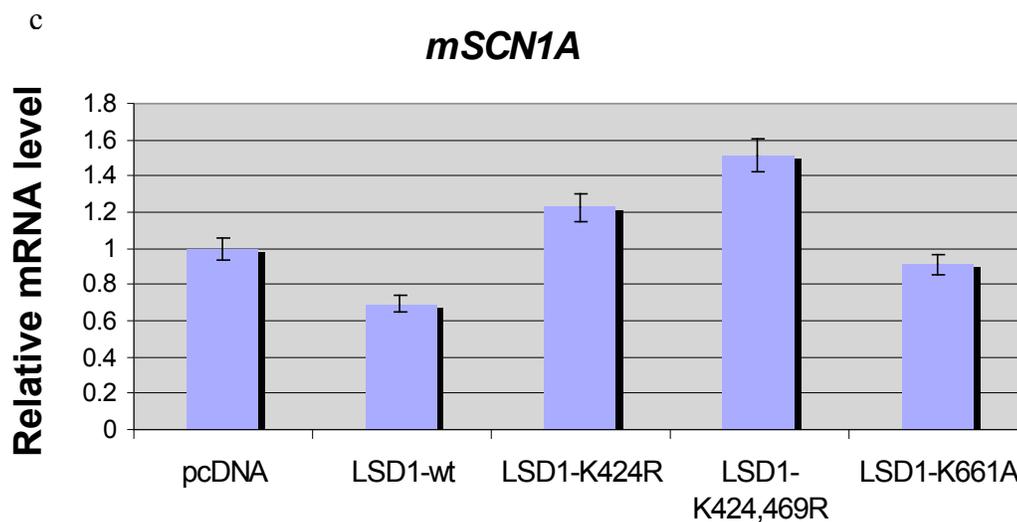
2007). Interestingly, significant up-regulation of both mRNA and protein levels of LSD1 has been shown to associate with high-risk prostate tumors, and LSD1 has been implicated to serve as a novel biomarker for prediction of aggressive prostate cancers (Kahl et al. 2006). SENP1 was also found overexpressed in human prostate cancer specimens but not in corresponding normal prostate tissues (Cheng et al. 2006). Transgenic mice with overexpressed SENP1 in the prostate gland exhibited evidence of early PIN formation (PIN refers to the disease progresses from benign hyperplasia to a prostate cancer precursor state, Cheng et al. 2006). These reports are consistent with our observations showing that both LSD1 and SENP1 act as coactivators of AR signaling and enhance AR-dependent transcriptional activation, a process correlated with prostate carcinogenesis. Future investigations of mechanisms underlying sumoylation-desumoylation as a putative LSD1 functional switch from repression to activation states as reported here shall shed light on designing novel drugs for therapeutic treatment of prostate cancers.



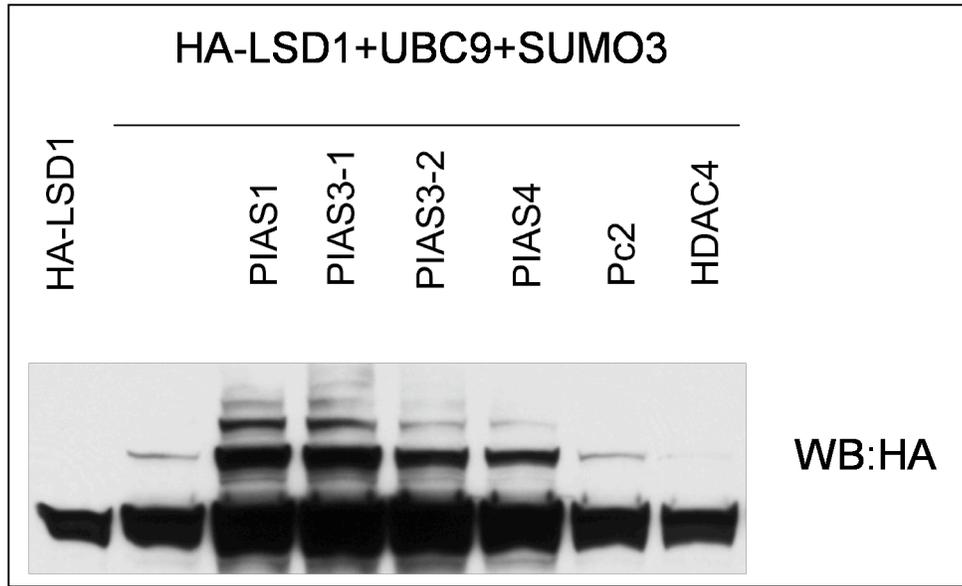
**Figure 4.1. In vitro and in vivo modification of LSD1 by SUMO.** a. In vitro modification of LSD1 by SUMO. In vitro translated LSD1 was incubated with a sumoylation assay kit (LAE Biotech) containing purified SAE1-SAE2, Ubc9, and ATP in the presence or absence of SUMO. b. Lysine 424 of LSD1 is a major SUMO conjugation site. 293T cells were cotransfected with plasmids expressing Flag-tagged either LSD1 wild-type (wt) or LSD1 K424R mutant (mt) in the presence of SUMO followed by western blot analysis using anti-Flag antibody.

**Figure 4.2. Sumoylation pathway is important for the repression function of LSD1.** a. 293T cells were cotransfected with a *cdc2* promoter driven luciferase reporter, LSD1-wt, LSD1- K424R mt, SENP1-wt, or SENP1-mt. SENP-mt represents the active site mutant that impairs the enzymatic activity. Extracts of transfected cells were subjected to luciferase assays. Luciferase activity was measured and normalized by  $\beta$ -galactosidase assay. Values are expressed as mean  $\pm$  s.d. (standard deviation) for three independent experiments. b. 293T cells were cotransfected with a partial growth hormone (GH) promoter driven luciferase reporter, LSD1-wt, or LSD1-K424R mt. Extracts of transfected cells were subjected to luciferase assays. Luciferase activity was measured and normalized by  $\beta$ -galactosidase assay. Values are expressed as mean  $\pm$  s.d. (standard deviation) for three independent experiments.

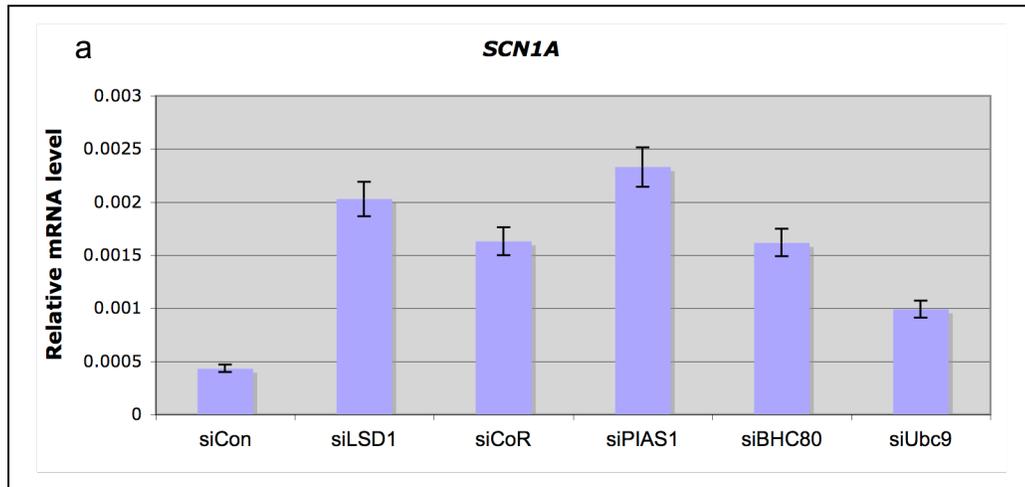




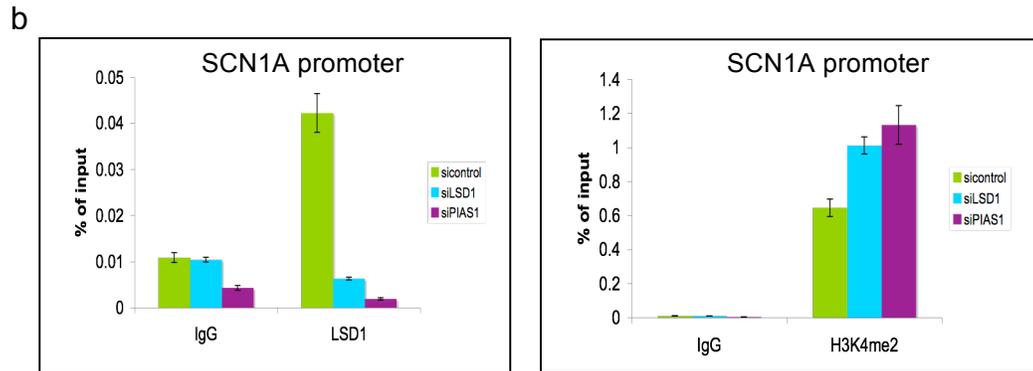
**Figure 4.2. continued.** c. Sumoylation enhances LSD1 repression function-Rescue experiment in LSD1<sup>-/-</sup> MEF cells. LSD1<sup>-/-</sup> MEF cells were transfected with plasmids expressing pcDNA, LSD1-wt, LSD-K424R mt, LSD1-K424, 469R mt or LSD1-K661A mt individually. Total RNA of transfected cells was isolated and amplified by real-time RT-PCR using transcript-specific primers. Values are normalized with GAPDH and expressed as mean  $\pm$  s.d. (standard deviation) for three independent experiments.



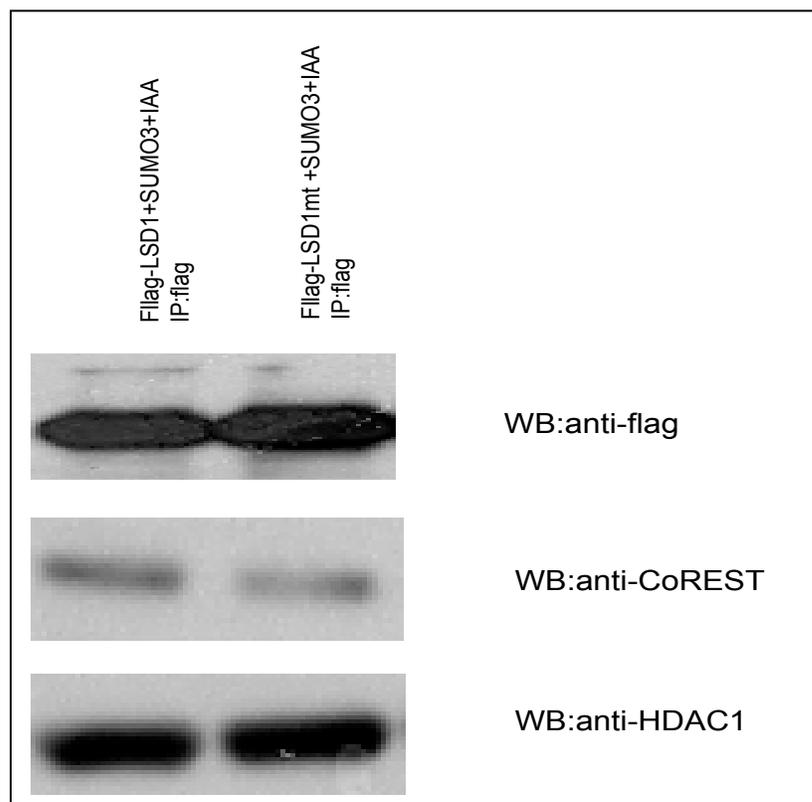
**Figure 4.3. PIAS family proteins act as LSD1 E3 SUMO ligases.** 293T cells were cotransfected with wild-type HA-LSD1, Ubc9 and SUMO together with each indicated plasmids PIAS1, PIAS3-isoform1, PIAS3-isoform2, PIAS4, Pc2 or HDAC4. Whole cell extracts were assessed by western blotting analysis against anti-HA antibody. All of the tested PIAS family members increase LSD1 sumoylation.



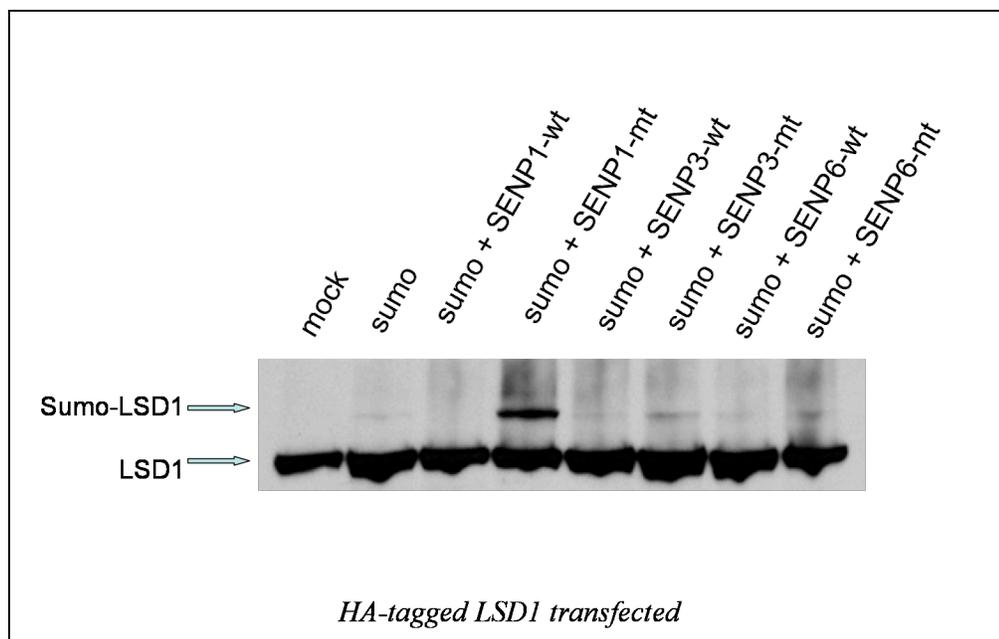
**Figure 4.4. PIAS1 plays a critical role in LSD1 mediated Rest target gene repression.** a. The knockdown of endogenous LSD1, PIAS1, CoREST (CoR), BHC80 or Ubc9 in HeLa cells affected the expression level of SCN1A gene. HeLa cells were transfected with siRNA specifically targeting LSD1, PIAS1, CoREST (CoR), BHC80, Ubc9 or a control siRNA individually. After transfection, total RNA was isolated and amplified by real-time RT-PCR using transcript-specific primers. Values are normalized with GAPDH and expressed as mean  $\pm$  s.d. (standard deviation) for three independent experiments.



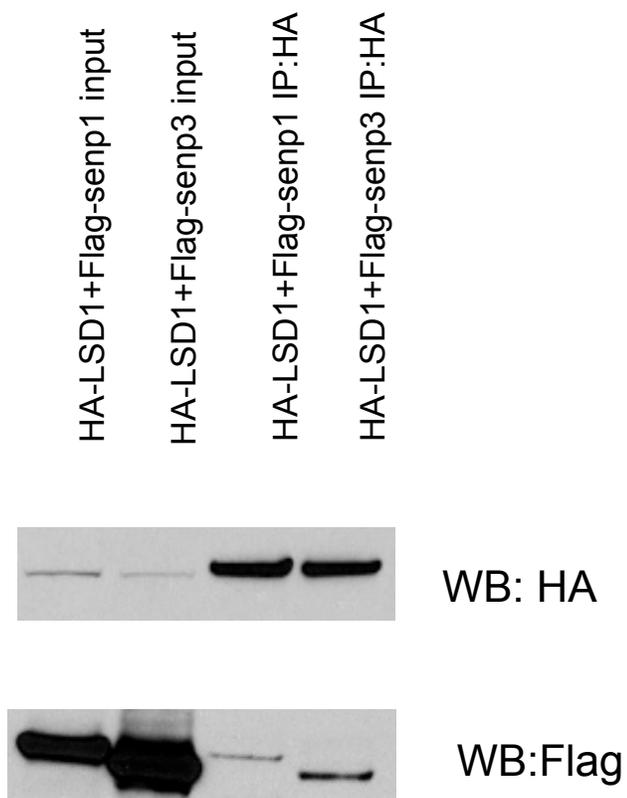
**Figure 4.4. continued.** b. Effects of silencing LSD1 and PIAS1 on the recruitment of LSD1 and the levels of dimethylated histone H3 Lys4 marks (diK4) to the SCN1A promoter. HeLa cells were transfected with siRNA specifically target LSD1, PIAS1 or control. After 48hrs transfection, transfected cells were subjected to CHIP assay with the indicated antibodies. The precipitated chromatin was amplified by PCR using primers flanking the SCN1A promoter region. Values are expressed as mean  $\pm$  s.d. (standard deviation) for three independent experiments.



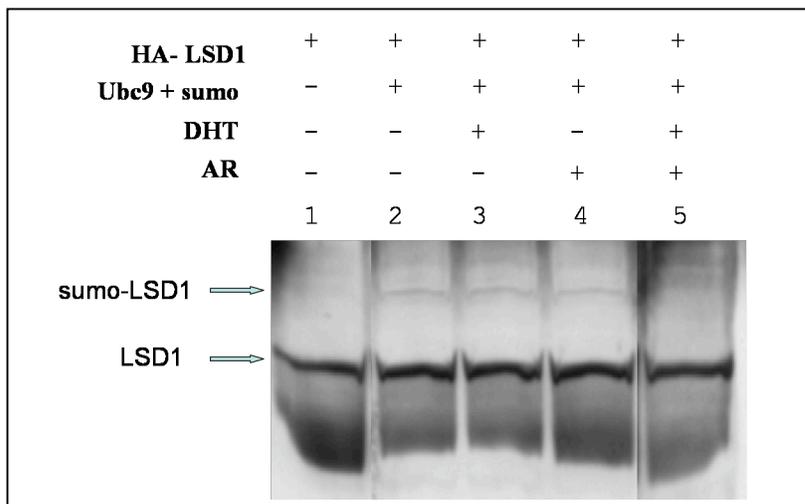
**Figure 4.5. Sumoylation affects LSD1 and CoRest interaction.** Flag-tagged LSD1 wt or LSD1 mt (K424R) and SUMO3 were cotransfected into 293T cells. Transfected cell lysates were subjected to immunoprecipitation with anti-Flag antibody, and the resultant precipitates were subjected to immunoblotting against anti-Flag (upper panel), anti-CoREST (middle panel) or anti-HDAC1 (bottom panel).



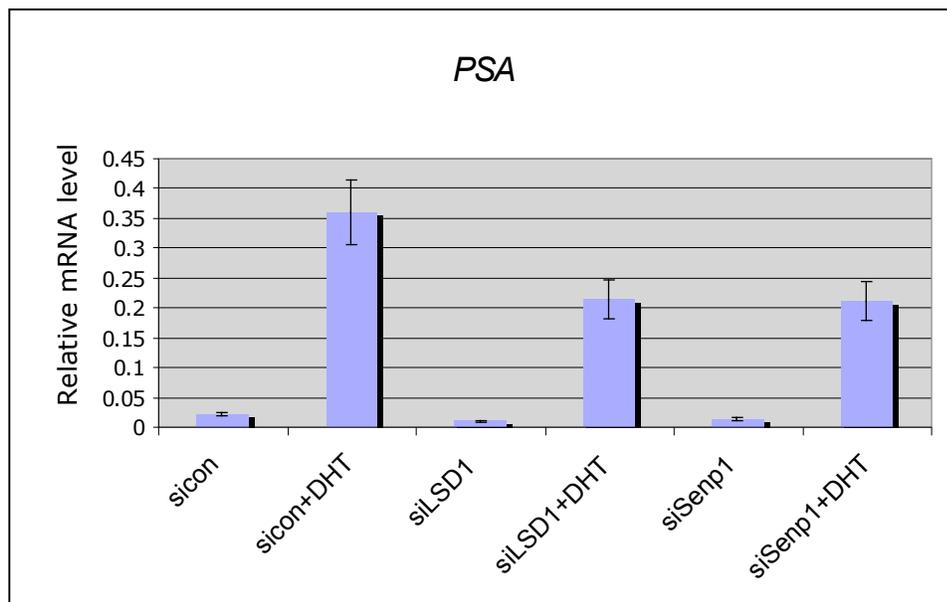
**Figure 4.6. SENP1 desumoylates LSD1 in vivo.** 293T cells were cotransfected with wild-type(wt) HA-LSD1 and SUMO together with each indicated plasmids SENP1-wt, SENP1-mutant (mt), SENP3-wt, SENP3-mt, SENP6-wt, or SENP6-mt. SENP-mt indicates the active site mutant that has impaired enzymatic activity. Whole cell extracts of transfected cells were subjected to western blotting analysis using anti-HA antibody.



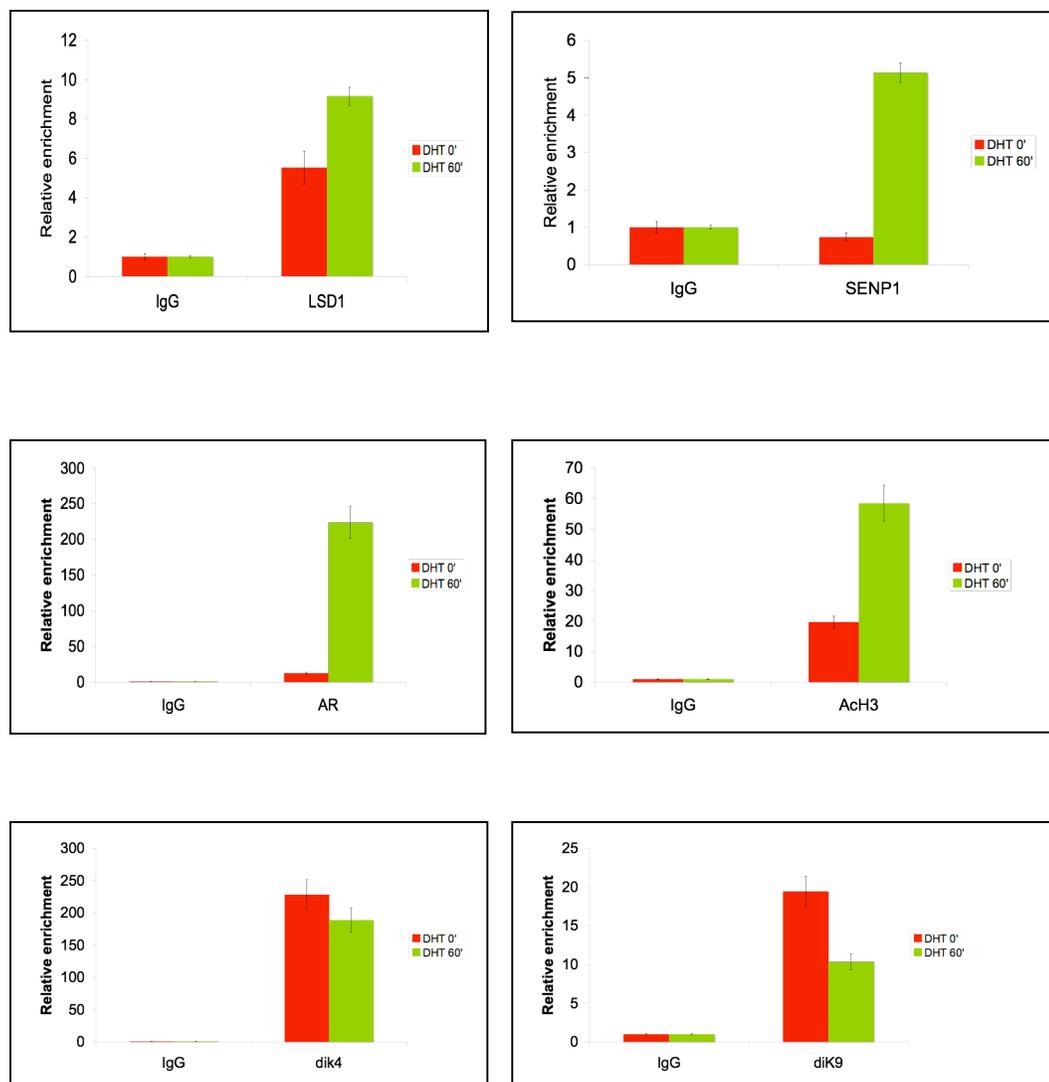
**Figure 4.7. LSD1 interacts with both SENP1 and SENP3.** HA-LSD1 and Flag-SENP1 or Flag-SENP3 plasmids were cotransfected into 293T cells by using lipofectamine 2000. Whole cell lysates of transfected cells were immunoprecipitated with antibody against either HA or control IgG, and the resultant precipitates were immunoblotted with either anti-HA (upper panel) or anti-Flag antibody (bottom panel).



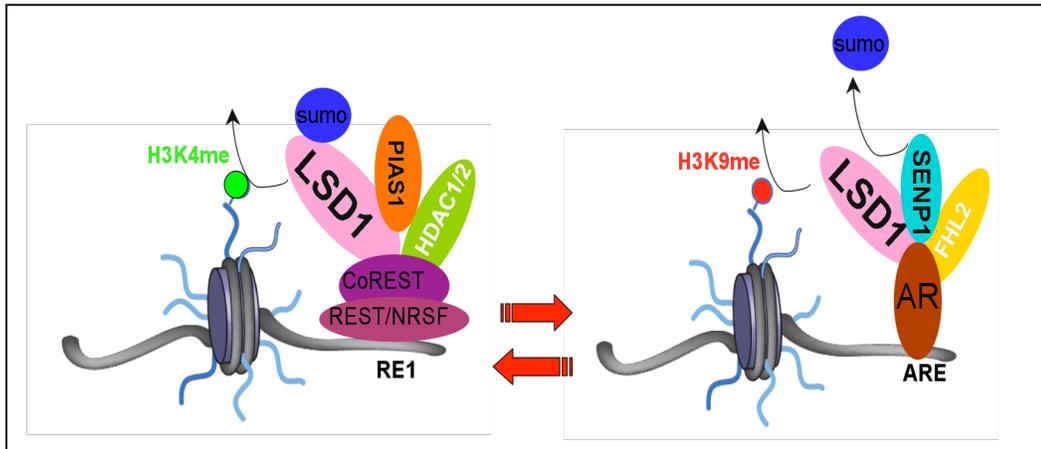
**Figure 4.8. Androgen receptor ligand treatment induces desumoylation of LSD1.** Hela cells were cotransfected with HA-LSD1, Ubc9 and SUMO in the presence or absence of androgen receptor (AR). After transfection, cells were treated with or without AR ligand DHT overnight and harvested for western blot analysis by using antibody against HA.



**Figure 4.9. Knocking down endogenous LSD1 or SENP1 in LNCaP cells affected the expression level of AR target PSA gene.** LNCaP cells were transfected with siRNA targeting SENP1, LSD1 or a control siRNA individually. After transfection, cells were treated with or without AR ligand DHT overnight. Total RNA was isolated and amplified by real-time RT-PCR using transcript-specific primers. Values are normalized with GAPDH and expressed as mean  $\pm$  s.d. (standard deviation) for three independent experiments.



**Figure 4.10. SENP1 and other cofactors were directly recruited to PSA enhancer in LNCaP cells.** LNCaP cells treated with or without DHT for 1hr were subjected to ChIP assay with the indicated antibodies. The precipitated chromatin was amplified by PCR using primers flanking the PSA gene promoter regions. Values are expressed as mean  $\pm$  s.d. (standard deviation) for three independent experiments.



**Figure 4.11. Model of sumoylation mediates LSD1 functional switch.**

## **Chapter V**

### **Conclusions and future directions**

**Mechanism underlying the downregulation of tumor metastasis suppressor gene *KAI1* in prostate cancers mediated by a  $\beta$ -catenin-Reptin corepressor complex.**

It was estimated that more than half a million people would die from cancer this year (Taylor et al. 2008). The majority of these patients die as the consequences of tumor metastasis (Steeg et al. 2006). Cancer metastasis is a complicated process that begins with dissemination of cells from the primary tumor and culminates in the formation of clinically detectable, overt metastases at one or more discontinuous secondary sites (Taylor et al. 2008). Surgery and adjunct therapies have been found to effectively control many localized cancers, however, only very limited methods are available for the treatment of metastatic cancers. Identifying factors that regulate the ability of cancer cells to form metastases helps to uncover unique targets for therapeutic intervention and disease management. Since the first metastasis suppressor gene, *MM23*, was identified in 1988, >20 metastasis suppressor genes have been identified including *KAI1* (Rinker-Schaeffer et al. 2006).

*KAI1*, a member of the tetraspanin family, has been shown to be capable of inhibiting the progression of tumor metastasis without affecting primary tumorigenicity (Dong et al. 1995). Indeed, as shown in Chapter II, we demonstrated that restoring *KAI1* expression to LNCaP prostate cancer cells, which normally do not express *KAI1*, significantly suppresses *in vivo* incidence of lung metastases (Kim and Cai et al. 2005). The expression of *KAI1* has been found significantly down regulated in many types of advanced and metastatic

tumors including prostate cancers, and such specific down-regulation does not seem to involve either allelic loss or gene mutations (Rinker-Schaeffer et al. 2006; Dong et al. 1996). *KAI1* gene contains methylated CpG islands in the promoter region, however, treatment with 5-aza-2-deoxycytidine or trichostatin-A failed to increase *KAI1* expression (Sekita et al. 2001). Thus, the expression of *KAI1* appears to be regulated by actions of unknown transcriptional cofactors. It has been suggested that p53, junB and AP2 bind to their sequence-specific sites in *KAI1* promoter, which has been shown positively correlated to *KAI1* expression (Marreiros et al. 2005). For instance, treatment with etoposide dramatically induced *KAI1* expression through p53 and c-Jun pathways (Mashimo et al. 2000). *KAI1* is also a NF- $\kappa$ B target gene and it has been shown that treatment with IL-1 $\beta$  induced *KAI1* expression (Li et al. 2001). Interestingly, we found that the induction of *KAI1* expression by IL-1 $\beta$  only occurs in normal prostate or tumorigenic prostate cancer cells, but not in metastatic prostate cancer cells, suggesting a metastatic cancer cell specific mechanism for *KAI1* induction. In Chapter II, we dissected the underlying mechanism for such cell type specific gene ON/OFF switch of *KAI1*. We found that, in metastatic prostate cancer cells, a  $\beta$ -catenin-Reptin transcriptional repressive complex mediates a repressive state of *KAI1*, serving to antagonize the Tip60 transcriptional coactivator complex that is required for *KAI1* activation. The balance of these two opposing complexes controls the expression of *KAI1* and metastatic potential among metastatic cancer cells. We further showed that the inhibitory activity of this  $\beta$ -

catenin-Reptin complex at least partially comes from the recruitment of a histone deacetylase HDAC1 by Reptin, an ATP-dependent helicase.

In chapter III, we further investigated how Reptin confers its repressive function to its target genes. By utilizing yeast two-hybrid screening, we identified Reptin-interacting factors, including UBC9, ASXL1, MLL5, FHL2, ID2 and etc. Interaction between Reptin and UBC9, the sole sumoylation E2 conjugating enzyme led to identification of Reptin as a sumo modification target, and we further showed that sumoylation conferred the repressive function to Reptin. Similar to our observation, Kim et al. has recently co-purified two sumo proteases, SENP1 and SENP6, in a Reptin-containing complex (Kim et al. 2006). In this report, they found that desumoylation of Reptin abrogated the repressive function of Reptin and its association with HDAC1, which resulted in the up-regulation of *KAI1* expression in metastatic cancer cells and inhibition of their metastasis.

Notably, a number of oncogenes and tumor suppressor genes have been shown to be substrates of sumoylation. Altered expressions of components of the sumo pathway have also been frequently observed in tumors. For example, increased expression of UBC9, the only E2 conjugating enzyme for sumo modification, has been detected in ovarian cancer, lung adenocarcinoma, and metastatic prostate cancer cell line LNCaP cells (Mo et al. 2005a, 2005b; McDoniels-Silvers et al. 2002; Kim et al. 2006). SENP1 has been shown to be upregulated in thyroid oncocytic adenomas and in human prostate cancer specimens (Jacques et al. 2005; Cheng et al. 2006). Elucidation of coordinated

sumoylation-desumoylation status of Reptin and other cancer-related proteins in the context of human diseases where the expression level and/or activities of sumo pathway components (including enzymes involved in both sumoylation and desumoylation) altered, will provide useful information for diagnosis and the development of potential therapeutic targets.

**The role of Reptin and its interacting partners in *Hox* gene activation in human embryonic carcinoma cell lines.**

In the second part of chapter III, we found that, when associated together with ASXL1 and LSD1, Reptin acted as a transcriptional coactivator in *Hox* gene expression in human embryonic carcinoma cell line NTera2 cells. Interestingly, Reptin and Pontin have been reported to function antagonistically with polycomb and trithorax complexes to control *Hox* gene expression in *Drosophila*, where Reptin was suggested to act as a transcriptional repressor in regulating *Hox* loci (Diop et al. 2008). Apparently distinct regulatory effects on *Hox* gene expression mediated by Reptin in fly and in human might be explained by the usage of different Reptin-containing complexes in these two species. In *Drosophila*, Reptin was co-purified with PRC1 complex, a well-known repressive complex (Saurin et al. 2001; Diop et al. 2008). In human embryonic carcinoma cell line NTera2, we found that Reptin interacts with ASXL1 and LSD1. ASXL1 is the human homolog of *Drosophila* ASX. Genetic studies indicated that ASX is an enhancer of both trithorax and polycomb factors and ASX is required for both the activation and repression of *Hox* gene clusters, as *Asx* mutation caused both anterior and

posterior homeotic transformations in flies (Milne et al. 1999). mASXL1, the mouse homolog of ASX/ASXL1, exhibited a spatial expression pattern along the anterior-posterior axis, similar to that of *Hox* genes (Chen et al. 2004). In human, ASXL1 has recently been shown to act as a co-activator of RAR through a functional cooperation with SRC-1 (Cho et al. 2006). Initial yeast two-hybrid screening also identified FHL2, a four and half LIM domain containing protein, as a Reptin interacting partner. I confirmed such interaction between FHL2 and Reptin by co-immunoprecipitation assays (data not shown). FHL2 has been reported as a transcriptional coactivator for AR target genes in LNCaP cells (Müller et al. 2000). The presence of LSD1 in a purified FHL2 complex in LNCaP cells led to initial identification of an activation function mediated by LSD1 (Metzger et al 2005). Similar to the function of LSD1 in LNCaP cells, we found LSD1 also acted as transcriptional activator in regulating *Hox* gene clusters in human NTera2 cells. We also investigated SENP3, a sumo protease, in Reptin-mediated activation of *Hox* genes, as knocking down SENP3 in HeLa cells caused a dramatically decreased expression of a subset of *Hox* genes (Cai et al. unpublished data). In chapter III, I further showed that Reptin interacts with SENP3, which acts as a sumo protease for Reptin. It is likely that SENP3 plays gene activation roles in these cells through desumoylation of Reptin.

### **Reptin and Pontin**

Reptin and Pontin are two closely related ATP-dependent helicases of opposite polarity (Kanemaki et al. 1999; Makino et al. 1999). They are very

similar in primary sequences and share residence in many types of protein complexes. Gene disruption of either Reptin or Pontin was lethal in all species examined so far, indicating their essential and nonredundant functions in cell growth and early development (Bauer et al 1998; Bauer et al 2000; Lim et al. 2000). Recent studies from Dr. Baek's lab and our group proved that both Reptin and Pontin are able to be sumoylated. Sumoylation is essential for Reptin-mediated gene repression and for Pontin-mediated gene activation in a context-dependent and promoter-specific manner (Kim et al. 2006, 2007; Cai et al. unpublished data). However, questions remain to be answered regarding the functions of Reptin and Pontin. For instance, how does Reptin act as a repressor while Pontin acts as an activator when both are present in the same protein complex? How can the same protein (e.g., Reptin) contribute to gene repression on one promoter and to activation on a different one? Is the ATPase/helicase activity of Reptin required for its activational or repressive function? Does sumoylation affect the ATPase/helicase activity of Reptin? How does sumoylation play a role in Reptin-mediated activation on *Hox* genes? Does Pontin regulate *Hox* gene expression in a similar way to Reptin? What complex does Reptin belong to when it acts as a coactivator? Is it the canonical Tip60 coactivator complex? What are the upstream signaling pathways to regulate the sumoylation-desumoylation switch of Reptin and Pontin? Are Reptin and Pontin subjected to other posttranslational modifications such as phosphorylation? Future studies are needed to resolve the above problems.

## **Transcriptional regulation by a histone lysine demethylase LSD1 and its functional switch induced by post-translational modifications.**

The core histones are predominantly globular except for their unstructured N-terminal tails, which are subjected to modifications. Histone lysine methylation occurs on histone H3 lysine (K) K4, K9, K27, K36, K79 and histone H4 K20. With few exceptions, methylation of H3K4, H3K36, H3K79 is correlated with transcriptional activation, while methylation of H3K9, H3K27, H4K20 is linked to transcriptional repression (Kouzarides et al. 2007). Histone lysine methylation is proven to be a dynamic process upon recent discovery of histone lysine demethylases (Shi et al. 2004; Tsukada et al. 2006). The first identified histone lysine demethylase LSD1, also called KDM1, belongs to the flavin-dependent amine oxidase family. LSD1 either demethylates mono- and dimethylated H3K4, as a repressor (Shi et al. 2004) or demethylates mono- and dimethylated H3K9, as an activator (Metzger et al. 2005).

In chapter IV, I reported that LSD1 was modulated by sumo modification and that changes in sumoylation-desumoylation status induced a switch of LSD1 functions in transcriptional regulation. Specifically, sumoylation enhanced LSD1 mediated-repression on CoREST target genes; Knockdown of PIAS1, a LSD1 sumo E3 ligase, abrogated the recruitment of LSD1 on these target genes, concomitant with subsequent de-repression of these genes. On the other hand, we also found that treatment with the AR ligand induced the desumoylation of LSD1 mediated by SENP1 on the AR target genes; Knocking down SENP1 decreased AR-mediated transcriptional activation. Thus, sumoylated forms of

LSD1 correlated with its gene repression function in CoREST complexes, and desumoylated forms of LSD1 correlated with its gene activation function when associated with AR. Our observations indicated that sumoylation-desumoylation cycling played an important role in regulating LSD1 function. The recent characterized LSD1 crystal structure provides further support for our studies. At least three distinct structural identities exist within LSD1: the N-terminal SWIRM domain (named for its presence in the proteins Swi3, Rsc8, and Moira), the C-terminal FAD-binding amine oxidase domain, and the insertion tower domain (Stavropoulos et al. 2006; Chen et al. 2006; Yang et al. 2006; Forneris et al. 2007). The SWIRM domain and the amine oxidase domain closely pack against each other and form a globular core structure from which the tower domain protrudes as an elongated helix-turn-helix motif. The major sumo-conjugating site that we identified in LSD1 is located in the tower domain, which gave an indication that sumo may be able to change LSD1 substrate specificity by inducing a conformational change of LSD1 (Stavropoulos et al. 2006; Chen et al. 2006; Yang et al. 2006; Forneris et al. 2007). Before we successfully perform *in vitro* demethylase assay towards methylated H3K9 by using purified sumoylated LSD1 versus nonsumoylated LSD1, we could not exclude other possibilities that also give reasonable explanations for LSD1 acting as a coactivator. First, in addition to sumoylation, other post-translational modification such as phosphorylation might also be involved in regulating LSD1 demethylase activity. Actually, proteomic analysis has already identified phosphorylated residues on LSD1 (Olsen et al. 2006). Second, the observed H3K9 demethylation could be

catalyzed by an unknown H3K9 demethylase associated to LSD1 complexes. Indeed, a recent report showing that LSD1 cooperates with the H3K9 demethylase JMJD2C to activate AR target genes supported this hypothesis (Wissmann et al. 2007). In addition, LSD1 may induce H3K9 demethylation indirectly through modifying non-histone substrates, which then may functionally oppose H3K9 methyltransferases and decrease methylated H3K9 levels. Future investigation needs to be done to give a clear answer to these questions of LSD1 functional switches from repression to activation.

Loss of H3K9 methylation and increased levels of H3K4 methylation have been reported to be associated with several types of solid tumors (Wang et al. 2007). MLL, which methylates histone H3K4, is found to be frequently involved in chromosomal translocation in both acute lymphoid and myeloid leukemia (Ayton et al. 2001). SMYD3, another histone H3K4 methyltransferase, has been shown to be upregulated in colorectal and hepatocarcinoma cells (Hamamoto et al. 2004). Inactivation of a histone H3K9 methyltransferase RIZ1/PRDM2 by mutations or silencing via promoter hypermethylation is observed in hepatocellular, breast, colon and gastric cancers (Kim et al. 2003; Gibbons et al. 2005). JMJD2C, which removes the methyl group from di- and tri-methylated H3K9, is frequently amplified and overexpressed in esophageal squamous cell carcinoma, lung sarcomatoid carcinoma and desmoplastic medulloblastoma (Yang et al. 2000; Ehrbrecht et al. 2006; Italiano et al. 2006). Furthermore, significant up-regulation of both mRNA and protein levels of LSD1 has been

shown to associate with high-risk prostate tumors, and LSD1 has been implicated to serve as a novel biomarker for prediction of aggressive prostate cancers (Kahl et al. 2006). These findings suggest that H3K4 and H3K9 methylation-demethylation regulation plays a crucial role in tumorigenesis. In addition, the sumo protease SENP1 was also found over-expressed in human prostate cancer specimens but not in corresponding normal prostate tissues (Cheng et al. 2006). Transgenic mice with overexpressed SENP1 in the prostate gland exhibited evidence of early PIN formation (PIN refers to the disease progresses from benign hyperplasia to a prostate cancer precursor state, Cheng et al. 2006). These reports are consistent with our observations showing that both LSD1 and SENP1 act as coactivators of AR signaling and enhance AR-dependent transcriptional activation, a process correlated with prostate carcinogenesis. Future investigations of mechanisms underlying lysine methylation and sumoylation-desumoylation in tumorigenesis shall shed light on designing novel drugs for therapeutic treatment of human cancers.

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