UNIVERSITY OF CALIFORNIA, SAN DIEGO

LSD1 Complex Controls Cell Type Terminal Differentiation During Mammalian Organogenesis

A dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy

in

Molecular Pathology

by

Jianxun Wang

Committee in charge:

Professor Michael G. Rosenfeld, Chair
Professor Christopher K. Glass
Professor Anthony Wynshaw-Boris
Professor Xiang-Dong Fu
Professor Robert G. Oshima

2006

The dissertation of Jianxun Wang is approved, and it is acceptable in quality and form for publication on microfilm.

Chair

University of California, San Diego

2006

DEDICATION

To Mom and Dad,

Without their endless love and their constant support none of this would have ever been possible

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LIST OF SYMBOLS

LSD1	**Lysine Specific Demethylase 1**
ChIP	**Chromatin Immunoprecipitation**
HDAC	**Histone Deacetylase**
REST	**Restricted Element 1 Silencing Transcription Factor**
CoREST	**Co-Repressor of REST**
CoREST3	**CoREST Homology 3**
LCoR	**Ligand-Dependent Co-Repressor**
ER	**Estrogen Receptor**
T_3R	**Thyroid Hormone Receptor**

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ACKNOWLEDGEMENTS

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CURRICULUM VITAE

Education

1991-1995: Nankai University, P.R. China

Bachelor Degree in Biochemistry and Molecular Biology

1995-1998: Peking Union Medical College, P.R. China

Master Degree in Biochemistry and Molecular Biology

1998-1999: Biological Chemistry Graduate Program, University of Utah, USA

1999-2006: Molecular Pathology Graduate Program, University of California, San Diego, USA

Doctor Degree of Philosophy in Molecular Pathology

ABSTRACT OF THE DISSERTATION

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Precise control of transcriptional programs that underlie metazoan development is modulated by enzymatically-active co-regulatory complexes, coupled with epigenetic strategies, but how specific histone modification enzymes are utilized to orchestrate distinct developmental programs remains unclear. Here, we report that LSD1, a histone lysine demethylase originally identified as a component of the CoREST/CtBP corepressor complex, functions *in vivo* as a required cofactor for both gene activation and

repression programs that dictate cell type determination and differentiation during pituitary organogenesis. Remarkably, LSD1 function can be converted from activation to repression in a temporal- and cell type-specific fashion by the induced expression of two additional components of the LSD1/CoREST/CtBP complex - ZEB1, a Krüpple-like zinc finger protein and LCoR, an agonist-dependent nuclear receptor corepressor. These findings reveal critical developmental roles of a specific histone lysine demethylase and provide a potential molecular mechanism for sequential, cell type-specific gene activation and restriction events during mammalian organogenesis.

INTRODUCTION

Mammalian organogenesis is orchestrated by precise control of gene activation and repression programs driven by diverse DNA binding transcription factors, and stringently regulated by epigenetic events (reviewed in Jaenisch and Bird, 2003; Edlund and Jessell, 1999; Hsieh and Gage, 2005; Turner, 2002). Developmental programs involve serial alterations in histone marks, often referred to as the "histone code" (reviewed in Fischle et al., 2003; Wang et al., 2004; Sims et al., 2003; Margueron et al., 2005; Bannister and Kouzarides, 2005; Martin and Zhang, 2005; Berger, 2002). Investigation of NRSF/REST-mediated repression of a broad neurogenic program (Schoenherr et al., 1995; Chong et al., 1995), led to discovery of the corepressor CoREST (Andres et al., 1999), a SANT domain protein that interacts with specific HDACs (Aasland et al., 1996). CoREST was subsequently found to be a component of a large complex including CtBP and the FAD binding protein KIAA0601/BHC110/LSD1 (Tong et al., 1998; You et al., 2001; Humphrey et al., 2001; Shi et al., 2003), which can function as a histone diMe H3-K4 demethylase (Shi et al., 2004). In addition, CoREST/LSD1 have been identified as the core components of several other complexes (Nakamura et al., 2002; Hakimi et al., 2002; Hakimi et al., 2003; Yamagoe et al., 2003). The activity of LSD1 has been suggested to be modulated or dependent on CoREST as a cofactor (Lee et al., 2005; Shi et al., 2005), suggesting that LSD1 may exert distinct functions depending on the associated coregulators *in vivo*. The narrow substrate specificity displayed by LSD1 (mono- and di-methylation of H3-K4 and H3-K9) (Shi et al., 2004; Metzger et al., 2005), in contrast to the large

number of SET-domain methyltransferases (>50) (Kubicek and Jenuwein, 2004; Jenuwein and Allis, 2001), suggested that other demethylases might exist (Trewick et al., 2005). Indeed, very recently, a member of the large JmjC domain-containing protein family, JHDM1 (JmjC domain-containing histone demethylase 1), has been reported to function as a specific histone H3-K36 demethylase (Tsukada et al., 2005) and several additional penultimate members of this family have been identified as histone H3-K9/K36 demethylases (Yamane et al., 2006; Whetstine et al., 2006). The presence of a broad array of histone lysine methyltransferase/demethylases makes it critical to identify the potential roles of individual enzymes in regulation of specific gene expression programs during development and homeostasis. Specifically, these observations impel an *in vivo* definition of the biological roles of LSD1 during mammalian development.

Development of the anterior pituitary gland, in which five specific hormone secreting cell types arise from a common primordium (reviewed in Sheng and Westphal, 1999; Cushman and Camper, 2001; Keegan and Camper, 2003; Scully and Rosenfeld, 2002; Zhu and Rosenfeld 2004; Dattani, 2005), has provided a powerful, well-defined model for investigating the underlying epigenetic regulatory mechanisms that drive cell type-specific gene expression programs during organogenesis. Initial organ commitment is controlled by the induction of specific regulators, including the homeodomain factors Lhx3 and Lhx4 (Sheng et al., 1996; Sheng et al., 1997). Determination of distinct cell-lineages is driven by highly orchestrated transcriptional programs. For the POMC-producing corticotropes, Tbx19 serves as a determining factor (Lamolet et

al., 2001; Liu et al., 2001); for the LHβ-producing gonadotropes, SF1 serves as a critical regulator (Zhao et al., 2001; Kaiserman et al., 1998; Seminara et al., 1999); and for the lineage which ultimately gives rise to thyrotropes (produce thyroid stimulating hormone TSHb), somatotropes (produce growth hormone GH), and lactotropes (produce prolactin Prl), Pit1 serves as a determining factor (Li et al., 1990; Camper et al., 1990; Ingraham et al., 1990). Cell lineage determination is followed by terminal differentiation events with cell type-specific hormone production. The terminal differentiation program of prolactin-producing lactotropes is distinct from other cell types, and involves initial expression of *GH* in precursors (GH^+/Prl^-) at e17.5, with subsequent restriction of *GH,* coincident with activation of *Prl* gene transcription at p5-p10 (GH^-/Prl^+) (Hoeffler et al., 1985; Behringer et al., 1988; Borrelli et al., 1989). The molecular mechanism underlying the restriction event, is however, unclear (Scully et al., 2000).

Here, we report the biological functions of the first identified histone lysine demethylase, LSD1, using a conditional gene deletion strategy in a well defined model of mammalian-organogenesis, finding that LSD1 controls both gene activation and repression programs critical for late cell-type determination events, but not for early organ commitment events. A temporally delayed induction of specific components of the LSD1/CoREST/CtBP complex, has proved critical in switching LSD1 function from activation to repression of specific gene expression. This molecular switch imposes cell type-specific

restriction in terminal differentiation, providing a plausible solution to a long-standing question of the mechanism of cell-type-specific target gene restriction during mammalian organogenesis.

Chapter 1

Studying the LSD1 function: Genetic deletion of LSD1

A: Introduction

LSD1 is initially identified as a component of CoREST/HDAC1/2/CtBP complex, and is highly expressed during development and become ubiquitously expressed in adult tissue. LSD1 has been suggested to function as a co-repressor for REST mediated repression, its biological function *in vivo*, however, is unclear. Given the importance of CoREST complex-mediated repression events in transcription regulation (Andres et al., 1999; Ballas et al., 2001; Lunyak et al., 2002; Roopra et al. 2004; Lee et al., 2005; Shi et al., 2005; Ballas et al., 2005), and the functional requirement of REST, HDACs and CtBP in early mouse development (Chen et al., 1998; Lagger et al., 2002; Hildebrand and Soriano, 2002), it becomes important to elucidate the roles of LSD1 in mammalian development.

B: Results

LSD1 expression is ubiquitous in adult tissue, abundant in brain and testis (**Figure 1-1**), suggesting its broad roles *in vivo*. In addition, LSD1 expression appears to be regulated during development (**Figure 1-1**).

We employed an *in vivo* approach for deletion of the LSD1/KIAA0601 genomic locus, with a strategy that permitted conditional gene deletion by removal of an essential coding region of *LSD1* (Binda et al., 2002), based on insertion of two Lox P sites flanking exon 6 corresponding to the N-terminal of the amine oxidase domain (Gu et al., 1994) **(Figure 1-2)**, the deletion of which would result in an alteration of the reading frame of *LSD1* transcript.

Using standard gene targeting technology in mouse embryonic stem (ES) cells, we obtained homologous recombination and generated both type I and type II recombinant alleles by using *PMC/Cre* electroporation *in vitro* **(Figure 1-2)**.

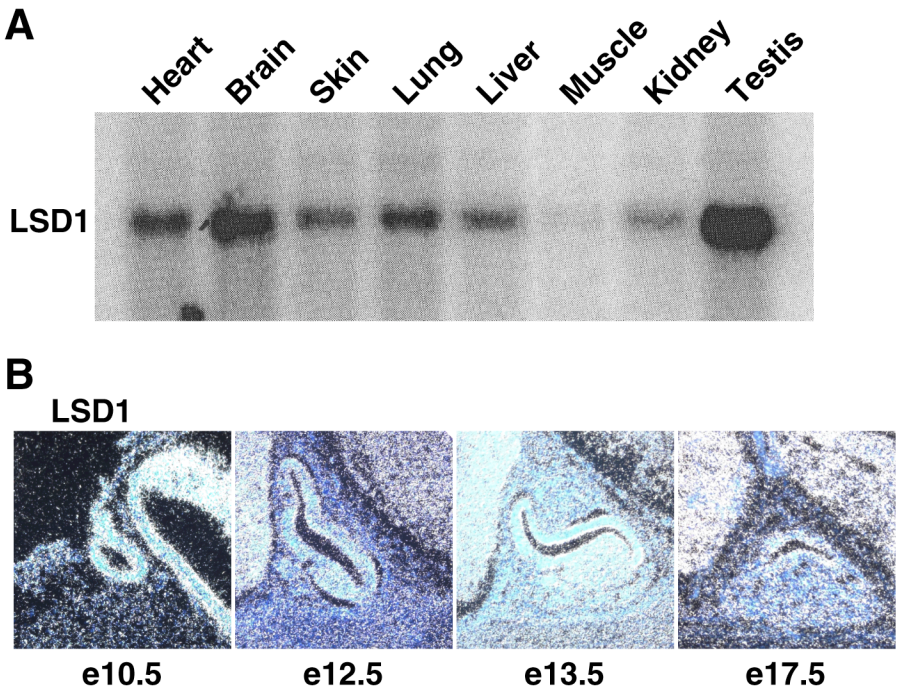


Figure 1-1: Tissue distribution of LSD1 transcript by Northern blot and *in situ* hybridization. **(A)** LSD1 gene expression in mouse adult tissue by Northern blot **(B)** LSD1 gene expression during pituitary development by *in situ* hybridization.

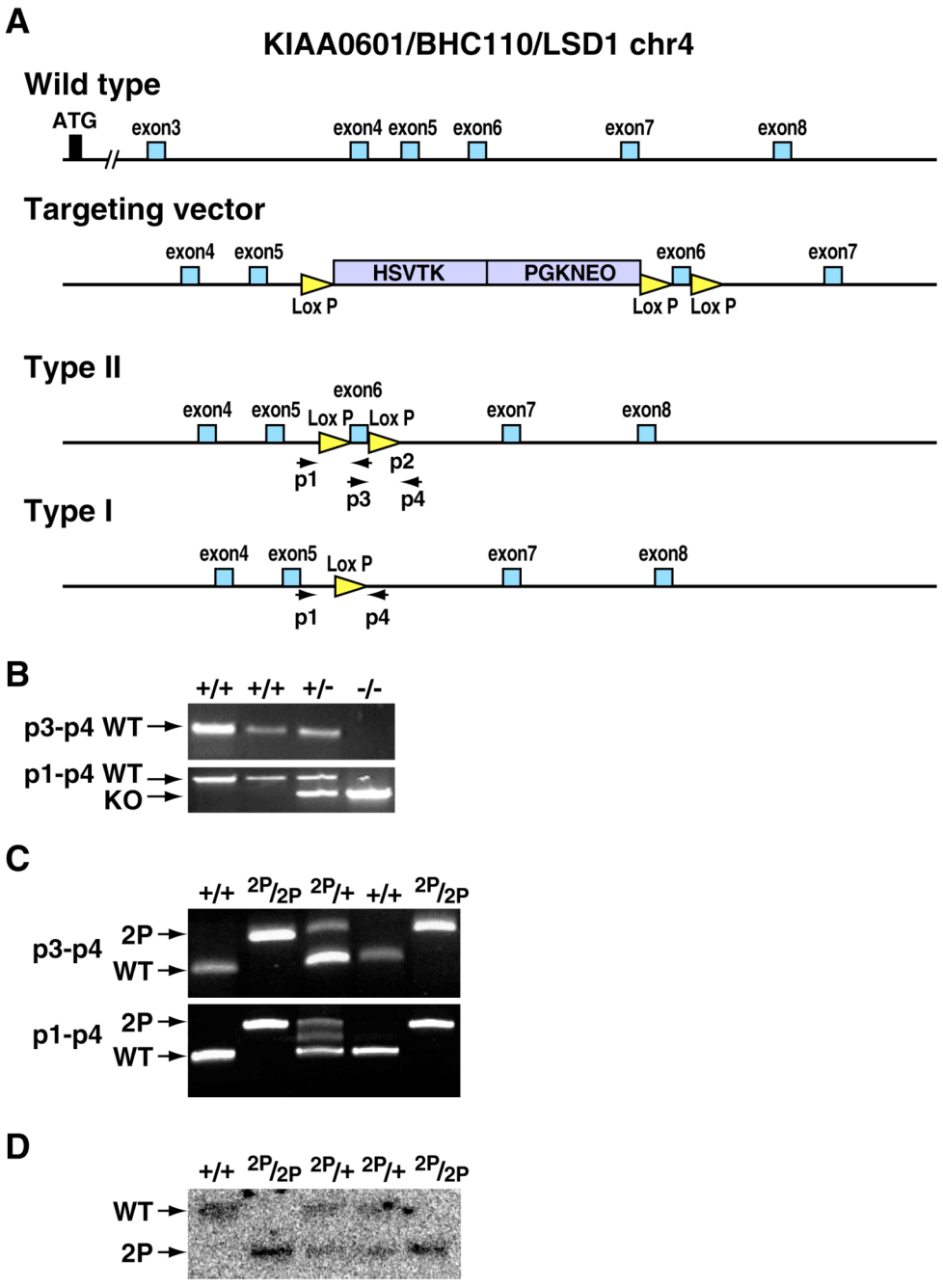
Germ-line transmissions were ultimately achieved for both types of recombination. Homozygous *LSD1*$^{Flox/Flox}$ (type II recombinant, conditional allele) mice were fertile and exhibited normal expression of *LSD1*, indicating that insertion of the two Lox P sites did not significantly affect *LSD1* transcription or RNA processing (**Figure 1-2**).

While mice heterozygous for conventional *LSD1* gene deletion (type I recombinant, conventional allele) appeared normal and fertile, no viable homozygous *LSD1*$^{-/-}$ embryos could be detected after e7.5, and the few homozygous *LSD1*$^{-/-}$ embryos obtained had not progressed beyond e5-e6 and exhibited severe growth retardation (**Figure 1-2** and data not shown). *LSD1* mRNA was markedly reduced in *LSD1*$^{-/-}$ embryos and no truncated protein was detectable (data not shown), indicating that the LSD1 knockout strategy resulted in a null phenotype.

C: Discussion

A common difficulty in investigation of histone modifying enzyme during mammalian development is early embryonic lethality. Here, employing a conditional knockout strategy to circumvent the early embryonic lethality caused by *LSD1* deficiency, we have the opportunity to study LSD1 function in specific developmental programs in a well-investigated model of mammalian organogenesis, pituitary development.

Figure 1-2: Generation of LSD1 conditional knockout mice.
(**A**) Gene targeting strategy of LSD1: schematic diagram of wild type, type I, type II LSD1 allele and targeting vector. (**B)** Genotyping of wild type, heterozygous and
homozygous LSD1 type I (null) allele in e7.5 embryos by PCR, PCR products generated from wild type (WT) and type I (KO) are separated using 1% agarose gel. No p3-p4 PCR product (WT) can be detected in $LSD1^{-/-}$ embryos. (**C)** Genotyping of wild type, heterozygous and homozygous LSD1 type II (conditional) allele in adult mice by PCR, PCR products generated from wild type (WT) and type II (2P) are separated using 1% agarose gel. No p3-p4 PCR product (WT) and p1-p4 PCR product (WT) can be detected in $LSD1^{Flox/Flox}$ mice. (**D)** Genotyping of LSD1 type II allele (2P) in adult mice by southern blot: wild type (WT) and type II (2P) allele are digested and separated using 1% agarose gel before hybridization with 5' external probe.


A
KIAA0601/BHC110/LSD1 chr4
Wild type
ATG
exon3
exon4 exon5 exon6
exon7
exon8
Targeting vector
exon4 exon5
HSVTK
PGKNEO
exon6
exon7
Lox P
Lox P Lox P
Type II
exon4 exon5
exon6
Lox P Lox P
exon7
exon8
p1
p2
p3
p4
Type I
exon4 exon5
Lox P
exon7
exon8
p1
p4
B
+/+ +/+ +/- -/-
p3-p4 WT
p1-p4 WT
KO
C
+/+ 2P/2P 2P/+ +/+ 2P/2P
p3-p4 2P
WT
p1-p4 2P
WT
D
+/+ 2P/2P 2P/+ 2P/+ 2P/2P
WT
2P

D: Materials and Methods

Generation and Genotyping of LSD1 deficient mice

LSD1 conditional allele mice were generated by targeted mutagenesis in ES cells to insert two LoxP sites flanking exon6 of LSD1 (**Figure 1-2**), correct targeting was established by southern blotting with 5' and 3' external probes. Embryos were genotyped by PCR method. Pitx1 Cre generated as described previously. $LSD1^{Flox/Flox}$ mice were crossed with Pitx1 Cre lines. Pituitary-specific *LSD1* gene-deleted mice were generated through breeding between $LSD1^{Flox/Flox}$ mice with $LSD1^{Flox/+}$ / Pitx1 Cre mice.

Chapter 2

Studying LSD1 function in pituitary organogenesis

A: Introduction

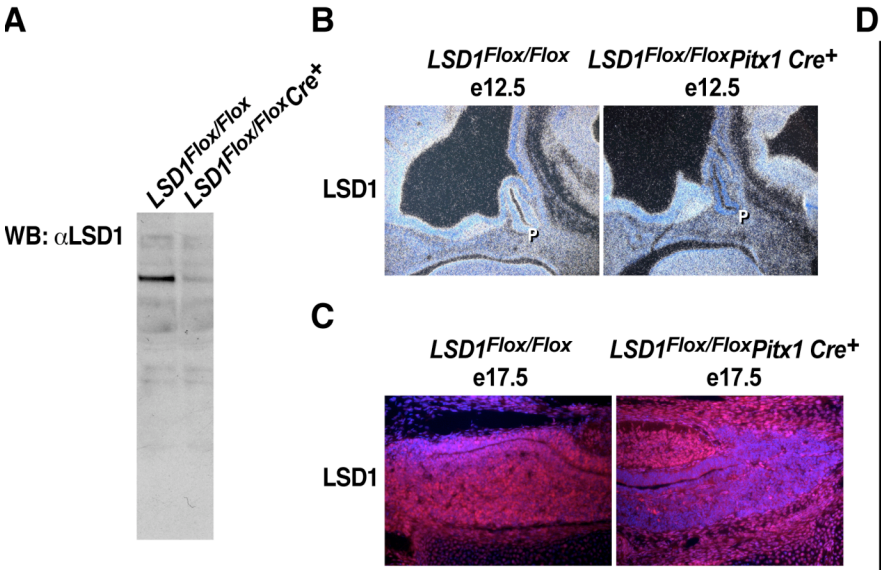
Although the *LSD1*-gene deletion leads to early embryonic lethality, *LSD1*$^{-/-}$ embryonic stem cell appears to be normal. When a protocol for neurogenesis is applied, *LSD1*$^{-/-}$ embryonic stem cell failed to generate neuron, while wild type embryonic stem cell can generate Tuj1 positive neurons. However, when a protocol for adipogenesis is applied, both ES cells can generate adipocytes efficiently according to lipid accumulation assay (data not shown), indication LSD1 plays different roles in organogenesis. Therefore, we decide to investigate LSD1 function in pituitary organogenesis.

B: Results

LSD1 is required for terminal differentiation events

To examine the specific role of LSD1 during mammalian organogenesis, which is expressed throughout pituitary development **(Figure 1-1)**, we generated a *LSD1*$^{Flox/+}$*/Pitx1/Cre* line by crossing with *Pitx1/Cre* mice. Here, Cre is selectively expressed in the oral ectoderm pituitary primordium and can execute effective recombination of the Rosa26 locus in virtually all pituitary cells by e9-e9.5 (Olson et al., 2006). Pituitary-specific *LSD1* gene-deleted embryos were generated through crossing *LSD1*$^{Flox/+}$ */Pitx1/Cre* mice with *LSD1*$^{Flox/Flox}$ mice, with approximately 25% embryos exhibiting homozygous deletion of the *LSD1* locus, and the mutant mice appeared fully viable throughout embryogenesis. The pituitary gland in the conditional *LSD1* gene-deleted embryo

exhibited complete loss of *LSD1* transcripts starting from e9-e9.5 (**Figure 2-1** and data not shown**),** but intact pituitary glands throughout development, retaining both anterior and posterior pituitary **(Figure 2-2)**. However, at e17.5, markers of terminal differentiation of *Pit1* lineage cells — growth hormone (*GH*) and thyroid-stimulating hormone *b* (*TSHb*) — were undetectable in *LSD1* gene-deleted pituitaries, while robustly expressed in pituitaries of littermate controls **(Figure 2-2)**. Luteinizing hormone *b* (*LHb*) and pro-opiomelanocortin (*POMC*), markers of gonadotropes and corticotropes, werealso reproducibly decreased in *LSD1* gene-deleted pituitaries **(Figure 2-2)**. Intriguingly, although *POMC*- and *aGSU*-encoding mRNAs were still present in *LSD1* gene-deleted pituitaries, very few if any POMC or aGSU protein positive cells could be detected by immunohistochemistry **(Figure 2-2)**, suggesting that LSD1 may regulate *POMC* and *aGSU* expression at both the transcriptional and translational or protein stability levels.

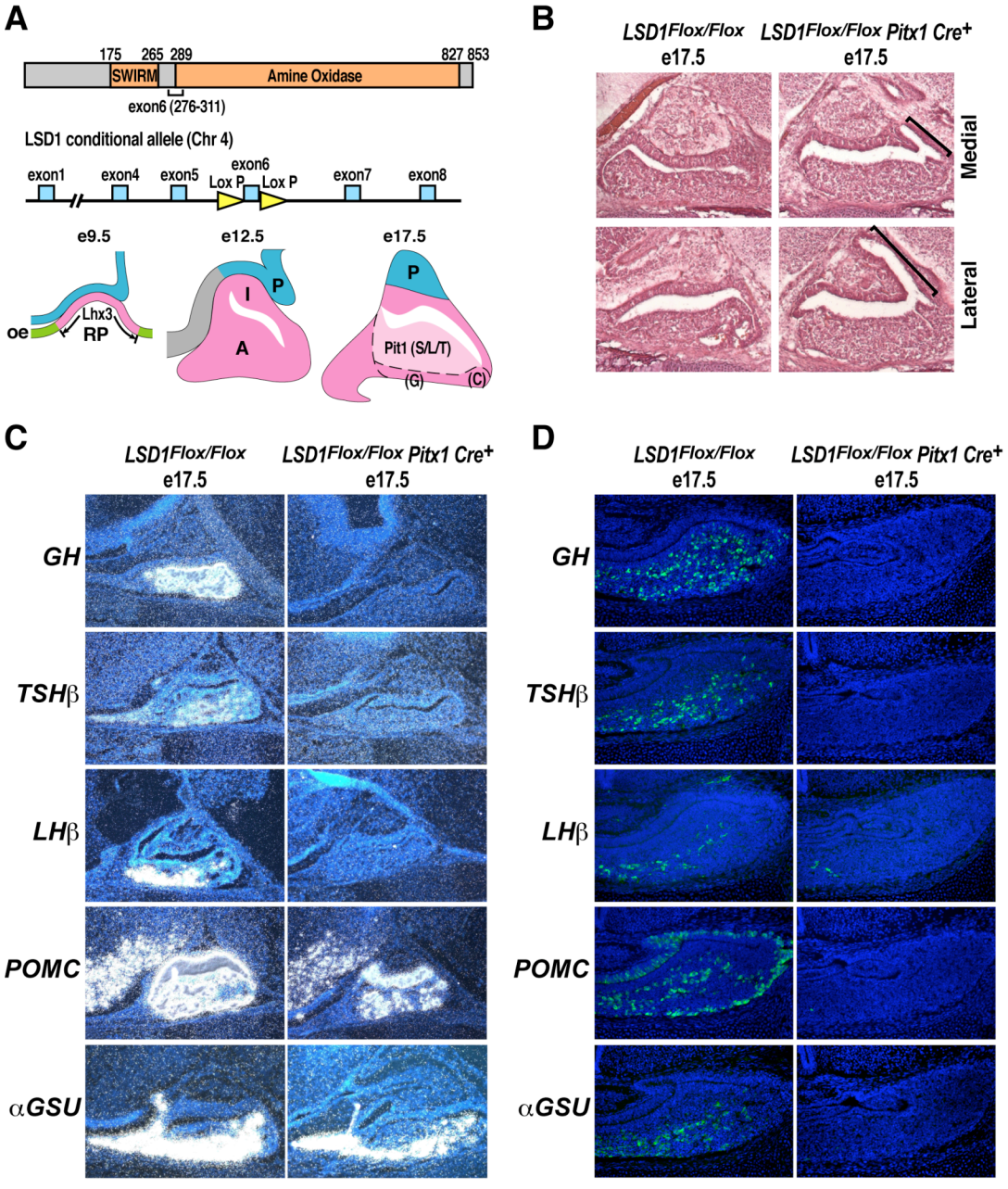


D

Gene	LSD1 wt	LSD1 KO
Foxg1	1	11.84
CCNE1	1	4.48
Hey1	1	2.39
Jmjd1a	1	2.19
ID2	1	2.19
GAPDH	1	1
Lhx3	1	0.76
Tbx19	1	0.29
FSH	1	0.27
Pit-1	1	0.19
Gpr30	1	0.18
LSD1	1	0.15
SF1	1	0.14
JunB	1	0.14
LSD1(exon6)	1	0.14
c-Fos	1	0.08
GSU	1	0.06
POMC	1	0.06
Math3	1	0.03
Tsh	1	0.002
LH	1	0.002
PRL	1	<0.001
Ghrhr	1	<0.001
GH	1	<0.001

Figure 2-1: **Cre recombinase mediated LSD1 gene deletion results in reduction of LSD1 mRNA and protein expression.** (**A**) Expression of full-length LSD1 protein in *LSD1*$^{Flox/Flox}$ and *LSD1*$^{Flox/Flox}$/Cre MEF cells revealed by western blot. (**B**) Pituitary-specific gene-deletion of *LSD1* results in reduction of *LSD1* transcripts in the e12.5 pituitary gland (P) revealed by in situ hybridization. (**C**) Pituitary-specific gene-deletion of *LSD1* resulted in reduction of *LSD1* protein in e17.5 pituitary gland, revealed by in immunohistochemistry. (**D**) Confirmation of LSD1 target gene expression from RNA profiling analysis by RT-qPCR.

Figure. 2-2. LSD1 is required for cell type specific pituitary hormone expression. (**A**) Strategy for generation of conditional allele of *LSD1*. Schematic diagrams of pituitary during development. Oral ectoderm= (oe), Rathke's Pouch = (RP), anterior lobe=(A), intermediate lobe=(I), posterior lobe=(P), cell types: gonadotropes = (G), corticotropes = (C), lactotropes = (L), somatotropes = (S), thyrotropes = (T) (**B**) H&E staining of control *vs LSD1* gene-deleted pituitaries, exhibiting representative medial and lateral para-sagital section of e17.5 pituitaries (**C**) Gene expression of *GH*, *TSHβ*, *LHβ*, *POMC* and *aGSU* in control *vs LSD1* gene-deleted pituitaries at e17.5 by *in situ* hybridization. (**D**) Protein expression of GH, TSHβ, LHβ, POMC* and aGSU in control *vs LSD1* gene-deleted pituitaries at e17.5 by immunohistochemistry. (*POMC expression was detected using αACTH IgG).


A
175 265 289
827 853
SWIRM
Amine Oxidase
exon6 (276-311)
LSD1 conditional allele (Chr 4)
exon1
exon4
exon5
exon6
Lox P
Lox P
exon7
exon8
e9.5
e12.5
e17.5
oe
Lhx3
RP
I
P
A
P
Pit1 (S/L/T)
(G)
(C)
B
LSD1Flox/Flox
e17.5
LSD1Flox/Flox Pitx1 Cre+
e17.5
Medial
Lateral
C
LSD1Flox/Flox
e17.5
LSD1Flox/Flox Pitx1 Cre+
e17.5
GH
TSHβ
LHβ
POMC
αGSU
D
LSD1Flox/Flox
e17.5
LSD1Flox/Flox Pitx1 Cre+
e17.5
GH
TSHβ
LHβ
POMC
αGSU

Pit1 expression was diminished, but remained easily detectable in *LSD1* gene-deleted pituitary **(Figure 2-3)**. Similarly, other cell lineage determining transcription factors, *Tbx19* and *SF1*, were detectable, but much diminished in LSD1 gene-deleted pituitary compared to littermate controls at e17.5 **(Figure 2-3).** In addition, growth hormone releasing hormone receptor (*Ghrhr*) expression, which is also a marker of somatotrope lineage (Lin et al., 1992; Godfrey et al., 1993), was not detected on e17.5 in *LSD1* gene-deleted pituitaries. Thus LSD1 controls late cell lineage determination and terminal differentiation events during pituitary development.

Based on RNA profiling analysis on three gender-matched pairs of dissected e17.5 pituitaries, significant difference in experiments was confirmed by SAM analysis (Tusher et al., 2001) and gene ontology analysis of these changes **(Figure 2-3)** revealed the most significant categories, that include cell type-specific pituitary hormones and peptide hormone processing machinery as well as secretion pathways; regulation of cell cycle and cellular morphogenesis as well as cell differentiation. These target genes were confirmed by RT-qPCR analysis and/or by *in situ* hybridization (**Figure 2-1**, and data not shown).

LSD1 is not required for pituitary early cell lineage commitment events

To investigate the specific developmental stage at which LSD1 modulates pituitary development, we examined the expression of early pituitary markers by *in situ* hybridization. Analyses of e12.5-e13.5 embryos revealed a normal morphology of the anterior pituitary gland, normal expression of transcripts encoding the initial cell lineage determination factor, the LIM homeodomain transcription factor, *Lhx3*, and the paired homeodomain transcription factor *Prop1*, in *LSD1* gene-deleted pituitaries **(Figure 2-3)**, indicating that LSD1 exerts little effect on pituitary early cell lineage commitment events. In addition, while *GATA*2 and *LEF1* expression were reduced, expression of other pituitary-specific transcription factors such as *Pitx2*, *Mash1* and *Hes1* was equivalent to that of littermate controls **(Figure 2-4** and data not shown**)**, indicating the specificity of LSD1 on transcriptional controls.

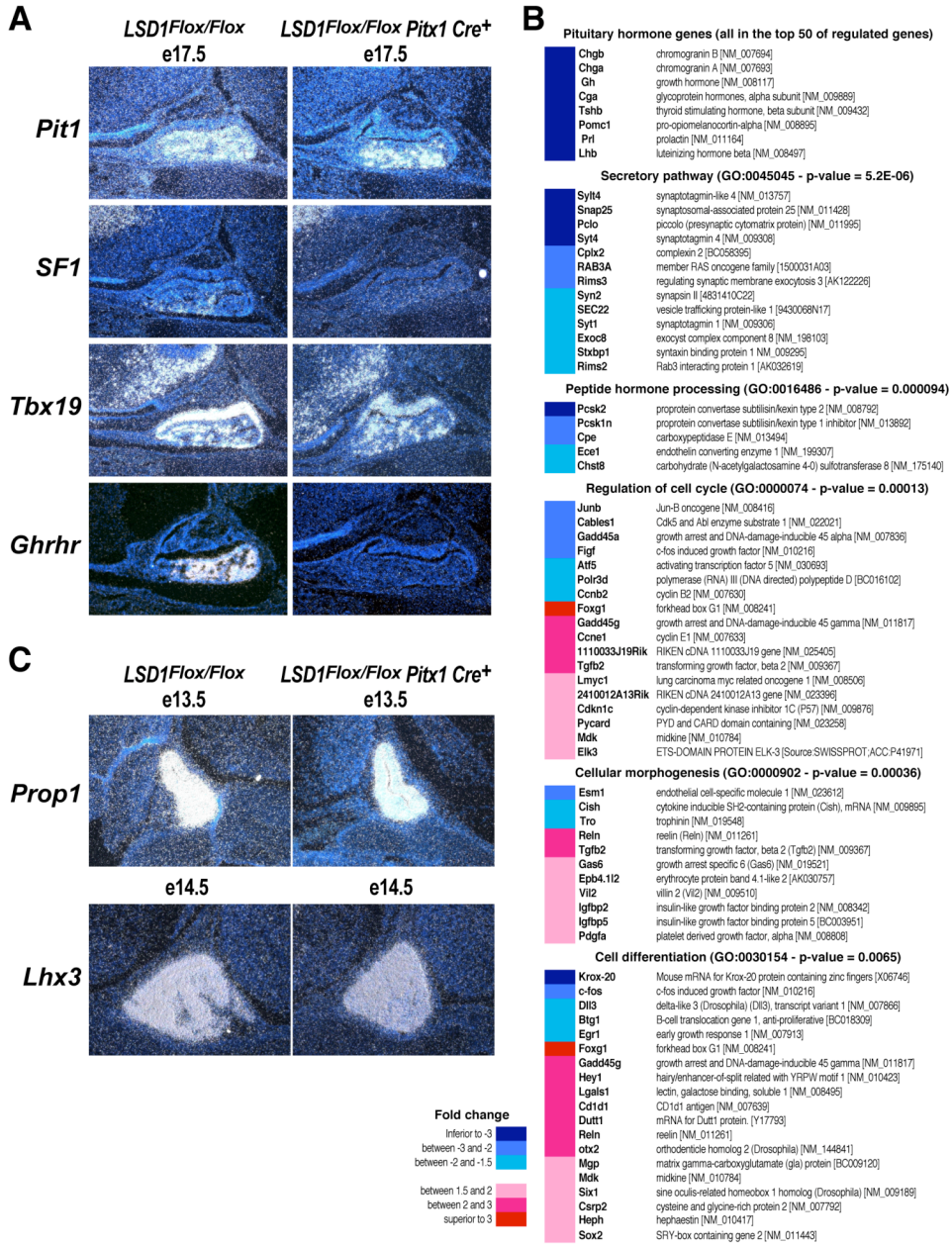


Figure 2-3. LSD1 in regulatory programs in pituitary development. (**A**) Gene expression of *Pit1*, *SF1*, *Tbx19* and *Ghrhr,* in control *vs LSD1* gene-deleted pituitaries at e17.5 by *in situ* hybridization. (**B**) RNA profiling analysis on control *vs LSD1* gene-deleted pituitaries at e17.5, with statistical analysis by SAM. (**C**) Gene expression of *Prop1* and *Lhx3* in control *vs LSD1* gene-deleted pituitaries at e13.5-e14.5 by *in situ* hybridization.

During pituitary development, LSD1 appears to regulate gene repression programs as well. For example, RNA profiling analysis and *in situ* hybridization revealed markedly increased of expression of *Hey1*, a direct downstream target of Notch signaling pathway (Maier and Gessler, 2000), in e17.5 *LSD1* gene-deleted pituitary, suggesting that LSD1 may play a critical role in CtBP-mediated repression of Notch signaling pathways (Oswald et al., 2005) **(Figure 2-4)**. Co-immunoprecipitation experiments revealed a potential interaction between the NICD interacting transcription factor, RBP-J/CSL, and LSD1 (**Figure 2-4**). Furthermore, ChIP analysis in e17.5 pituitary revealed recruitment of LSD1 to the *Hey1* promoter (**Figure 2-4**). Therefore, in pituitary development, LSD1 specifically controls terminal differentiation rather than early cell lineage commitment events.

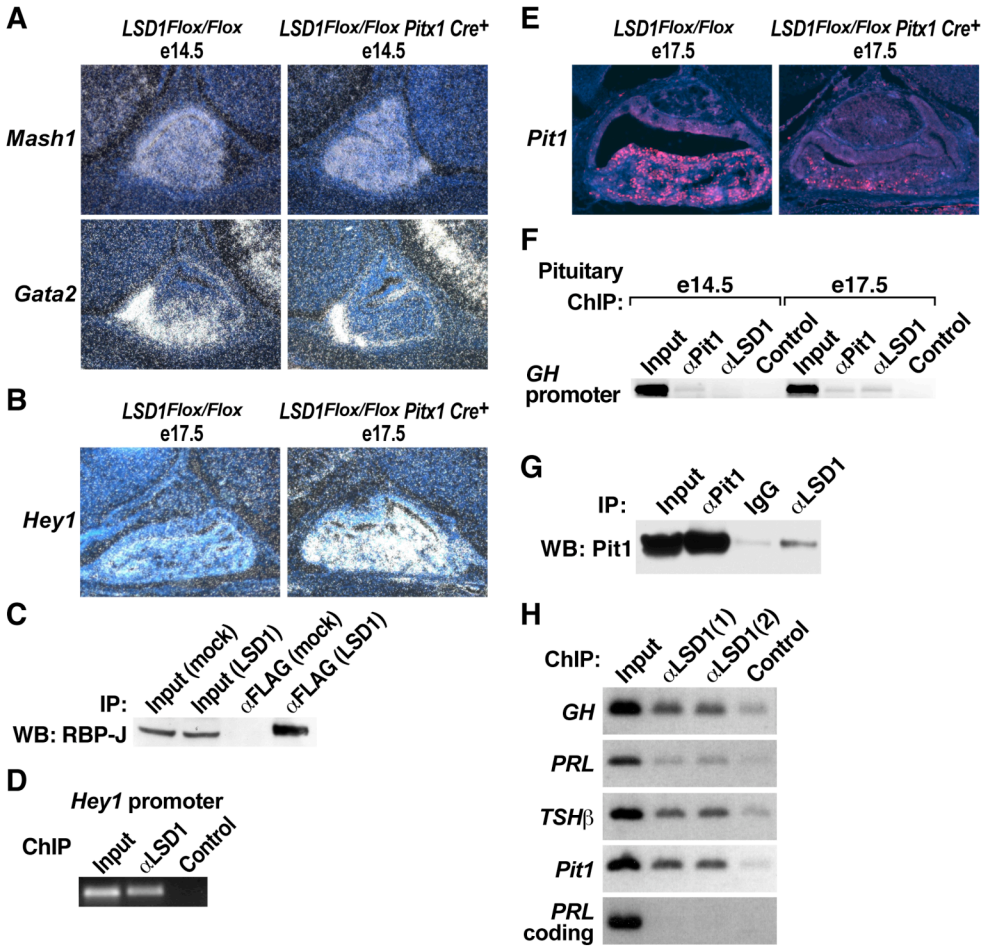


Figure 2-4. LSD1 is required for GH gene expression (A) Gene expression of *Mash1* and *Gata2* in control vs *LSD1* gene-deleted pituitaries at e14.5 by *in situ* hybridization. (**B**) Gene expression of *Hey1* in control vs *LSD1* gene-deleted pituitaries at e17.5 by *in situ* hybridization (**C**) Coimmunoprecipitation assay of RBP-J and flag-tagged LSD1 in transfected HEK293 cells. (**D**) ChIP on *Hey1* promoter in e17.5 pituitary with αLSD1 or preimmune control IgG. (**E**) Immunohistochemical analysis of Pit1 lineage precursors in control vs *LSD1* gene-deleted pituitaries at e17.5 using αPit1 IgG. (**F**) Chromatin immunoprecipitation (ChIP) with αPit1 and αLSD1 IgG analyzing, the *GH* gene promoter in e13.5-14.5 *vs* 16.5-17.5 pituitaries. (**G**) Coimmunoprecipitation assay of Pit1 with LSD1 in pituitary cells (**H**) ChIP was performed on p10 pituitaries using two independent, specific αLSD1 IgGs on the *GH*, *Prl*, *TSHβ* and *Pit1* promoters with a Prl coding region providing the negative control.

LSD1 positively regulates *GH* gene expression

Although *Pit1* expression exhibits a 5-fold reduction, *Pit1*$^+$ cells representing the somatotrope, thyrotrope and lactotrope precursors were all present at e17.5 in *LSD1* gene-deleted pituitaries **(Figure 2-4)**. The failure of *GH* gene expression in *Pit1*-expressing cells, suggests a direct requirement for LSD1 in *GH* gene expression, as the *GH* gene can remain expressed even when *Pit1* levels are ~10% of those of wild-type mice (C.J. Lin and M.G.R., unpublished data). Because *Pit1* directly regulates expression of *GH* gene expression, we hypothesized that LSD1 may function as a required "co-activator". Intriguingly, the critical transcriptional factor for *GH* gene expression, Pit1 was detected on the *GH* promoter regulatory region by ChIP analysis of pituitary tissue as early as e13.5-e14.5, significantly before *GH* mRNA levels are initially detected. However, recruitment of LSD1 to the same regulatory region of *GH* promoter occurred on e16.5-e17.5 **(Figure 2-4**), coincident with *GH* gene activation, demonstrating that LSD1 is temporally and spatially positioned to exert critical roles in specific gene activation programs that are required for terminal cell type differentiation. Co-immunoprecipitation experiments using pituitary cells revealed Pit-1/LSD1 interaction **(Figure 2-4)**. In addition, LSD1 was recruited to the promoters of other Pit1-dependent genes, including *Prl*, *TSHb* as well as *Pit1* using ChIP analysis, with similar results using either of two independently-

derived, specific anti-LSD1 antibodies **(Figure 2-4)**. Therefore, LSD1 appears to regulate gene activation programs during pituitary organogenesis, consistent with our findings that LSD1 is required for estrogen receptor (ER) mediated gene expression by ChIP-DSL.

LSD1 represses *Cyclin E1* gene expression during pituitary terminal differentiation

In addition to its roles in gene activation events, LSD1 functions as a required corepressor on several transcription units as well (**Figure 2-2**), including several cell cycle control genes, suggesting potential roles of LSD1 on pituitary growth control. Examination of cell proliferation in *LSD1* gene-deleted pituitaries revealed an increased number of luminal cells in S phase, beginning on e13.5 based on two hour BrdU pulse-labeling experiments (Li et al., 2002) **(Figure 2-5)**, with no evidence of change in apoptosis (Ward et al., 2005) (data not shown). *LSD1* gene-deleted pituitaries exhibit some dysmorphogenesis with a convoluted lumen at e17.5, resembling that of *Propl* gene-deleted pituitaries (Sornson et al., 1996;Gage et al., 1996;Nasonkin et al., 2004; Ward et al., 2005; Olson et al., 2006) and exhibit more Ki67-positive luminal cells **(Figure 2-5)**, indicating increased proliferation in the lumen. Among LSD1-regulated cell cycle control genes revealed by RNA profiling analysis, we confirmed the up-regulation of

Cyclin E1 (*CCNE1*) and *Id2* at e17.5 by *in situ* hybridization **(Figure 2-5)** and by RT-qPCR (**Figure 2-1**).

Chromatin immunoprecipitation revealed that LSD1 was recruited to the *Cyclin E1* promoter in the e17.5 pituitary **(Figure 2-5)**, and a small, but highly reproducible increase in histone diMe H3-K4 on the *Cyclin E1* promoter at e17.5 in *LSD1* gene-deleted pituitaries measured by q-PCR **(Figure 2-5)**. Therefore, *Cyclin E1* appears to be a direct target of LSD1 *in vivo*. It has been reported that *Rb* represses *Cyclin E1* gene expression by targeting *E2F* mediated repression (Geng et al., 1996; Botz et al., 1996; Hwang and Clurman, 2005). In addition, *Rb* represses *Id2* gene expression and the derepression of *Id2* appears to play a critical role in pituitary tumor progression in $Rb^{+/-}$ mice (Hu et al., 1994; Lasorella et al., 2005). Therefore, we hypothesized that LSD1 might function as a required corepressor of *Rb*/*E2F* dependent gene repression. To determine whether LSD1 may play a role in *Rb*/*E2F* repression in a cellular context, we used $LSD1^{-/-}$ embryonic fibroblast cell lines (MEF) generated from $LSD1^{Flox/Flox}$ MEFs (**Figure 2-5**), finding that *Cdc2* and *Cyclin E1* promoter-driven reporters were inhibited by re-expression of wild-type, but not enzymatically-inactive *LSD1* **(Figure 2-5)**. Consistent with the role of LSD1 in *E2F* mediated repression, the pocket protein family, *Rb*, *p107* and *p130* all proved to be capable of associating with LSD1 *in vivo* by immunoprecipitation assay **(Figure 2-5)**.

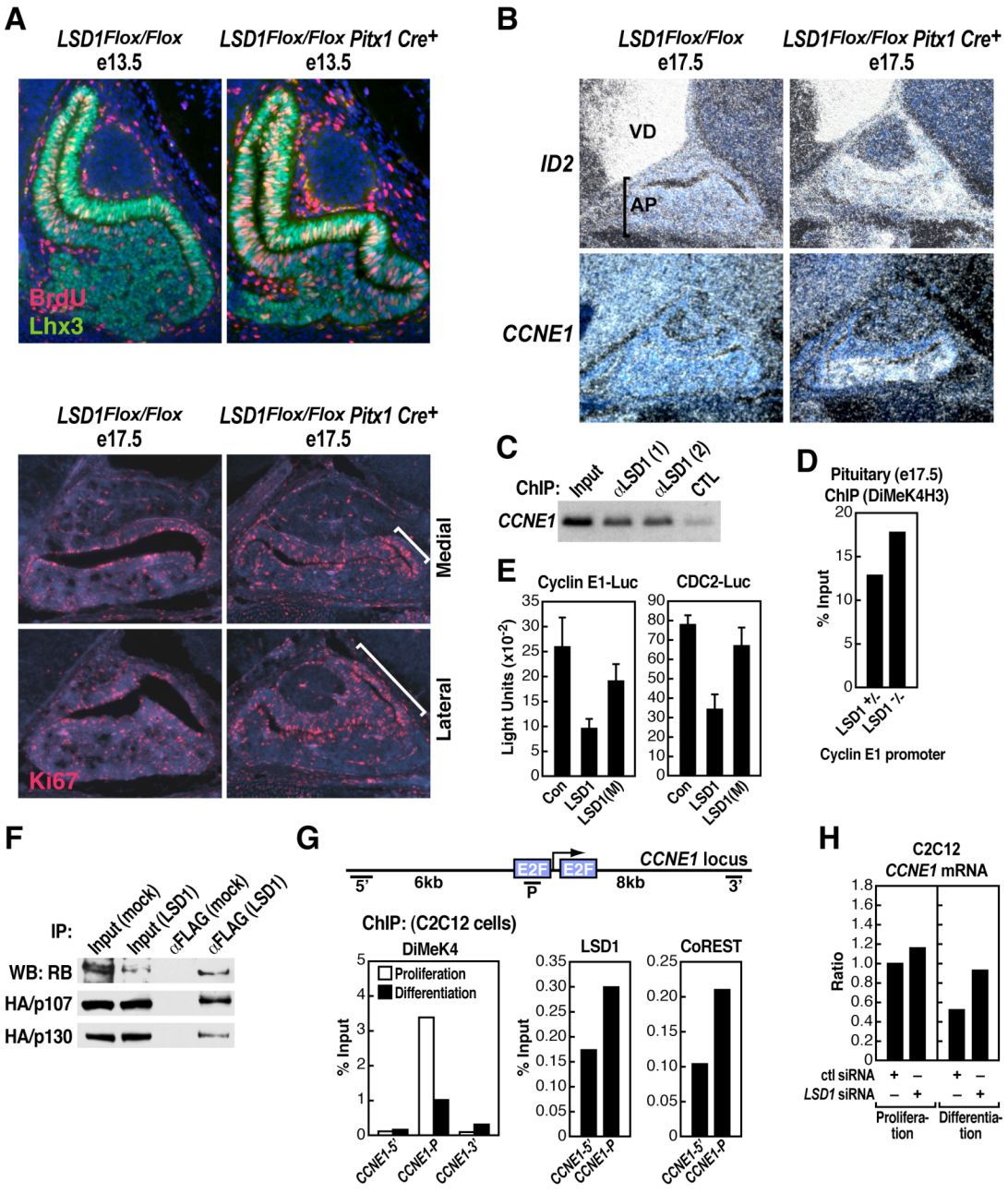
Previously, using NIH3T3 and C2C12 cells as models, *E2F*-mediated repression was found to be targeted by histone deacetylation in both proliferation and differentiation events, while histone H3-K9 methylation was observed only in differentiation, but not in proliferation events (Nielsen et al., 2001; Nicolas et al., 2003; Ait-Si-Ali et al., 2004), suggesting distinct requirements of histone modification on *E2F*-mediated repression. To determine whether *E2F*-mediated repression is targeted by histone H3-K4 demethylation, we evaluated NIH3T3 and C2C12 cell models, finding that histone diMe H3-K4 remained constant in proliferating NIH3T3 cells (**Figure 2-6)**, but was significantly decreased on the *Cyclin E1* and *Cdc2* promoters in differentiating C2C12 cells **(Figure 2-5)**. These data indicated that *E2F*-mediated repression is regulated by histone H3-K4 demethylase activity only during differentiation, potentially by LSD1 or other demethylases. ChIP analysis revealed that *LSD1* and *CoREST* were recruited to the *Cyclin E1* and *Cdc2* promoters during C2C12 differentiation **(Figure 2-5** and data not shown**)**. To further determine whether LSD1 may be responsible for repression of *E2F* target genes during differentiation, we knocked down LSD1 expression in C2C12 cells using specific siRNAs, finding that LSD1 siRNA selectively abolished repression of *Cyclin E1* or *Cdc2* in differentiating, but not in proliferating, C2C12 cells **(Figure 2-5** and data not shown**)**, suggesting that the LSD1 H3-K4 demethylase activity is required for the pocket protein-dependent

repression of *E2F* targets during differentiation events. Together, these data demonstrate that LSD1 functions in both repression of genes associated with cell proliferation and simultaneous activation of transcriptional programs required for terminal differentiation.

C: Discussion

LSD1 is initially identified as a co-repressor, however, it functions *in vivo* as a required cofactor for both gene activation and repression programs that dictate cell type determination and differentiation during pituitary organogenesis.

Figure 2-5. LSD1 control of pituitary progenitor cell proliferation. (A) Examination of proliferation in the e13.5 pituitary by BrdU pulse labeling (2 hours) and in the e17.5 pituitary by Ki67 staining, comparing *LSD1* $^{Flox/Flox}$ and *LSD1* $^{Flox/Flox}$*/Pitx1/Cre,* pituitary. (**B)** Gene expression of *Cyclin E1* and *Id2* in control *vs LSD1* gene-deleted pituitaries at e17.5 by *in situ* hybridization (**C)** ChIP on *Cyclin E1* promoter in e17.5 pituitary with αLSD1 or preimmune control IgG. (**D)** qPCR of ChIP of *Cyclin E1* promoter using α diMe H3-K4 in control *vs LSD1* gene-deleted pituitaries at e17.5 (**E)** Wild-type LSD1 represses *Cdc2* and *Cyclin E1* promoters in reporter assay in LSD1$^{-/-}$ MEF cells, (mean ± SEM). Enzymatically-inactive mutant LSD1 (LSD1 K661A) was unable to mimic this function. (**F)** Rb, p107 and p130 interacted with LSD1 in co-immunoprecipitation assay: western blot detecting expression of Rb or HA-tagged p107, p130 (**G)** Schematic diagram of *Cyclin E1* locus, with two *E2F* binding sites. Primers used in qPCR analysis: 5', P, 3' regions of *Cyclin E1* locus. Decreased diMe H3-K4 by ChIP was noted on the *Cyclin E1* promoter on differentiated C2C12 cells. LSD1 and CoREST were recruited to C2C12 promoter during differentiation by ChIP assay. (**H)** LSD1-dependent repression of *Cyclin E1* during C2C12 differentiation, but not in proliferating cells using RT-qPCR assay (mean of duplicates differing by <10%). Similar results were observed in three independent experiments.


A
LSD1Flox/Flox e13.5
LSD1Flox/Flox Pitx1 Cre+ e13.5
BrdU
Lhx3
LSD1Flox/Flox e17.5
LSD1Flox/Flox Pitx1 Cre+ e17.5
Medial
Lateral
Ki67
B
LSD1Flox/Flox e17.5
LSD1Flox/Flox Pitx1 Cre+ e17.5
ID2
VD
AP
CCNE1
C
ChIP: Input αLSD1 (1) αLSD1 (2) CTL
CCNE1
D
Pituitary (e17.5)
ChIP (DiMeK4H3)
% Input
LSD1 +/- LSD1 -/-
Cyclin E1 promoter
E
Cyclin E1-Luc
CDC2-Luc
Light Units (x10-2)
Con LSD1 LSD1(M)
F
IP: Input (mock) Input (LSD1) αFLAG (mock) αFLAG (LSD1)
WB: RB
HA/p107
HA/p130
G
CCNE1 locus
5' 6kb E2F E2F 8kb 3'
P
ChIP: (C2C12 cells)
DiMeK4
Proliferation
Differentiation
% Input
CCNE1-5' CCNE1-P CCNE1-3'
LSD1
CoREST
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C2C12
CCNE1 mRNA
Ratio
ctl siRNA
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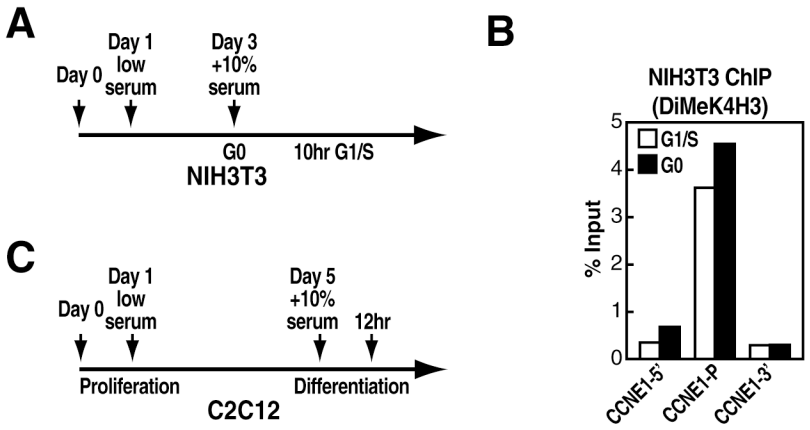

Figure 2-6: Static histone diMe H3-K4 on *Cyclin E1* promoter in proliferating NIH3T3 Cells. (**A**) Schematic diagram of protocol for synchronizing NIH3T3 cells by serum starvation. (**B**). Histone diMe H3-K4 ChIP of *Cyclin E1* locus in G0 and G1/S phase of synchronized NIH3T3 cells. (**C**) Schematic diagram of protocol for C2C12 cells differentiation assay by serum starvation.

D: Materials and Methods

In situ hybridization and immunohistochemistry

In situ hybridization was performed as described previously (Simmones et al 1990). For immunohistochemistry, 10% neutral buffer formalin fixed, 20% sucrose penetrated embryos were embedded with paraffin and sectioned as 15 micron histology slides. Unstained sections were post fixed with 10% neutral buffer formalin for 10 minutes and washed twice with PBS. Antigen was retrieved through boiling in 10mM Citrate buffer (pH 6.0) for 10 minutes. Immunostaining was carried out with standard immunochemistry protocols.

Immunoprecipitation and chromatin Immunoprecipitation

3X FLAG tagged LSD1 and mock control were transfected into HEK293 cells with standard Lipofectmin 2000 transfection protocols. 48 hours after transfection, nuclear extracts from collected cells were incubated with M2-FLAG matrix for 2 hours in 4ºC. Interacting proteins were after washed 4 times with 300mM NaCl, 0.1% NP-40 50mM Tris-HCl (pH8.0), eluded with 250mM Glycine (pH2.5), separated by SDS-PAGE electrophoresis, transferred to nitrocellulose membrane, and visualized by western blot. Chromatin immunoprecipitation were performed as previously described (Kioussi et al., 2002) on manually dissected embryonic or adult pituitaries. Anti-LSD1 antibodies were generated in rabbits and guinea pigs against recombinant N-terminal and C-terminal of LSD1 expressed in bacteria and affinity-purified. Anti-LCoR and Anti-ZEB1 antiserum were generated in guinea pigs against recombinant LCoR and ZEB1 expressed in bacteria. Anti-dimethyl H3-K4 antibody is obtained from Upstate Biotechnology.

Generation of LSD1 null MEF cells and Reporter Assay

LSD1$^{Flox/Flox}$ mouse embryonic fibroblasts (MEF) were generated from e13.5 embryos. Primary MEF cells were immortalized with standard 3T3 methods. Immortalized MEF cells were transduced with retrovirus for Cre expression to generated LSD1$^{-/-}$ MEF cells. For reporter assays, 200-400ng reporters, 50ng LacZ control and 100-200ng LSD1 expression vector were co-

transfected according to standard Lipofectmin2000 transfection protocols. Luciferase activities were determined and normalized according to LacZ control. All reporter assays were repeated at least three times and representative results were shown.

In vitro C2C12 differentiation assay

In vitro C2C12 differentiation assay was performed as described previously (Ait-Si-Ali et al., 2004). Briefly, proliferation C2C12 cells were cultured with DMEM 0.5% fetal bovine serum for 4 days to induced muscle differentiation.

RNA Profiling Analysis

The Agilent whole genome arrays were scanned using the Agilent scanner and analyzed using "Feature Extraction" program. We used the lowest option with spatial detrend. Then the normalized data were imported in Focus (Cole et al., 2003) to extract interesting genes with more confidence than using fold change only. This gene list was then run through a program that uses hypergeometric distribution to extract over-represented GO terms and pathways compared to a random sampling.

Chapter 3

Studying LSD1/ZEB1/LCoR complex on

control of GH gene restriction

A: Introduction

In the pituitary, a developmental switch of a subset of GH^+/Prl^- cells to GH^-/Prl^+ lactotropes occurs at p5-p10 (Hoeffler et al., 1985; Behringer et al., 1988; Borrelli et al., 1989), but molecular events underlying this switch have remained unknown. Previous *in vivo* experiments (Scully et al., 2000) suggested that a sequence (-161/-146) in the rat *GH* promoter was required with other DNA sites, for *GH* gene restriction events in developing lactotropes (**Figure 3-2**). Therefore, a pituitary cDNA phage expression library was constructed and screened using -161/-146 region as probe. cDNA expression clones obtained encoded three independent and overlapped N-terminal portions of ZEB1 (Tcf8/dEF1/BZP/zfhx1a/zfhep/AREB6) , a 130kDa protein containing one homeodomain and seven Cys/His Zinc finger (Williams et al., 1991; Higashi et al., 1997; Takagi et al., 1998; Postigo et al., 1999). ZEB1 contains three PLDLS *CtBP* recognition sequences (Furusawa et al., 1999), consistent with it being identified as a component of the LSD1/CoREST/CtBP complex in Hela cells (Shi et al., 2003). Because ZEB1 is as a component of the LSD1/CoREST/CtBP corepressor complex (Shi et al., 2003), we were particularly intrigued to investigate whether the function of LSD1 on the *GH* gene might be involved in the switch from activation to repression as a component of the post-partum restriction of *GH* expression out of the subset of GH^+ cells that begin to express *Prl* during "switching" to the lactotrope phenotype.

B: Results

Previously, we found LSD1 is required for GH expression during pituitary development. Surprisingly, we found LSD1 represses GH expression in reporter assay in cell lines (**Figure 3-1**). Therefore, we next wished to address whether the role of LSD1 on a given target might itself be altered during development.

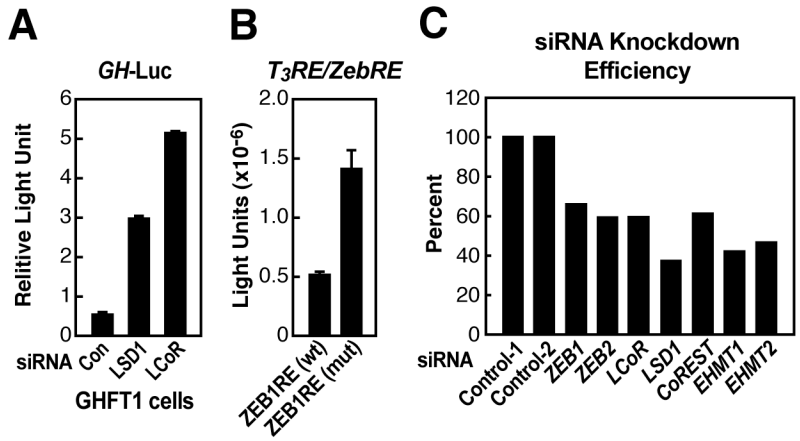

Figure 3-1: Restriction of GH expression by LSD1.

(**A**) LSD1 and LCoR siRNA derepressed expression of the *GH* reporter in GHFT1 cells. (**B**) ZEB1 repressed a T_3RE/ZEB1RE (-191 to -146)-dependent reporter in HEK293 cells, when treated with T3. (**C**) Confirmation of siRNA knockdown efficiency in MMQ cells by RT-qPCR, relative percentage of remaining mRNA level in specific siRNA treated cells compared to mRNA level in control siRNA treated cells are shown.

Examination of the ontogeny of *ZEB1* expression during pituitary development by *in situ* hybridization, revealed only minimal expression prior to birth, but a marked induction between p5-p10 **(Figure 3-2)**, a temporal pattern consistent with a potential role in *GH* gene restriction events. The same pattern of post-partum expression proved to be the case for a second component of the LSD1/CoREST/CtBP complex (Shi et al., 2003) **(Figure 3-3)**, LCoR/KIAA1795/MLR2, which was initially described as an agonist-dependent estrogen receptor corepressor, recruited by nuclear receptors based on an LXXLL motif (Fernandes et al., 2003). Hence, two components of the CtBP corepressor complex initially isolated in HeLa cells actually appear to be expressed only post-partum during pituitary development.

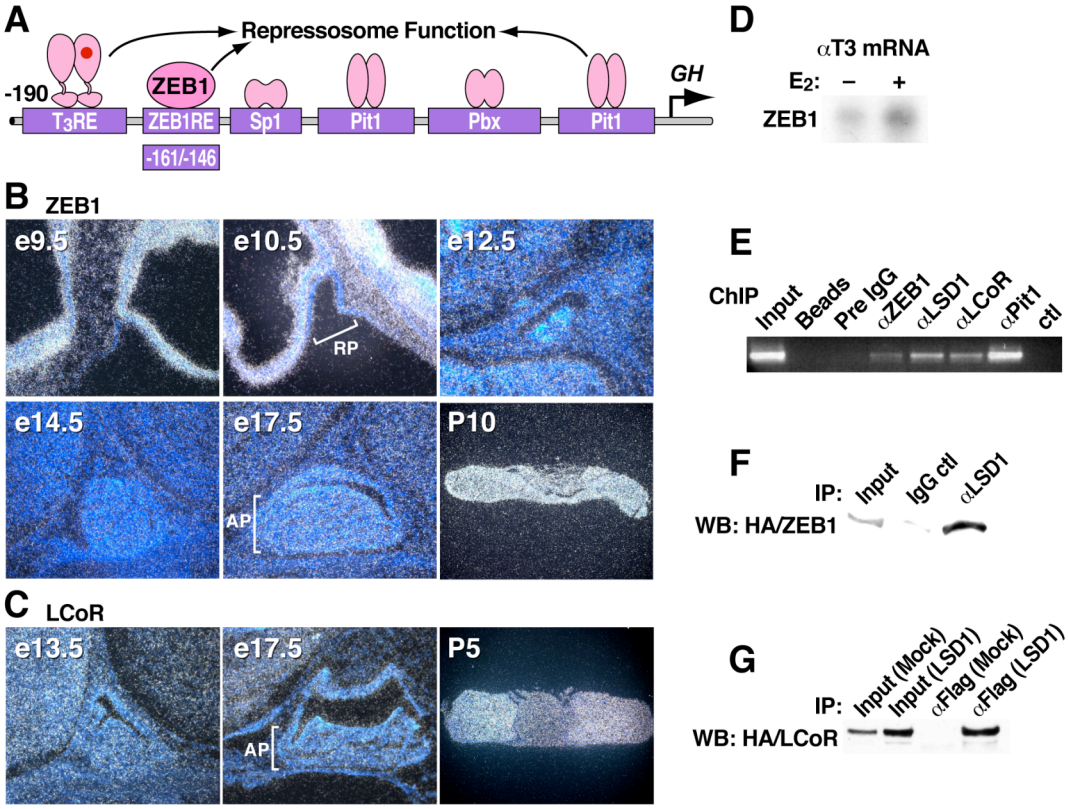

Figure 3-2. LSD1 in restriction of *GH* expression in lactotropes. (**A**) Schematic diagram of *rGH* promoter with regulatory elements, including the -161 to -146 repressive element that bound *ZEB1*. (**B**) Ontogeny in *ZEB1* expression in pituitary development. Rathke's Pouch = (RP); Anterior Pituitary = (AP) (**C**) Ontogeny of *LCoR* expression in pituitary development. (**D**) Induction of *ZEB1* expression by estrogen in aT3 pituitary cells, analyzed by northern blots. (**E**) ZEB1/LSD1/LCoR/Pit1 is recruited to the *GH* promoter by ChIP assays on p10 pituitaries. (**F**) HA/ZEB1 and LSD1 coimmunoprecipitation in HEK293 cells. (**G**) HA/LCoR interacted with Flag/LSD1 in co-immunoprecipitation assays in HEK293 cells.

In concert with the suggestion that prolactin-producing cells appear in response to a postpartum estradiol-17b (E_2) surge (Lieberman et al, 1983; Day et al., 1990; Simmons et al., 1990; Scully et al., 1997), we note that the estrogen-dependent induction of *ZEB1* transcripts could be observed in aT_3 pituitary cells **(Figure 3-2),** consistent with an observation of estrogen induction of *ZEB1* gene expression in chicken oviduct (Chamberlain and Sanders, 1999). Chromatin immunoprecipitation analysis of adult pituitaries revealed that ZEB1 is recruited with LSD1 and LCoR to the *GH* promoter **(Figure 3-2)**. These data suggested the model that the late expression of ZEB1/LCoR acts as the molecular "beacon" that mediates recruitment of an LSD1/ CoREST/CtBP and LCoR-containing complex to the *GH* promoter, and thereby "switches" off *GH* expression during initial appearance of the lactotrope.

Interestingly, a thyroid hormone receptor response element T_3RE (-190 to -167 region of the rat *GH* promoter) is located immediately adjacent to the ZEB1 binding site ZEB1RE (-161 to -146 region of the rat *GH* promoter) **(Figure 3-2)** and is required for both *GH* gene activation in somatotropes and repression in lactotropes (Scully et al., 2000). Since LCoR was identified as an agonist-dependent nuclear receptor corepressor and as a potential component of the LSD1/CoREST/CtBP complex, we also tested its role in ZEB1-mediated events. We found that LSD1 could be coimmunoprecipitated with ZEB1 and LCoR **(Figures 3-2)** and that ZEB1 was capable of repressing the T_3RE/ZEB1RE-driven reporter (-191 to –146 region of the *GH* promoter), but not one with a mutation in

the ZEB1 regulated element **(Figure 3-1).** Intriguingly, ZEB1 and ZEB2 have been reported being sumoylated on two conserved lysine residues (Long et al., 2005), with one (ZEB1 K698) located adjacent to the CtBP interaction motif of ZEB1. Using the T_3RE/ZEB1RE reporter (-191 to –146 region of the *GH* promoter) in HEK293 cells, we found that mutation of the these potential sumoylated lysine residues (K271R, K698R) actually caused a decrease of the repressor function of ZEB1 **(Figure 3-3)**, suggesting sumoylation of ZEB1 may be required for repression function of ZEB1. In addition, we found that LCoR was capable of exerting repression function on *GH* promoter reporter as well as a thyroid hormone receptor-regulated *Dio1* promoter reporter in the presence of T3 **(Figure 3-3** and **Figure 3-1)**. The repressor function of LCoR depended on both its LXXLL motif and PLDLS motifs, arguing that LCoR represses *Dio1* expression through interactions with both T3R (via LXXLL) and a LSD1/CoREST/CtBP/ZEB1 (via PLDLS) complex (**Figure 3-3)**. Therefore, recruitment by ZEB1/LCoR of a LSD1- and CtBP-containing complex might be capable of "locking" the liganded T3R/LCoR interaction and inhibiting the normal cycling of coactivators on T3R (reviewed in Dennis and O'Malley, 2005; Malik and Roeder, 2005; Perissi and Rosenfeld, 2005), thereby causing repression. Indeed, ZEB1 has been suggested as a repressor of T_3R-induced *GH* gene expression by transient transfection experiments (Cabanillas et al., 2001).

To test the putative ability of LSD1/CoREST/CtBP/ZEB1/LCoR-containing complexes in *GH* gene repression, we took advantage of an available

lactotrope cell line model, the *prolactin* gene-expressing MMQ cells (Judd et al., 1988; Ooi et al., 2004). Employing specific *ZEB1-, LCoR-, CoREST*-, and *LSD1*-specific siRNAs, we found that *GH* gene expression was specifically enhanced by each siRNA, but not by control or by *ZEB2* siRNAs (**Figure 3-3)**. Thus, LSD1 appeared to be a component of a corepressor complex in "lactotropes", imposing repression on the *GH* gene expression. This is conceptually similar to the role of LSD1/CoREST complex in REST-mediated repression (reviewed in Ballas and Mandel, 2005). ChIP experiments revealed that the histone diMe H3-K4 mark was enriched on the *GH* promoter in "somatotrope" GH^+/Prl^+ (GC) cells but not in the "prolactin" GH^-/Prl^+ MMQ cells **(Figure 3-3)**, suggesting that LSD1-dependent demethylation of histone diMe H3-K4 may contribute to restriction of *GH* gene expression out of lactotrope, with ZEB1 functioning in a fashion analogous to REST, as a DNA binding factor recruiting the LSD1/CoREST/CtBP complex. In this case, we speculate that CtBP thereby serves as a bridging factor as apposed to CoREST in the case of REST. In addition, the histone H3-K9 methyltransferases EuHMT1 and EuHMT2/G9a are also found in this CtBP complex (Shi et al., 2003), and siRNA-mediated knockdown of these methyltransferases also derepresses *GH* gene expression in MMQ lactotropes **(Figure 3-3)**, suggesting that histone H3-K9 methylation also contributes to the repression event.

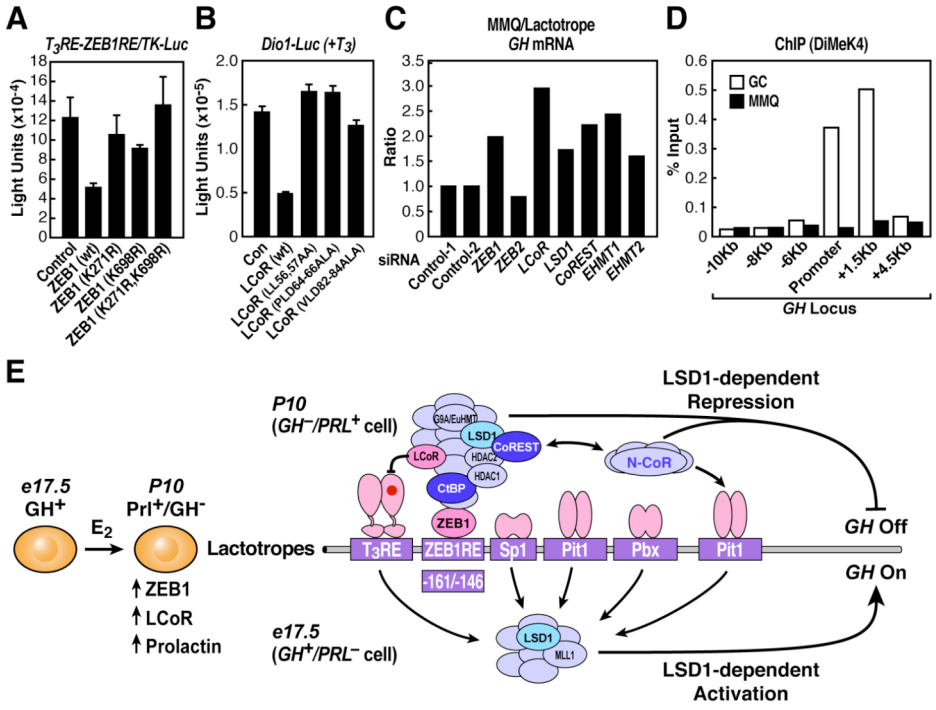

Figure 3-3. LSD1 can switch from a coactivator to a corepressor in *GH* gene expression. (**A**) Effects of wild-type or mutant ZEB1 on the activity of the *GH* T3RE/ZEB1RE (-191 to -146) region of the *GH* promoter in reporter assay. (**B**) LCoR represses T3R-mediated *Dio1* activation in reporter assay in HEK293 cells. (**C**) Various siRNA specifically depress *GH* expression in MMQ lactotropes cells by RT-qPCR assay. (**D**) Loss of histone diMe H3-K4 marks on the *GH* promoter in MMQ lactotropes by ChIP assay. (**E**) Model of the "switch" in LSD1 function during pituitary organogenesis. Initial activation of the *GH* gene required the coactivator functions of LSD1; however, a subsequent induction of *ZEB1* and *LCoR* expression nucleated the recruitment of the LSD1/CoREST/CtBP complex to the T_3RE/ZEB1RE of *GH* promoter, with EuHMT1/2 and HDAC1/2 participating in repression, synergizing with the N-CoR complex recruited to Pit1 sites on *GH* promoter in lactoropes.

C: Discussion

Therefore, the delayed induction of LCoR and ZEB1 can cause a switch in LSD1 function from activation to repression, "restricting" *GH* gene expression as a critical component of lactotrope terminal differentiation events during pituitary organogenesis.

D: Materials and Methods

siRNA knockdown and RT-qPCR

siRNAs against LSD1 and control were obtained from Qiagen. siRNA: LSD1-01: AACTGGCCAAGATCAAGCAAA; LSD1-02: AATGGACAAGCTGTTCCAAAA are targeted to the identical sequence between mouse and rat LSD1. 20nM siRNA was used in experiments according to standard Lipofectamin 2000 siRNA transfection protocols. siRNA knockdown efficiency was determined by western blot or RT-qPCR. Primers are designed for specific targets and available on requests. All qPCR for ChIP and RT-qPCR were repeated at least three times and representative results were shown.

Chapter 4

Studying CoREST3 function: genetic deletion of CoREST3

A: Introduction

CoREST was initially identified by yeast two-hybrid screen for interacting-protein of REST/NRSF (Andres et al., 1999) and functions as a required corepressor. However, its functional role in development is unclear.

Three homolog of CoREST in human and mouse have been identified through genome sequence data mining (our unpublished data). They share strong homology in N terminal ELM2 domain and the dual SANT domain. Recently, CoREST has been proved as the required coregulator of LSD1 (Lee et al., 2005). We decide to use gene-targeting strategy to investigate the *in vivo* function of CoREST during mammalian development.

B: Results

Unlike CoREST1 and CoREST2, which expression are ubiquitous in adult tissue, CoREST3 expression is restricted to embryonic stage, mainly in developing brain and pituitary (**Figure 4-1**), suggesting its restricted roles in neurogenesis.

We employed an *in vivo* approach for deletion of the CoREST3/RCoR2/1A13 genomic locus, with a strategy that permitted conditional gene deletion by removal of an essential coding region of *CoREST3* (Ballas et al., 2001), based on insertion of two Lox P sites flanking exon 5-9 corresponding to

the C-terminal of the SANT domain (Gu et al., 1994) **(Figure 4-2)**, the deletion of which would result in an alteration of the reading frame of *CoREST3* transcript.

Using standard gene targeting technology in mouse embryonic stem (ES) cells, we obtained homologous recombination and generated both type I and type II recombinant alleles by using *PMC/Cre* electroporation *in vitro* **(Figure 4-3)**. Germ-line transmissions were ultimately achieved for both types of recombination. Homozygous *CoREST3*$^{Flox/Flox}$ (type II recombinant, conditional allele) mice were fertile and exhibited normal expression of *CoREST3*, indicating that insertion of the two Lox P sites did not significantly affect *CoREST3* transcription or RNA processing (**Figure 4-3**).

While mice heterozygous for conventional *CoREST3* gene deletion (type I recombinant, conventional allele) appeared normal and fertile, and e9.5 homozygous *CoREST3*$^{-/-}$ embryos reveal no obvious difference from heterozygous or wild type littermates, no viable homozygous *CoREST3*$^{-/-}$ embryos could be detected after e13.5, and the few homozygous *CoREST3*$^{-/-}$ embryos obtained had not progressed beyond e12.5 and exhibited severe growth retardation (**Figure 4-3** and data not shown), suggesting *CoREST3* play important roles during development.

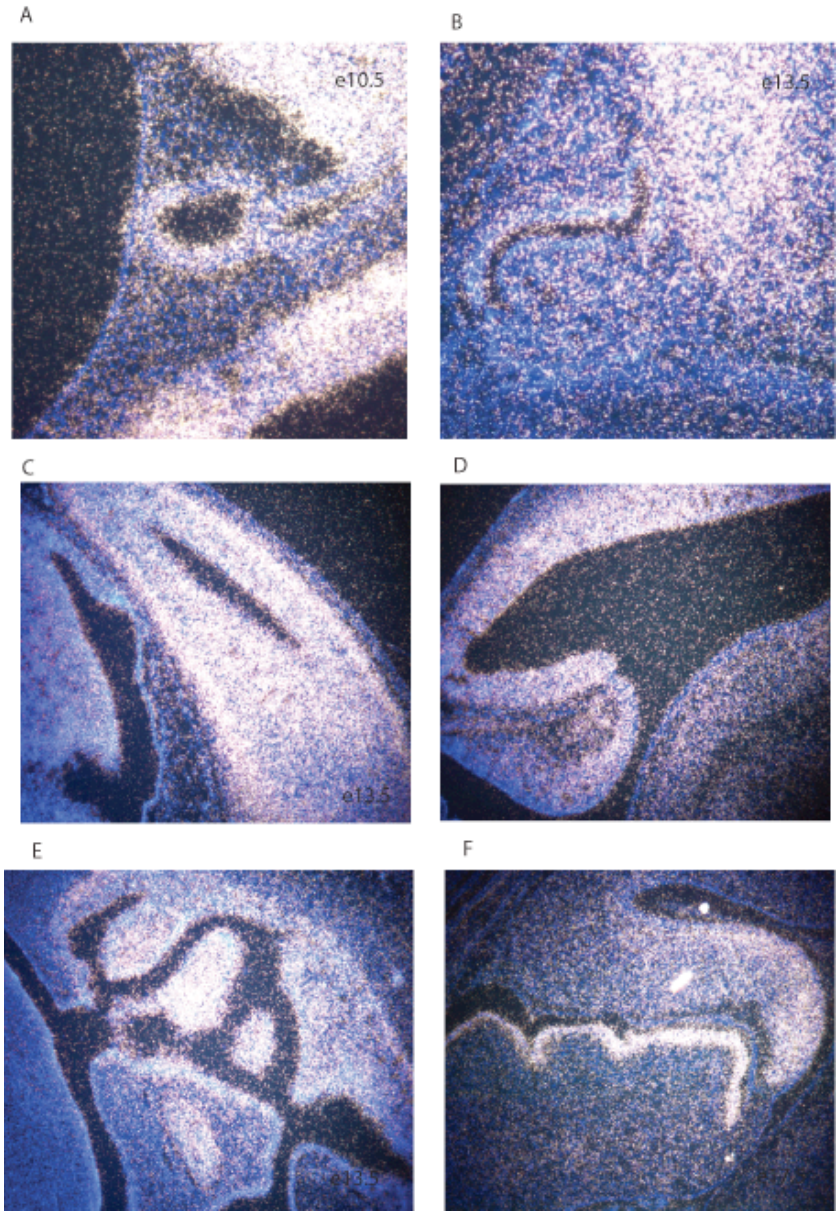

Figure 4-1: CoREST3 expression in development. *In situ* hybridization of CoREST3 at (A) e10.5 pituitary; (B) e13.5 pituitary; (C) e13.5 telecephalon; (D) e13.5 mesen cephalon; (E) e13.5 nasal epithelium; (F) e17.5 cerebellum

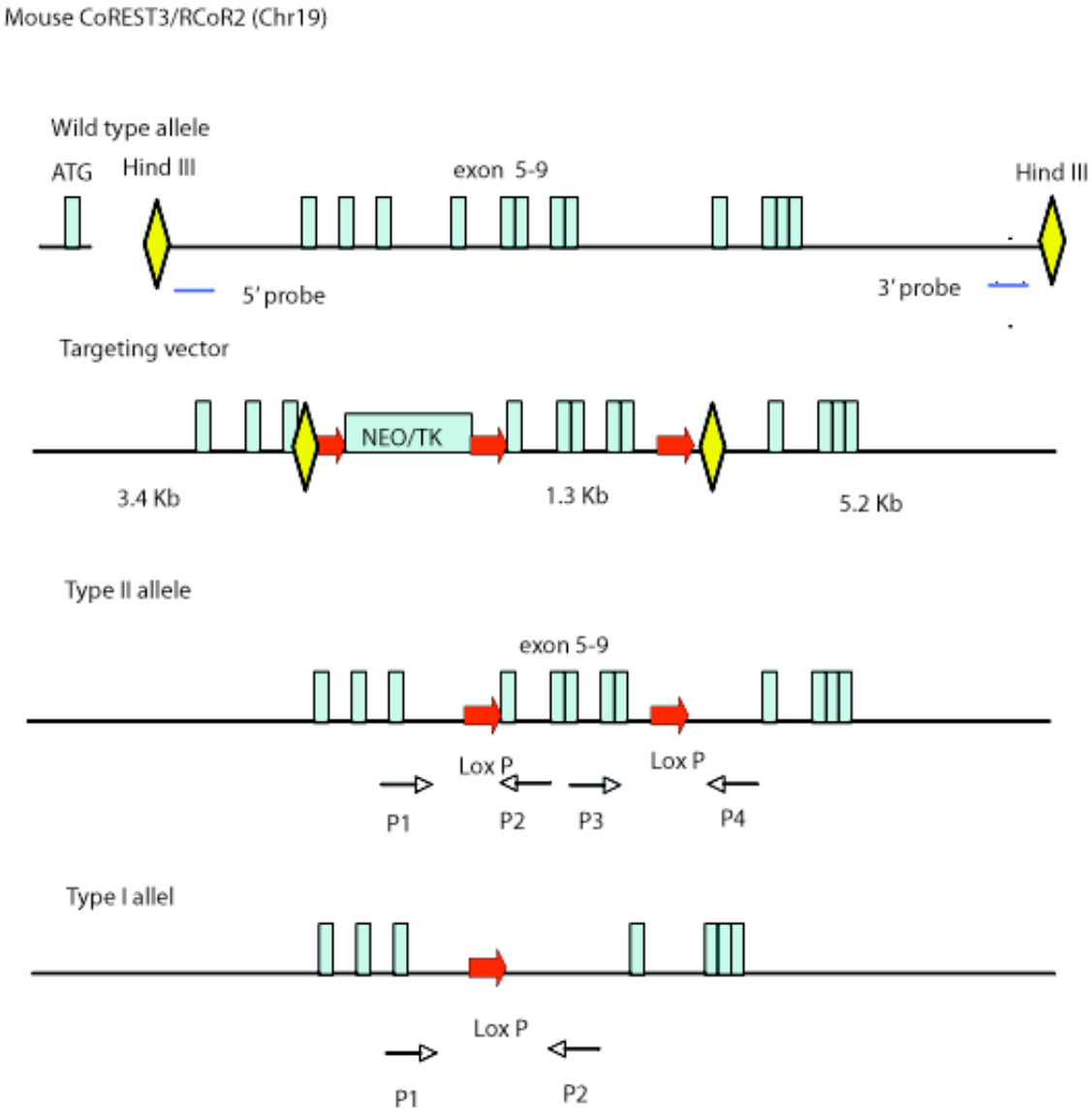

Figure 4-2: Gene targeting strategy for CoREST3

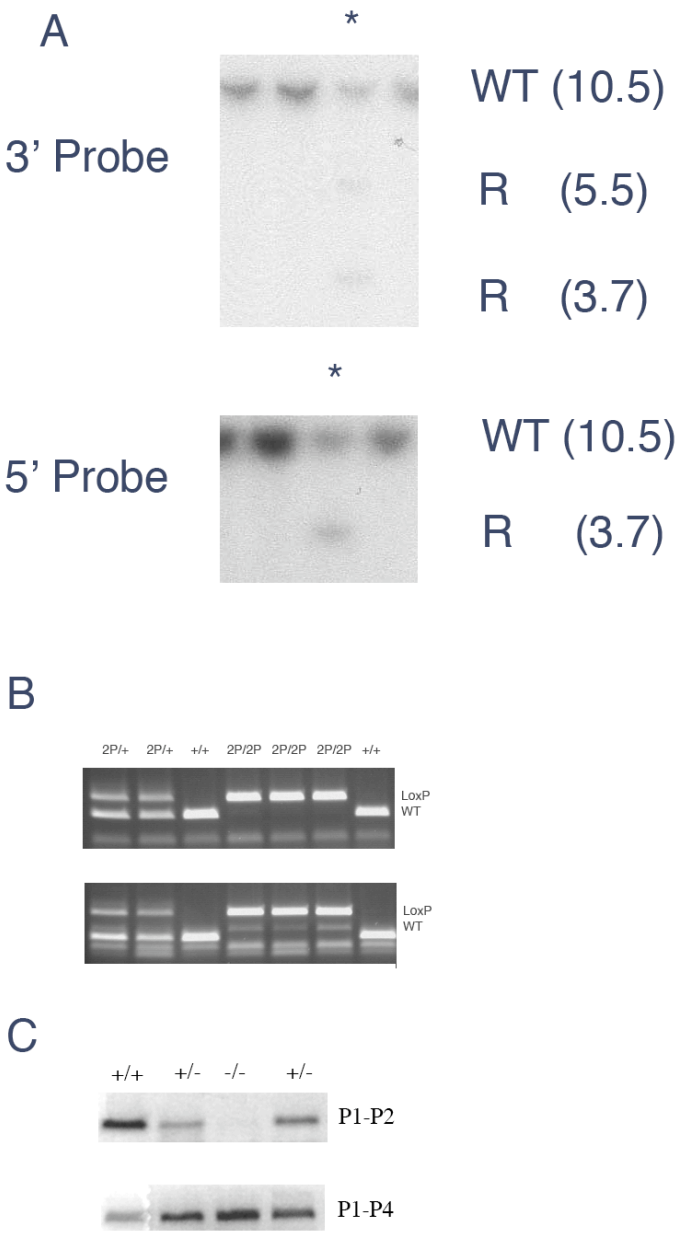

Figure 4-3 Generation of CoREST3 gene-targeting mice. (A) Identification of recombinant allele by southern blot. (B) Genotyping of type II allele from tail DNA of adult mice. (C) Genotyping of type I allele from yolk sac DNA of e9.5 embryos.

C: Discussion

REST gene-deletion result early embryonic lethality. Here, employing a conditional knockout strategy to circumvent the early embryonic lethality caused by *CoREST3* deficiency, we have the opportunity to study CoREST3 function in specific developmental programs such as pituitary organogenesis and neurogenesis.

D: Materials and Methods

Generation and Genotyping of CoREST3 deficient mice

CoREST3 conditional allele mice were generated by targeted mutagenesis in ES cells to insert two LoxP sites flanking exon 5-9 of CoREST3 (**Figure 4-2**), correct targeting was established by southern blotting with 5' and 3' external probes. Embryos were genotyped by PCR method.

DISCUSSION

As the regulatory roles of histone modification receive increasing experimental support in vertebrates (Yu et al., 1995; Xu et al., 1998; Yao et al., 1998; Peters et al., 2001; Zhang et al., 2002; Vega et al., 2004; Solter et al., 2004; Mager and Bartolomei, 2005; Solter et al., 2004; Mager and Bartolomei, 2005), and as there are multiple enzymes that can mediate site-specific histone modification, it is of particular interest to define the *in vivo* roles of each histone-modification factor. Here, employing a conditional knockout strategy to circumvent the early embryonic lethality caused by *LSD1* deficiency, we have demonstrated that LSD1 regulates specific developmental programs after organ commitment and prior to cell type differentiation in a well-investigated model of mammalian organogenesis. Together, our data suggests a model of dynamic developmental regulation in which LSD1 serves as a key component of opposing activation/repression complexes required for cell-specific gene expression during mammalian organogenesis **(Figure 3-3)**. These results are in agreement with our findings of broad recruitment of LSD1 on gene promoters in MCF7 cells by ChIP-DSL.

Although expressed throughout pituitary organogenesis, LSD1 proved to be required only in late embryonic development, during cell lineage determination and cell type-specific differentiation events. Early organ commitment regulators such as *Lhx3*, and cell lineage determination regulators such as *Prop1* are not

affected, while, *GH, Ghrhr, Prl and TSHb* gene, expression driven by Pit1, are severely reduced (e.g., $>10^6$-fold for *GH*) in LSD1 gene-deleted pituitaries. Intriguingly, while the critical DNA binding transcriptional activator of *GH* expression, Pit1, is bound by e13.5-e14.5, it is the recruitment of LSD1 that actually coincides temporally with initial *GH* gene activation (e16.5-e17.5), arguing for its critical biological role in specific gene activation programs that are required for terminal cell type differentiation. In addition, LSD1 is recruited to *Pit1* gene regulatory regions on e13.5 (G.Prefontaine and M.G.R., unpublished data), indicating that this delayed event on the *GH* promoter reflects a promoter-specific temporal difference in the recruitment of LSD1. This temporal specificity of LSD1 recruitment to the *GH* promoter might reflect post-translational modifications of Pit1 (Kapiloff et al., 1991**;** Caelles et al., 1995). In addition, the recent finding of lysine methylation on non-histone substrates, such as TAF10 and p53 (Kouskouti et al., 2004; Chuikov et al., 2004), suggests that LSD1 is also likely to target non-histone substrates. LSD1 is a component of Mll1/All1 complex (Nakamura et al., 2002), which has been identified as coactivitors in many transcription programs (Guenther et al., 2005; Wysocka et al., 2005a; Dou et al., 2005), suggesting Mll1 may be a component of the LSD1 activation complex. Indeed, we found that LSD1 interacts with Mll1 in pituitary by co-immunoprecipitation assays (data not shown). LSD1 enhances AR-mediated

activation by histone H3-K9 demethylation and exerts a broad role in ERa transcriptional activation programs (Metzger et al., 2005; Yamane et al., 2006; Garcia-Bassets et al., accompanying manuscript, 2006), and we consider it is also likely that LSD1 activates *GH* gene expression by similar removal of repressive histone marks. Interestingly, the recent discovery of additional histone di-Me H3-K9 demethylases — the JHDM2/JMJD1 family (Yamane et al., 2006), as well as the histone tri-Me H3-K9/K36 JMJD2 family demethylases (Whetstine et al., 2006), indicates multiple demthylases for removal of repressive mark histone H3-K9, therefore, LSD1 function can be compensated by other demethylases during pituitary development. However we find that, all *Jhdm2*/*Jmjd1* and *Jmjd2* family members are expressed during pituitary development (our unpublished data); and also find that *Jhdm2a*/*Jmjd1a* appears to be up-regulated in *LSD1* gene-deleted pituitary at e17.5 revealed in RNA profiling analysis, and further confirmed by RT-qPCR (**Figure 2-1**). It is therefore tempting to suggest that there might be an early redundancy of LSD1 function by other histone demethylases. Thus, our *in vivo* data reveals a unique function of LSD1 in the sequential transcriptional control during mammalian organogenesis required for emergence of mature cell types.

LSD1 homologues have been identified through genetic screens in worms and plants (Eimer et al., 2002; He et al., 2003). Interestingly, a CoREST mutation

was also identified in the same genetic screen in *C. elegans* and has been suggested to serve as a negative regulator of Notch signaling pathways (Jarriault and Greenwald, 2003), consistent with our finding of over-expression of *Hey1*, a direct downstream target of Notch signaling pathways, in the *LSD1* gene-deleted pituitary gland. Interestingly, several essential signaling pathways, including Wnt and Notch, which functions are required in the early stages of pituitary development, can actually block terminal differentiation events if their expression are not appropriately turned off in the later stages (X. Zhu. and M.G.R., unpublished data). Here, we find that LSD1 is required for repression of *Hey1,* a Notch target and *Tcf3* (data not shown), a Wnt target in e17.5 pituitary, arguing that down-regulation of key signal-dependent gene expression programs required for normal development may be specifically controlled by LSD1-dependent histone diMe H3-K4 demethylation. Both histone diMe H3-K4 and diMe H3-K9 binding proteins have been identified to exert effects on transcription regulation through modulating chromatin structure and accessibility of transcription factors (Nielsen et al., 2002; Jacobs and Khorasanizadeh, 2002; Wysocka et al., 2005; Martin and Zhang 2005). LSD1 demethylase activity may participate in the functional balance between histone H3-K4 and H3-K9 methylation/acetylation, a fine-tuning mechanism for transcriptional control. The first-described histone

lysine demethylase thus exerts critical functions in mammalian organogenesis, controlling both gene activation and gene repression programs.

ZEB1/LCoR-dependent switch in LSD1 functional effects

A second intriguing aspect of LSD1 function in a biological context is the apparent change in its function during the temporally-regulated, cell type-specific, restriction of *GH* gene expression. LSD1 actions are initially critical for *GH* gene activation, but later the function of LSD1 "switches", now becoming required to suppress *GH* gene expression in the emerging lactotropes.

After birth, the Krüpple-like zinc finger protein, ZEB1, and LCoR, components of LSD1/CoREST/CtBP complex are induced in the pituitary, with ZEB1 nucleating the binding of the LSD1/CoREST/CtBP/LCoR corepressor complex to the *GH* promoter. Thus, ZEB1-mediated recruitment of a potent corepressor complex to a DNA site that has previously been determined to be required *in vivo* for the silencing of *GH* gene expression in lactotropes (Scully et al., 2000) provides a molecular mechanism for restriction of *GH* expression out of lactotropes. The parallel induction of LCoR would permit repression of the liganded T3R based on its presence in the complex for which ZEB1 serves as molecular "beacon", by the cognate ZEB1 DNA binding element immediately adjacent to the T3R. As the *GH* promoter exhibits histone H3-K4 demethylation

in lactotropes, the loss of H3-K4 methylation may serve as one aspect of the observed *GH* gene restriction events.

The allosteric effect of a specific Pit1 binding site on the *GH* promoter causes recruitment of the N-CoR complex, which is also required for lactotrope-specific restriction events (Scully et al., 2000). Thus, LSD1/CoREST/CtBP and NCoR/SMRT complexes appear to synergize in forming the "repressosome" that causes cell type-specific restriction of *GH* gene expression (**Figure 3-3**). Intriguingly, we do found that both CoREST and LSD1 can interact with N-CoR and SMRT (T. Zhou and M.G.R., unpublished data). Recently, a component of NCoR/SMRT complex (Yoon et al., 2003), JMJD2A has been demonstrated as a histone triMe H3-K9/K36 demethylase (Whetstine et al., 2006), and thus might contribute the *GH* restriction, synergizing with LSD1-dependent histone demethylation. Recently, ZEB2 has been reported to be a substrate for sumoylation by PC2 (Kagey et al., 2003; Long et al., 2005), another component of LSD1/CoREST/CtBP complex (Shi et al., 2003), causing it to lose binding to CtBP and hence its repression function (Long et al., 2005). This is in contrast to the sumoylation of N-CoR, which enhances its corepressor function (Tiefenbach et al., 2006). Here, we have shown that ZEB1 repression function may, instead, require specific sumoylation events, suggesting sumoylation events may contribute to the synergism between LSD1/CoREST/CtBP complex and

NCoR/SMRT complex. Therefore, postpartum induction of ZEB1, LCoR and their recruitment to the *GH* promoter provides a potential molecular mechanism for an estrogen-dependent switch from LSD1-dependent activation to LSD1-dependent repression of the *GH* gene expression, coincident with estrogen-dependent induction of *prolactin* gene expression.

Hence, distinct LSD1 complexes are suggested to act as key modulators, first in the initial *GH* gene activation, and subsequently in cell type-specific *GH* repression events. The switch in LSD1 function from activation to repression is mediated by the temporally-delayed induction of at least two specific components of the LSD1/CoREST/CtBP complex, ZEB1 and LCoR, acting to restrict *GH* expression in *GH*-producing precursor cells as they become *Prl*-producing lactotropes (**Figure 3-3**). The dual functions of LSD1 are likely to control gene expression in development and homeostasis in many tissues.

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