UNIVERSITY NAPOLI "FEDERICO II"

PhD Program

Molecular Pathology and Physiopathology

School of Molecular Medicine
XXVII Cycle

cAMP-PKA regulate the interaction between histone demethylase, LSD1, estrogen receptor-α and the transcription initiation complex

Supervisor
Chiar. mo Prof. Antonio Porcellini

CANDIDATE
Antonia Feola

PhD COORDINATOR
Chiar. mo Prof. Vittorio Enrico Avvedimento

Academic Year 2013-2014

Ai miei genitori

Che, in ogni mia scelta,

Non mi hanno fatto mai mancare

Il loro sostegno e la loro fiducia.

Table of Contents

List of Publication

1. **The inhibition of p85αPI3KSer83 phosphorylation prevents cell proliferation and invasion in prostate cancer cells**. **Feola A**, Cimini A, Migliucci F, Iorio R, Zucchegna C, Rothenberger R, Cito L, Porcellini A, Unteregger G, Tombolini V, Giordano A, Di Domenico M. *J Cell Biochem*. 2013

2. **The p85 regulatory subunit of PI3K mediates cAMP–PKA and insulin biological effects on MCF-7 cell growth and motility.** Di Zazzo E.*, **Feola A**.*, Zuchegna C., Romano A., Donini C.F., Bartollino S., Costagliola C., Frunzio R., Laccetti P., Di Domenico M., Porcellini A. *The Scientific World Journal*. 2014

   * These authors have equally contributed

List of Abbreviations

Abbreviation	Definition
AC	Adenilate Cyclase
AF-1/2	Activation function-1/2
AKAP	PKA-anchoring Proteins
AOL	Amine-oxidase-like
AR	Androgen receptor
ATF1	activating transcription factor
BMP2	Bone morphogenetic protein 2
cAMP	cyclic Adenosine MonoPhosphate
CBP	CREB-binding Protein
CoREST	co-Repressor element silencing factor
CoRNR	Corner
CRE	cAMP-response element
CREB	cAMP-response element-binding
CREM	cAMP-response modulator
DNMT	DNA methyltrasferases
E_2	Estrogen
EOMES	Eomesodermin
ER	Estrogen receptor
ERE	Estrogen Responsive Element
FAD	Flavine-adedine-nucleotide
FOXA2	Forkhead box A2
H3	Histone 3
H3K4	Histone 3 Lysine 4
H3K4me2/3	Histone 3 Lysine 4 di- tri-methylated
H3K9	Histone 3 Lysine 9
H3K9me2/3	Histone 3 Lysine 9 di- tri-methylated
H3T6	Histone 3 Threonine 6
H4	Histone 4
HAT	Histone Acetyltransferase
HDAC	Histone demethylase
HDM	Histone methylase
HMT	Histone methyltransferase
K	Lysine
LBD	Ligand Binding Domain
LSD1	Lysine Specific Demethylase 1
MAPK	Mitogenic Protein Kinase
N-CoR1	Nuclear Receptor co-Repressor1
NuRD	Nucleosome Remodeling Deacetylase
OGG1	8-oxo-guanine DNA glycosylase-1
PDE	3',5'-cyclic nucleotide phosphodiesterases
PI3K	Phosphatidylinositol-3-kinase
PKA	Protein Kinase A
PKC	Protein Kinase c

PKI	Protein Kinase A Inhibitor
PLC	Phospholipase C
R	Arginine
RA	Retinoic acid
RNA Pol II	RNA Polimerase II
Src	proto-oncogenic tyrosine-kinase
SWIRM	Swi3p/Rsc8p/Moira

Abstract

ERα recruits co-activator and transcription factors to the Estrogen Responsive Elements (EREs) to induce transcription. Co-activator complexes facilitate transcriptional activation in part by interacting with chromatin remodeling and histone-modifying enzymes, which render the target chromatin permissive to transcriptional activation. One important protein is LSD1 or KDM1, a flavin adenine dinucleotide-dependent amine oxidase, that catalyzes the removal of methyl groups from di-methylated lysine 4 and lysine 9 in H3 histone, H3K4m2 and H3K9m2, to repress or induce transcription, respectively.

The scope of this study is to determine whether cAMP and protein kinase A (PKA) regulate LSD1 and the initiation transcription complex formation.

Our data demonstrate that cAMP-PKA phosphorylate LSD1 in threonine 110 and stimulate the interaction with active estrogen receptor α. This event is crucial for the assembly of the transcription initiation complex. We have mapped the region in the receptor necessary for this interaction and we have generated a mutant LSD1 that is not able to interact with the receptor and to stimulate estrogen-dependent transcription. PKA phosphorylates LSD1 at the mapped site (threonine 110) *in vitro*, and favors the recruitment of factors required for the transcription initiation complex formation.

These data illustrate the interplay between two major signaling pathways, estrogens and cAMP-PKA, and how they regulate transcription initiation induced by estrogens.

Introduction

Chromatin structure and regulation.

In the eukaryotic cells, the genetic information is packaged into a nuclear structure called chromatin that was first identified by Walther Flemming in 1882 (Flemming W., 1882). Chromatin is the tightly association between negatively charged DNA and positively charge histone proteins. The complex is defined nucleosome and consists of 146 base pairs (bp) of DNA wrapped in 1.75 turns around a histone octamer, all of which is organized as a central tetramer of H3/H4 histones surrounded by two histone H2A/H2B dimers (Figure 1). The DNA between nucleosomes is associated with the histone H1 to obtain the high-order compression of the chromatin. This classical view of chromatin as a simple structural entity has recently been superseded by evidences demonstrating that nucleosome deposition, subunit composition, and post-translational modification can profoundly affect how chromatin function is regulated. Chromatin conformations can be defined as: "euchromatin", with a low degree of compression associated with a relaxed conformation that allows to recognize DNA and regulate transcription by transcription factors (Agalioti T. et al., 2000), and "heterochromatin", with a high degree of compression that can be susceptible to changes in the degree of compression, which is observed during the phases of cell cycle. Epigenetics controls the variations in the structure or the degree of chromatin compression facilitating or preventing access of the transcription factors required for gene expression processes. This kind of regulation occurs via mechanisms that involve DNA methylation and oxidation; and post-translational modification of the N-terminal tails of histone proteins such as methylation, demethylation,

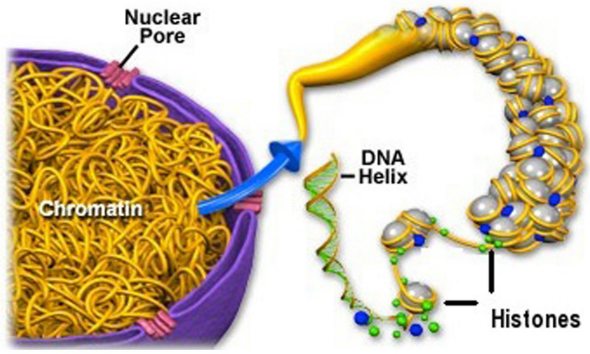

Figure 1. Chromatin structure. DNA in eukaryotic chromosomes is compacted in chromatin. Chromatin appears diffuse in a non-dividing cell and refers to DNA wropped around the histone proteins that form nucleosomes.

http://micro.magnet.fsu.edu/cells/nucleus/chromatin.html

acetylation, deacetylation, biotinylation, ubiquitination, and sumoylation and phosphorylation (Figure 2). Each of these modifications has a characteristic effect on the level of gene expression: the addition of acetyl groups to the histones is frequently related to transcriptional activation; the methylation-demethylation can be associated with transcriptional activation or repression depending on the function of the residue and the degree of methylation (Berger S. L. 2007; Latham J. A. and Dent S.Y. 2007). These modifications play a key role in regulating transcription, contributing to epigenetic memory, and maintaining genome integrity.

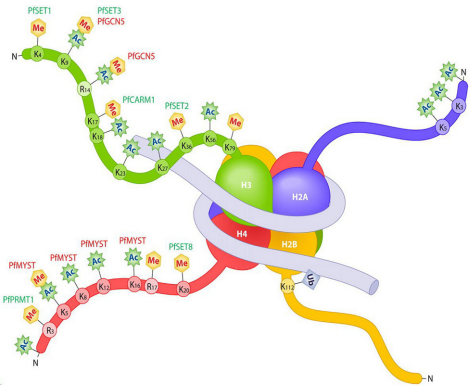

Figure 2. Histone modification. A schematic representation of the histone modifications involved in the regulation of eterocromatin / eucromatin change.
http://www.amsbio.com/Nucleosomes-and-Histone-Proteins.aspx

Histone Methylation.

Histone methylation occurs at the 3-amino group of lysine (K) and the guanidine group of arginine (R) and is catalyzed by enzymes that use S-adenosyl-L-methionine (SAM) as the methyl group donor (Paik W. K. and Kim S., 1971). Histones contain numerous lysine and arginine residues, many of which being methylated in vivo (Zhang Y and Reinberg D., 2001; Margueron R. et al., 2005). Histone methylation is considered as an important modification linked to both transcriptional activation and repression (Margueron R. et al., 2005). Histone methylations of arginine can occur on H3R2, R8, R17 and R26 and H4R3 and can active or repress transcription. Histones H4K20, H3K4, -9, -27, -36, and -79 were studied extensively and linked to transcriptional regulation and DNA damage response (Margueron R. et al., 2005; Martin C. and Zhang Y., 2005). Lysines can be mono-, di-, and tri-methylated (Bannister A. J. and Kouzarides T., 2004), whereas arginines can be both mono-methylated and dimethylated in asymmetrical or symmetrical

manner (Bedford M. T. and Richard S., 2005). In general, methylation at histone H3K4 and H3K36, including di- and trimethylation at these sites, has been linked to actively transcribed genes (Martin and Zhang, 2005). For the methylation at H3K9 there are different point of view: although the H3K9 methylation is considered a repressive mark for euchromatic genes (Nielsen S. J. et al., 2001), the H3K9 trimethylation (H3K9Me3) is shown associated with actively transcribed genes (Vakoc C. R. et al., 2005). All these differentially methylated lysine residues may serve as docking sites for different effector proteins and/or platforms for chromatin modifiers that perform "writer" and "reader" functions and act in a spatially and temporarily coordinated manner (Ruthenburg A. J. et al. 2007; Taverna S. D. et al. 2007). "Writers" usually have subunits harboring catalytic activity that add or remove marks on histones, while "Readers" often are characterized by domains that specifically recognize and bind to these marks (Ruthenburg A. J. et al. 2007). These chromatin-regulating complexes include histone acetyltransferases (HATs), DNA methyltransferases (DNMTs), histone deacetylases (HDACs), histone methyl-transferases (HMTs) and histone demethylases (HDMs) that act in a stepwise and/or combinatorial manner and engage in extensive cross talk.

Lysine Specific Demethylase: LSD1 or KDM1

In the last ten years with the development of new technologies for the analysis of the histone methylation pattern, the hypothesis that the link between cellular phenotypes and methylation-demethylation pattern is part of "normal" cell development and/or insurgence of different pathologies, such as cancers is making its way. Until 2004 histone methylation was considered a stable and irreversible mark of chromatin. In that year, the description of the action mechanism of the first enzyme endowed with lysine specific demethylase activity, opened new research outlook on these proteins and their association with epigenetic regulatory mechanisms, breaking the paradigm of irreversible methylation (Shi Y. et al., 2004).

LSD1/KDM1A (below named LSD1) is the first histone demethylase characterized, a nuclear amine oxidase as part of a multiprotein corepressor complex that contains both histone deacetylase-1 or -2 and demethylase activities (Shi Y. et al., 2004).

LSD1 consists of three major domains: an N-terminal SWIRM (Swi3p/ Rsc8p/Moira) domain (Yoneyama M. et al., 2007), a C-terminal AOL (amine oxidase-like) domain, and a central protruding Tower domain (Stavropoulos P. et al., 2006).

The C-terminal domain has high sequence homology to FAD-dependent polyamine oxidases family (Da G. et al., 2006; Qian C. et al., 2005). The AOL domain of LSD1 contains two sub-domains, a FAD-binding sub-domain and a substrate-binding sub-domain (Figure 3A). The interface of the two sub-domains forms a large cavity, in which catalytic center is localized. The N-terminal SWIRM domain reveals a compact helix-turn-helix-related fold found in several chromatin-associated proteins (Qian C. et al., 2005). Although some studies reveal that the SWIRM domains bind and anchor DNA and present their associated protein or protein complexes to nucleosomal substrates, the precise function of the LSD1 SWIRM domain is still unknown (Yoneyama M. et al., 2007). Additionally, the LSD1 SWIRM domain makes close interactions with the amine oxidase domain, forming a highly conserved cleft, that serve as an additional site to bind histones (Metzger E. et al., 2005). The Tower domain forms a long helix-turn-helix structure, into the AOL domain, and offers a surface for the interaction to protein co-repressor element silencing factor CoREST (Figure 3 B). CoREST binds LSD1 and modulates its activity. Biochemical studies have demonstrated that the demethylase activity of LSD1 on H3K4 is regulated by the association with CoREST (Lee M. G. et al., 2005). The C-terminal region of CoREST, which contains the SANT2 domain, confers to LSD1 the ability to demethylate nucleosomal substrates. Moreover, the co-crystal structure of the LSD1-CoREST complex displays that the region between the SANT1 and the SANT2 domains, in the C-terminal region of CoREST, sorrounds the LSD1 Tower domain with the SANT2 domain resting on the tip of the tower (Yang M. et al., 2006). The overall structure appears as

anchor to lock the complex into the nucleosomal surface. The main and well-characterized function of LSD1 is the catalyzation of the histone H3-Lys4 mono- and di-methylation (Forneris F. et al., 2005), even though after androgen stimulation the activity of LSD1 on H3K9 has also been reported (Metzger E. et al., 2005) (Figure 3C).

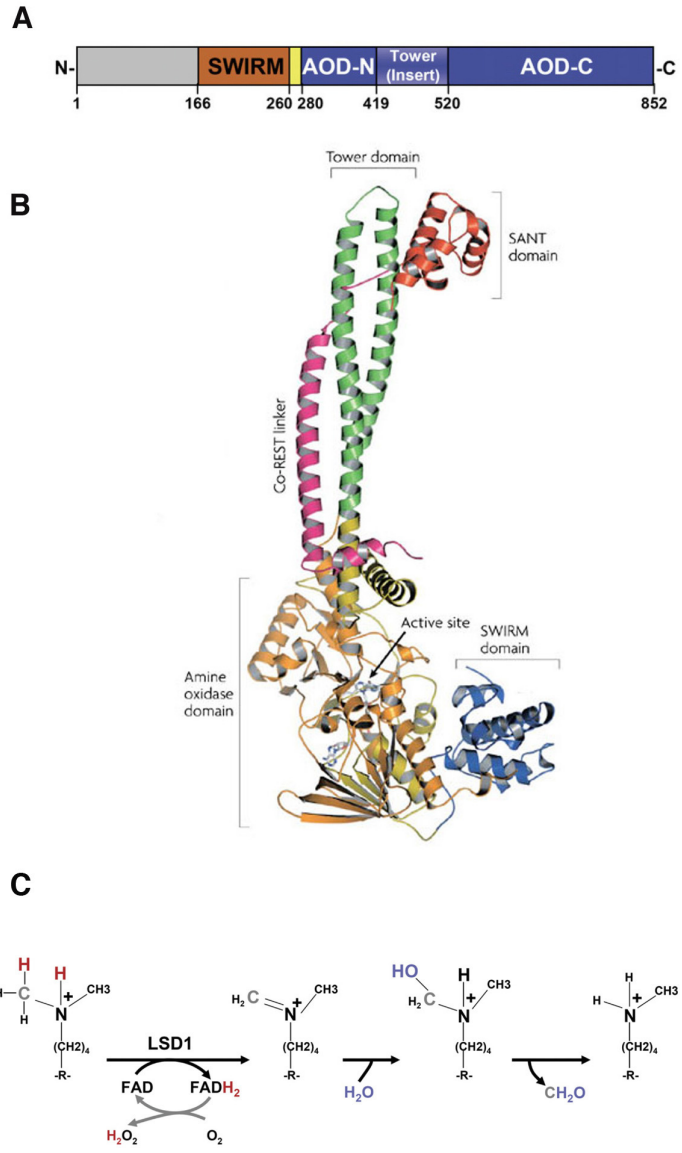

Figure 3. LSD1 Schematic and tridimentional structures and reaction. The panel A shows the schematic diagrams of LSD1 domains. LSD1 domains are indicated by different colors. AOD stands for amine oxidase domain. The 3D structures represented in the panel B are taken from two recent studies (Stavropoulos et al., 2006; Yang et al., 2006). LSD1 alone demethylates H3K4me1/Me2 (Shi et al., 2004). Co-REST interacts with the tower/insert region of LSD1 (lower interaction). The reaction mechanism, adapted from Shi et al. (2004), depicts LSD1 removing a methyl group from a mono- and di-methylated lysine residue, generating an unmethylated lysine (C). Modified from Shi Y. et al., 2006

Histone demethylase role in development and stem cell function.

LSD1 is considered to play cardinal functions in the formation and development of organs and tissues such as heart, brain and skeletal muscle. Its essential role in cellular energy expenditure, inflammatory responses and hematopoiesis is described. In human LSD1 is encoded by a single gene, KDM1A, located at the chromosome 1 p36.12. Conditional knockout of LSD1 in embryonic stem (ES) cells causes embryonic lethality at approximately day 6 (Foster C. T. et al., 2010). Mice embryos, in which the LSD1 expression is absent, have dimensions reduced compared to heterozygous controls suggesting a block to development shortly after implantation. The aberrant developmental program in these mice leads to embryonic lethality. LSD1 knockdown by shRNA, in human ES cells, causes a partial cell arrest in the G0/G1 phase with decreased growth rate and up-regulation of genes involved in the development processes such as FOXA2 (forkhead box A2), EOMES (eomesodermin), BMP2 (bone morphogenetic protein 2) and SOX17 (Adamo A. et al., 2011). LSD1 orthologs in *Drosophila melanogaster* and *Caenorhabdis elegans* are expressed in the germline. Inactivation of the *Drosophila melanogaster* ortholog of LSD1, Su(var)3–3 (suppressor of variegation 3–3), leads to a global reduction of H3K4me2/me1 and H3K9me levels in heterochromatic regions that could be the reason of sterility and tissue defects. The *Caenorhabdis elegans* SPR-5 (suppressor of presenilin defect) protein has also been implicated in the control of H3K4me2 levels in the germline, in fact increased levels of H3K4me2 are observed only in late generations, corroborating the ideas that LSD1 orthologs regulates the genes expression during spermatogenesis (reviewed in Amente S. et al., 2013). Together, these observations suggest a central role for H3K9 demethylation activity of LSD1 in the early development and maintaining stem cell function.

Histone demethylation leads to DNA oxidation

LSD1 belongs to flavin-dependent amine oxidase family that typically catalyzes the oxidation of an amine-containing substrate using molecular oxygen as the electron acceptor (Binda C. et al., 2002; Shi Y. et al., 2004; Forneris F. et al., 2005). The amino group of the methylated Lys is oxidized, this generates the corresponding imine compound, which is subsequently hydrolyzed, the final product is formaldehyde. The oxidation reaction leads to the reduction of the two-electron hold in protein-bound FAD cofactor, which is regenerated to its oxidized form by molecular oxygen to produce hydrogen peroxide.

Recently, Perillo et al. have shown that H_2O_2 produced during LSD1 mediated demethylation at estrogen receptor (ER) target genes results in production of 8-oxo-guanine lesions. This DNA damage event mediates the recruitment of the 8-oxo-guanine DNA glycosylase-1 (OGG1) and topoisomerase IIb repair enzymes at the regulatory regions of the gene. Interestingly, the single stranded breaks induced during the DNA repair process is required for estrogen-dependent genes transcription because may facilitate DNA bending permitting more efficient RNA Pol II loading onto the promoter during gene activation (Perillo B. et al., 2008) (Figure 4).

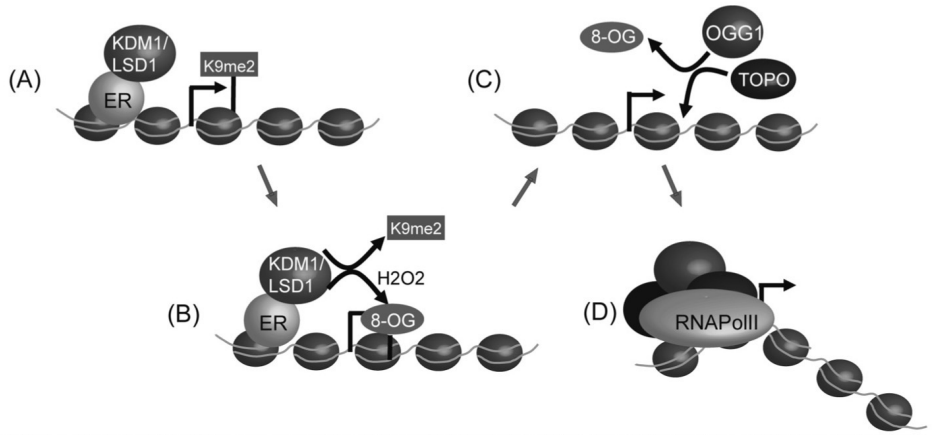

Figure 4. H2O2 produced by LSD1 contributes to transcriptional activation. (A) During estrogen-dependent transcriptional activation, LSD1 demethylates H3K9me2. (B) A reaction product is hydrogen peroxide (H2O2) which is reactive and causes 8-oxo-guanine (8-OG) lesions on DNA. (C) 8-OG is targeted by the 8-oxo-guanine DNA glycosylase-1 (OGG1). This repair process leads to single stranded DNA nicks that are a substrate for topoisomersase IIb (TOPO). Recruitment of topoisomerase IIb leads to alterations in DNA architecture. (D) Changes in DNA architecture may aid in RNApolII loading onto target genes by promoting chromatin accessibility and therefore contribute to transcriptional activation.
Image taken from S. S. Ng et al., 2008.

LSD1 is a transcriptional co-repressor or co-activator?

A key issue is to define the biological properties of LSD1, especially in relation to its ability to induce or repress the gene transcription. First of all, LSD1 is a cofactor of several molecular complexes, including CoREST and NuRD, acting as co-repressor, and consistent with this role in transcription repression, LSD1 demethylates monomethyl and dimethyl histone H3 lysine 4 (H3K4me1 and H3K4me2). Secondly, LSD1 was described with androgen (AR) or estrogen (ER) nuclear receptors acting as co-activator (Shi Y. et al., 200). During androgen receptor (AR)-activated gene expression, LSD1 removes mono- and dimethyl marks from H3K4me1/me2 to H3K9me1/ (Gargia-Basserts I. et al., 2007), promoting gene transcription (Figure 5). The key mechanisms that control this dual specificity of demethylation is the phosphorylation of histone H3 at threonine 6 (H3T6) by protein kinase C beta I that inhibit LSD1 demethylating H3K4 during AR-dependent gene activation. Moreover, after androgen treatment, protein kinase C is recruited to AR target promoters and phosphorylates H3T6. This modification switches LSD1 H3K4 demethylating activity from H3K4me2 to H3K9me1 and H3K9me2 (Imhof A. et al., 2010).

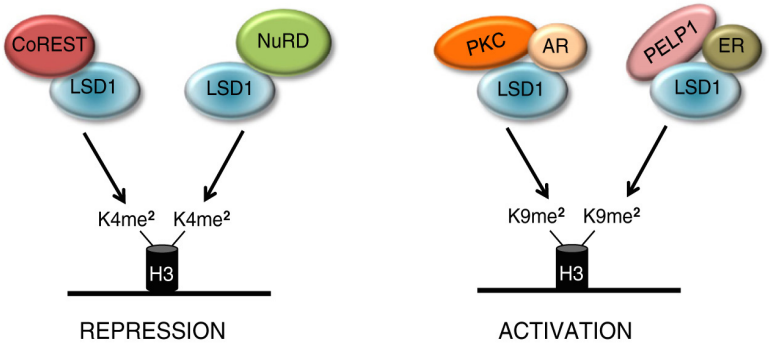

Figure 5. LSD1 as co-activator and co-repressor. LSD1 as part of the Co-REST complexes contributes to repression of neuronal genes in non-neuronal cells. LSD1 contributes to repression by removing H3K4 methylation. When bound to the androgen receptor (AR), LSD1 is converted from a transcriptional repressor to an activator by changing the substrate specificity of LSD1 so that it catalyses the removal of H3K9 methylation.
Modified from Amente S. et al., 2013

In addition, LSD1 is a chromatin-modifying enzyme, which serves as a docking module for the stabilization of the associated corepressor complexes on chromatin and is finely tuned and highly specific. Although the Lys4 is the main site of LSD1 oxidative action, the enzyme is also sensible to covalent modifications on neighboring residues. The histone modifications, in fact, are

important signature for the activity of LSD1. Lys9 acetylation affects enzyme catalysis, whereas phosphorylation of Ser10 totally abolishes its activity. LSD1 does not have a strong preference for mono- or dimethylated H3K4, it binds H3 independently of Lys4 methylation. The fact that LSD1 acts with similar efficiency on mono- and dimethylated substrates indicates that in vivo it works resetting H3K4 to its demethylated state.

The cascades of events that lead to transcriptional activation/repression should be:

- Phosphorylation/Dephosphorylation of Ser10 by a Kinase/phosphatase;
- Lys9 deacetylation by HDAC1/2;
- H3 demethylation catalyzed by LSD1 (reviewed by Forneris F. et al., 2005).

Role of transcription factors in LSD1 gene-recruitment

The regulation of gene expression within euchromatin requires the delivery of chromatin-modifying enzymes by DNA-bound transcription factors. Following a stimulus, transcription factors bind to their gene-promoter regions and induce a cascade of modification events that result in the expression or silencing of the gene. Nuclear receptors regulated transcription by binding ligands to the C-terminal domain, this causes conformational changes, that include a change in the position of the so-called AF2 helix. The shift of AF2 helix favors the nuclear receptor association with specific coactivator complexes, converting of the receptor into a transcription activator (reviewed in Rosenfeld et al., 2006). Thus, when nuclear receptors, such as the thyroid hormone (T3) and the retinoid acid (RA) receptors, lack of ligand, act as repressors recruiting specific corepressor complexes via the "CoRNR" domain (Horlein et al., 1995; Chen J. D. and Evans R. M., 1995; Heinzel T. et al., 1997), whereas, in the presence of ligand, they are functionally converted to activators by recruiting coactivator complexes. Nowadays there are many evidences that linked between LSD1 and the ERa-mediated gene activation program. One of these are the discovery that approximately 58% of ERa+ promoters also exhibiting LSD1 recruitment. The 80% of the 4200 LSD1-

positive promoters were associated with RNA polymerase II and gene activation (Garcia-Bassets I et al., 2007). Recently it is shown that LSD1 is an essential mediator of the interchromosomal interactions necessary for E2-dependent ERα-mediated transcription (Hu Q. et al., 2008). In particular, Julie A. Pollock et al. have elucidate that the physical presence of LSD1 both as a scaffolding protein, and its demethylase enzymatic activity on the ERE region, was important for ERα-regulated transcription, in fact E_2 treatment induces the recruitment of ERα and LSD1 to ERE of pS2, and influences the methylation status of histones at or close to the pS2 EREs (Pollock J. A. et al., 2012). Therefore, it is of particular interest to further explore the linkage between the recruitment of nuclear receptors and the coregulatory complexes that underlie ligand-dependent activation of transcriptional programs.

Estrogen receptors: "genomic" and "non-genomic" actions.

Steroid hormones, such as 17β-estradiol (E2), play pivotal roles in the regulation of sexual development and fertility in both males and females (Couse J. F. and Korach K. S., 1999; Nef S. and Parada L. F., 2000). Estrogens also regulate metabolic processes in fat, liver, and bone tissues (DeCherney A., 1993; Vaananen H. K. and Harkonen P. L., 1996). In addition, estrogens, not only influence different disease states, for example, cancers (e.g., breast, uterine) causing hormone-dependent growth and proliferation (Foster K.R., Ratnieks F. L., 2001; Prall O. W. et al., 1998), but also important physiological/pathological processes, such as inflammation, cellular differentiation, cardiovascular integrity and immunity. Estrogen elicits their actions through ER proteins. ERs exist as two isoforms, ERα and ERβ, with different functions and tissue expressions, they are members of a conserved superfamily of nuclear receptors that have the same conservative structure (Mangelsdorf D. J. et al., 1995).

ERα, like the other nuclear receptors, contains two C4-type zinc fingers and binds as a dimer to palindromic sequences known as estrogen response elements (ERE) (Schwabe J. W. R. et al., 1993). The ligand-binding domain

(LBD) is encoded within a region of about 300 amino acids and is bound by estrogens and anti-estrogens (Tanenbaum D. M. et al., 1998). The LBD also contains a ligand-activated transcription activation function, AF-2, as well as sequences required for ligand-dependent dimerization. The N-terminal 180 amino acids contain transcription activation function AF-1. Extensive studies have shown that AF-1 and AF-2 can act both independently and synergistically in a promoter- and cell-specific manner (Tsai M. J. and O'Malley B. W., 1994). A large number of studies have described the mechanisms underlying the inhibition of ERα activity by partial anti-estrogens such as tamoxifen and pure antagonists such as ICI 182, 780. These studies have shown that the activity of tamoxifen results in the inhibition of AF-2, whereas ICI 182, 780 prevents the activation both AF-1 and AF-2, increasing its turnover and causing the disruption of the estrogen receptor nucleo-cytoplasmic shuttling (Dauvois S. et al., 1992; Dauvois S. et al., 1993).

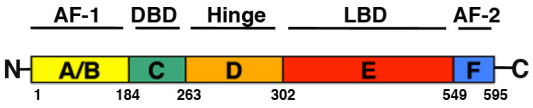

Figure 6. Schematic representation of estrogen receptor. With letters are represented the five domains: AF stands for activation function; DBD stands for DNA Binding Domain; Hinge for the hinge region; LBD for Ligand Binding Domain.

Mutational analysis and crystallographic studies have defined a region at the C-terminus of the ERα, LBD, originally referred to as AF-2 activating helix (AH) and now known to form part of an amphipathic helix, helix 12, of the LBD, which is essential for ligand-dependent transcriptional activity. Ligand binding results in the realignment of helix 12, inducing co-regulators to associate (Figure 6). Determination of the LBD structures for a number of other nuclear receptors indicates that ligand-induced H12 realignment is a common feature of the nuclear receptor LBD (Moras D. and Gronemeyer H., 1998).

The "genomic" pathway of estrogen consists in the activation of estrogen receptors after hormone stimulation; ERs dissociate from nuclear chaperone proteins, dimerize, and bind to DNA at specific sequences known as estrogen response elements (EREs), modulating the estrogen-dependent transcription of responsive genes (Deroo B. J. and Korach K. S., 2006).

In addition to the "genomic" pathway, it is recently described an alternative mechanism of action, "non-genomic", that is faster that the genomic one, and by which estrogen control the cell cycle progression, cell survival and cell migration. Some of these effects are mediated by estrogen receptors, but most of them are dependent by the activation of cellular kinases, that are the proto-oncogenic tyrosine-kinase (Src), the phosphatidylinositol-3-kinase (PI3K), the mitogenic protein kinase (MAPK), the protein kinase A (PKA) and C (PKC) through G protein-coupled receptors (GPCRs) or ionic channel (Castoria G. et al., 2008). All these signaling pathways culminate, depending on the cell context, in differentiated effects of steroid hormones, such as proliferation, survival, migration and differentiation, through the activation of several gene expression programs (Figure 7).

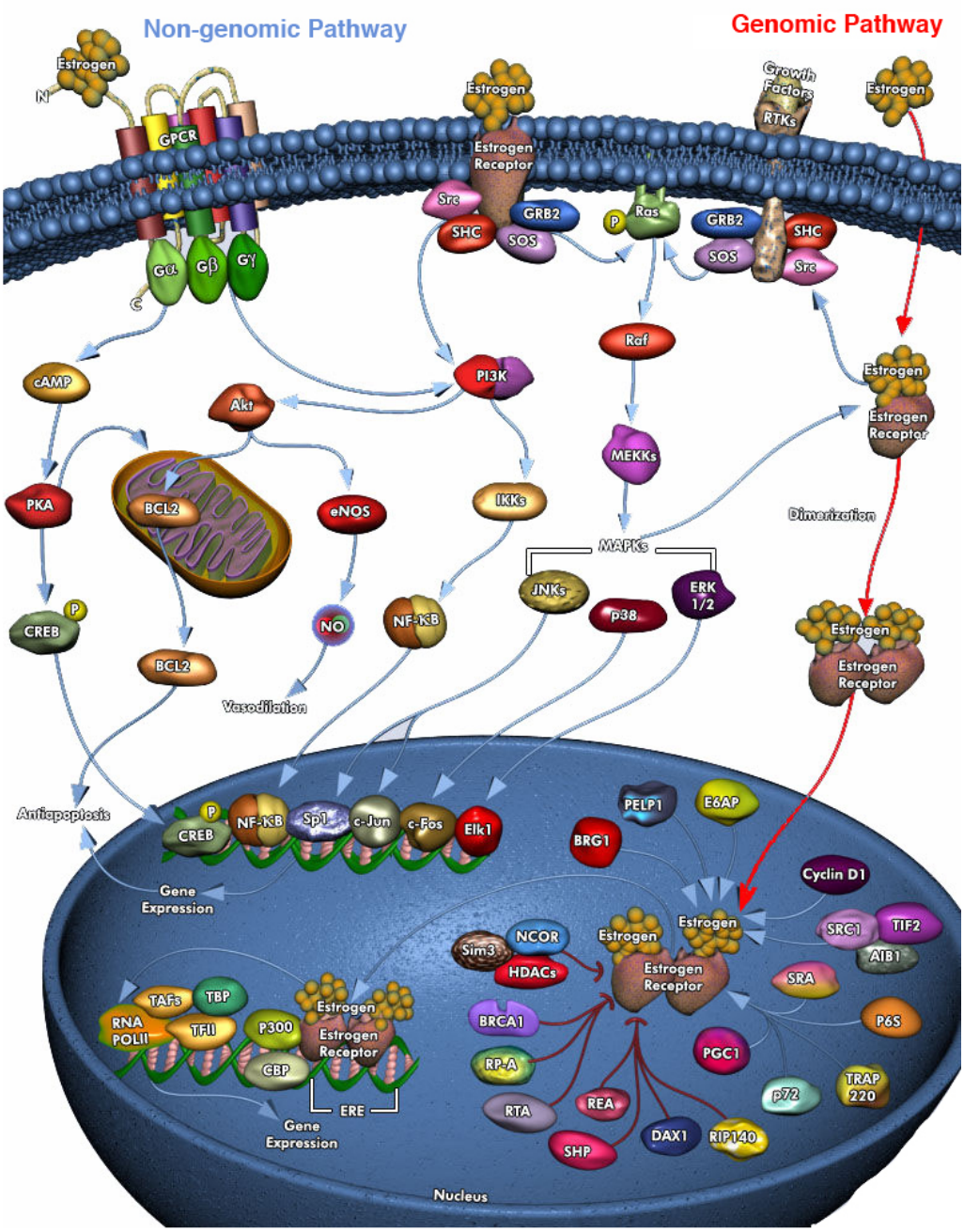

Figure 7. Genomic and non-genomic pathways of estrogen receptor. In the left part of the image, the schematic representation of the different signaling pathways activated by estrogen receptor via non-genomic is present (blue line), while in the right side the genomic pathway is depicted (red line). Modified from QIAGEN Sample and Assay Technologies.

cAMP/PKA signaling pathway

The cyclic adenosine monophosphate (cAMP) is an important intracellular signaling molecule, which acting as second messengers between extracellular stimuli such as hormones, elicits intracellular response. While the specific function of a given signal varies according to the cell type and the extracellular environment, stimulus activating the signal, generally activates the cyclase enzyme with the formation of the cyclic nucleotide (cNT). This, in turn affects the activity of downstream effectors including kinases, ion channels, transcription factors, and scaffolding proteins. Among these, PKA play an important role in different cellular processes, for example negative regulator of cAMP signaling, mediator of anti-apoptotic and pro-apoptotic cascades, etc. etc. (Rehmann, H. et al., 2007; Insel, P. A. et al., 2012).

The holoenzyme, PKA, is a tetramer consisting of two regulatory subunits (R) and two catalytic subunits (C): the formers contain two binding sites for cAMP, and upon its binding, the latters activate their substrates by phosphorylation (Gerits, N. et al., 2008). The catalytic activity of the C subunit is decreased by a protein kinase inhibitor (PKI) that can also acts as a chaperone and promotes nuclear export of the C subunit, decreasing nuclear functions of PKA. PKA-anchoring proteins (AKAPs) provide specificity in cAMP signal transduction by making closer interaction between PKA, specific effectors and substrates. They can also target PKA to particular subcellular locations and anchor it to ACs (for immediate local activation of PKA) or PDEs (to create local negative feedback loops for signal termination) (Wong and Scott 2004). A large number of cytosolic and nuclear proteins have been identified as substrates for PKA (Tasken et al., 1997). PKA phosphorylates numerous metabolic enzymes, such as glycogen synthase and phosphorylase kinase that inhibits glycogen synthesis and promotes glycogenolysis. It can phosphorylate also acetyl CoA carboxylase, which inhibits lipid synthesis. PKA regulates other signaling pathways too. For example, it inactivates phospholipase C (PLC) b2, while it activates MAP kinases through phosphorylation respectively. The activities of Raf and Rho can be decreased

PKA too, and it modulates ion channel permeability. In addition, it regulates the expression and activity of various ACs and PDEs, modulating itself its activity. PKA transcription regulation is mainly got to the direct phosphorylation of the transcription factors cAMP-response element-binding protein (CREB) and cAMP-responsive modulator (CREM) (Rehmann H. et al., 2007). Phosphorylation allows these proteins, once activated, to interact with the transcriptional coactivators CREB-binding protein (CBP) and p300 when bound to cAMP-response elements (CREs) of target genes (Mayr B. and Montminy M., 2001). The CREM gene encodes the cAMP-induced transcription repressor ICER, which makes a negative feedback on itself (Sassone-Corsi P., 1995). PKA can also influence the activity of other transcription factors, including some nuclear receptors (Figure 8).

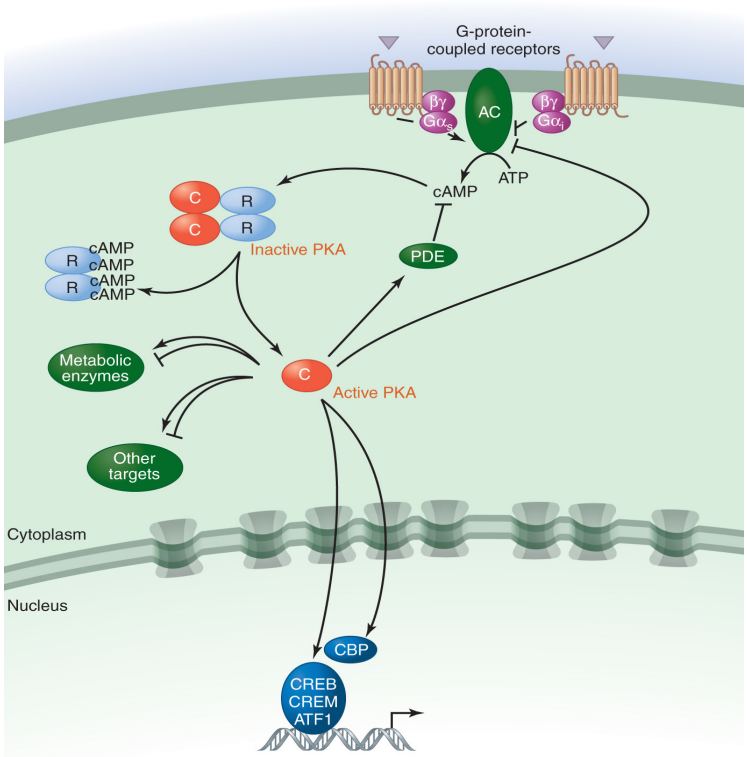

Figure 8. cAMP/PKA signaling pathway. Stimulus activating the signal, generally activates the Adenilate Cyclase enzyme (AC) that in turn produces cAMP. This affects the activity of downstream effectors including PKA.The catalytic C subunit is relised and acts phosphorylating several substrate. Among these CREB is the weel-characterized one. PDEs create local negative feedback loops for signal termination.
Taken from Sassone-Corsi P. 2015

Interplay between estrogen and cAMP/PKA transduction pathways in transcription factors activation

At this time it is evident that estradiol increases the intracellular cAMP levels both *"in vitro"* and *"in vivo"* (Szego C. and Davis J., 1967*;* Nakhla A. et al., 1994). It in turn induces adenylyl cyclase activation and stimulates cAMP response element (CRE)-mediated gene expression (Aronica S. M. et al., 1994). There is data that report a nonclassical effect of estrogen on the expression of different types of genes through cAMP-mediated mechanisms (Szego E'va M. et al., 2006; Hously M. D. Kolch W., 2000; Liu D. et al., 2009) Steroid hormones exert dramatic effects on neuronal expression of genes; in fact promote transcription of neurotensin/neuromedin (NT/N) by interactions with the cAMP cascade in a neuronal cell line, SK-N-SH, and in a mouse model (Watters J. J. and Dorsa D. M., 1998). Furthermore, cAMP/PKA signaling increases estrogen relate receptor α phosphorylation and nuclear localization, recruitment to the SP-A promoter, and interaction with PKAcat and SRC-2, resulting in the up-regulation of SP-A gene transcription (Liu D. et al., 2009).

Moreover, cAMP is involved in resistance to steroid antagonists through the activation of the steroid receptor coactivator-1 (SRC-1) (Bai W. et al., 1997; Rowan B. G. and O' Malley B. W. 2000).

All these observations highlight a role for cAMP/PKA-dependent pathway in the estradiol regulation of transcription. This "cross-talk" may represent a more generalized mechanism by which steroid hormones act through other signal transduction cascades to regulate the gene expression.

Aim of the study

Estrogens play important roles in the regulation of sexual development, fertility, metabolic processes and disease states. The biological effects of estrogens are mediated by the interaction with two intracellular estrogen receptors, ERα and ERβ. Upon interaction with ERs, estrogens induce a conformational change of the receptor, which favors receptor dimerization and recruitment to promoter elements either directly, through their DNA-binding domain or indirectly, through interaction with other transcription factors. ER complexes then recruit transcriptional co-regulators (co-activators and co-repressors) to increase or inhibit target gene transcription. Many transcriptional co-regulators of nuclear receptors exhibit enzymatic activities that participate in their mechanism of action, such as acetyltransferases (HATs) or histone deacetylases (HDACs), and methyltransferases. All these enzymes may act as regulators of gene expression effecting post-translational modifications at the N-terminal tails of histones.

Recently, LSD1 has been shown to play a role in transcription induced by androgens, estrogens or Myc (Metzger E. et al., 2005; Perillo B. et al. 2008; Amente S. et al., 2010). Mono aminoxidase activity of the enzyme is essential for the formation of the transcription initiation complex induced by androgens, estrogens and Myc. It is known that LSD1 demethylates selectively H3K4me2 "*in vitro*", while "*in vivo*" it binds the androgen or estrogen receptors and favors demethylation H3K9me2 (Shi Y. et al., 2004; Metzger E. et al., 2005). Notwithstanding, the mechanism by which the enzyme is recruited to the ERE sequences and activates the estrogen-dependent gene transcription, remains still elusive. Estrogens, through their non-genomic pathway, can activate many different signaling pathways, such as cAMP/PKA pathway, that in turn activate several gene transcription programs. During their transit from cytoplasm to nucleus, estrogen receptor genomic and the non-genomic actions converge in one or more points.

Our aim is to study both *"in vitro"* and *"in vivo"* the mechanism that regulates the recruitment of LSD1 to the ERE region following estrogen stimulation. We have focused our attention on the role cAMP/PKA-mediated

association of LSD1 with the transcriptional initiation complex and on the mechanism of induction of transcription of the estrogen-dependent genes.

We wish to demonstrate that modification of LSD1 at a specific site on the N-terminal domain facilitates the assembly of the transcription initiation complex driven by the estrogen receptor. We will analyze the recruitment of several co-factors, including the large subunit of RNA polymerase II, the single strand binding protein RPA, and the catalytic subunit of PKA.

We wish to identify the critical elements that regulate estrogen induction of transcription of target genes.

Material and Methods

Materials and Methods

Cell cultures, treatments and transfection

Human breast cancer MCF-7 cells were grown at 37°C in 5% CO2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with phenol red, L-glutamine (2 mM), insulin (10 μg/ml), hydrocortisone (3.75 ng/ml), and 10% fetal bovine serum (FBS, Invitrogen, Rockville, MD, USA). Cells were provided with fresh medium every 3 days. To evaluate the effect of specific treatment challenge, cells were grown in phenol red-free DMEM containing 10% dextran–charcoal-stripped FBS for 3 days, and then treated according to the experimental condition with 10 E_2 (Sigma-Aldrich, Co., St. Louis, MO, USA), 1 μM ICI 182.780 (Sigma-Aldrich, Co., St. Louis, MO, USA), 10 μg/ml PKA inhibitor P9115 (PKI) (Sigma-Aldrich, Co., St. Louis, MO, USA), 10 μM H89 B1427 (Sigma-Aldrich, Co., St. Louis, MO, USA), 10 μg/ml PKC inhibitor (PKCi) (Chalbiochem), 100 μM 8-Br-cAMP (Sigma-Aldrich, Co., St. Louis, MO, USA), 40 μM Foskiln (Sigma-Aldrich, Co., St. Louis, MO, USA).

To obtain LSD1 overexpression, MCF-7 cells were transiently transfected with plasmids carrying LSD1wt tagged with FLAG (p3xFLAG-pLSD1wt), or its T110A mutated form, LSD1-Ala (p3xFLAG-pAla) (Amente S. et al., 2010), using Neon^R Transfection System (Life Technologies) with the following settings: 1100V, 30ms and 2 pulses.

In all transfections, pEGFPC3 plasmid was included to determine and normalize transfection efficiency through FASC analyses. Experiments varying in the transfection efficiency above 20% were discarded. All data used derived from experiments in which transfection efficiency was greater than 55%.

co-Immunoprecipitation and Western Blot

MCF7 cells, transfected or not with LSDwt and mutant, were lysed with Lysis Buffer containing Tris-HCl (50 mM), EDTA (1mM), TRITON (1%), NaCl (150 mM), MgCl2 (5mM), EGTA (1mM), 1x protease inhibitor, 1x

PMSF and 1x Na_3VO_4. Lysates, clarified by centrifugation at 12000g for 30' at 4°C, were co-immunoprecipitated with antibodies against LSD1 (sc-67272), FLAG tag M1 (A-4596) or normal IgG (as control), 1μg of antibody each mg of total proteins, accordingly to the specific experimental needs. In the former case, the anti-LSD1 co-immunoprecipitation was performed in Lysis Buffer, while in the latter case, anti-FLAG tag co-immunoprecipitation, 1mM $CaCl_2$ was added to the same Buffer. The sample were separated by SDS-PAGE and subjected to western blot. The nitrocellulose membranes were immunoblotted with antibodies against LSD1, P-(Ser/Thr)PKA substrate, P-CREB, GAPDH, PKA, RPA, RNA Pol II, NCoR1, H1 at the dilution of 1:1000 in 3% BSA in T-TBS (0,1% tween in TBS) over night and antibodies against ER-α at the dilution of 1:5000 in 3% milk in T-TBS. Antibodies for the detection of ERα (sc-543 and sc-8005), PKA (sc-903), P-CREB (sc-101663), GAPDH (sc-59540), H1 (sc-393358), and LSD1 (sc-271720) were purchased from Santa Cruz Biotechnology; RNA polymerase II antibody (05623) was obtained from Upstate Biotechnology; RPA antibody (A303-874A) was from Bethyl Laboratories; anti-flag antibody (F-3136) was from Sigma Aldrich; P-(Ser/Thr) PKA subustrate antibody was from Cell-Signaling Technology (9621); finally fluorescein isothiocyanate anti-rabbit and texas red anti-mouse were from Jackson ImmunoResearch Laboratories, Inc (UK). Image analysis for all gels was performed with ImageJ software using the "Gel Plot" plug-in.

GST-ERα fusion proteins preparation

The DH5α bacteria cells expressing the following recombinant GST-ERα fusion proteins, GST-ERα wild type (Heg0); GST-ERα deleted of 1-280 amino acids (Heg14); GST-ERα deleted of 281-595 amino acids (Heg15); GST-ERα deleted of 1-241 amino acids (Heg241-595) (Abbondanza C. et al. 1998), were grown over night at 37°C. The day after each culture was diluted 1:10 and grown until OD 0.5_{595nm}, then 1mM IPTG was added to induce the expression of the recombinant proteins for 2h (see Figure 10 B).

Cells were lysed through freeze and thaw technique for three times in KCl 15mM; PBS 1x; N-laurosylsarcosine 1.5%; Triton 0.5%; DTT 5mM; PMSF 1mM; 1x proteases inhibitor. The lysates, purified by centrifugation for 10' at 14000g at 4°C, were incubated with Sefarose beads for 2h at 4°C. The GST-ERα fusion proteins, collected by centrifugation at 1700g for 2', were washed four times with PBS 1x. To test the fusion, the recombinant fusion proteins were subjected to SDS-PAGE and stained with Comassie blue.

Pull-down assay.

MCF7 Cells were lysed as above described. The cell extracts (2mg) were incubated, for 2h at 4°C with gentle rock agitation, with 2 μg of GST-Heg0, GST-Heg14, GST-Heg15, GST-Heg241 constructs respectively, in the presence or in the absence of 10nM estradiol. The pellets were washed in lyses buffer twice, and once in the lysis buffer with 200 mM NaCl and then processed for Western blot analysis as above described.

Confocal microscopy of ERα-LSD1.

MCF-7 cells, grown on glass slides, were hormone starved for 3 days, treated with E2 for the indicated times. Cells on coverslips were washed once with PBS, fixed for 20 min with paraformaldehyde (3%, w/v in PBS), permeabilized for 20 minutes with Triton X-100 (0.2%, v/v in PBS) and incubated for 1 h with PBS containing FCS (1%, v/v). For ER-α detection, coverslips were stained by incubation with anti-ER-α antibody diluted 1:100 in PBS for 1 h followed by three washings with PBS. Coverslips were then incubated in fluorescein isothiocyanate anti-rabbit (Jackson ImmunoResearch Laboratories, Inc, UK) diluted 1:200 in PBS. For LSD-1 detection, coverslips were stained by incubation with anti-LSD1 antibody diluted 1:100 in PBS for 1 h followed by three washings with PBS. Coverslips were then incubated in texas red anti-mouse (Jackson ImmunoResearch Laboratories, Inc, UK) diluted 1:200 in PBS. All coverslips were washed three times in PBS, incubated for 10

min with PBS containing Hoechst 33258 (Sigma) at a final concentration of 1 mg/ml and finally washed three times with PBS. The coverslips were inverted and mounted in Moviol (Calbiochem, CA) on glass slides. All images were captured with Zeiss confocal microscope 510. The microphotographs were analyzed with ImageJ software using the colocalization and colocalization finder plug-insand the Pearson's coefficient was calculated for each experimental point.

In vitro kinase assay

For LSD1 *in vitro* kinase assay, MCF7 cells transfected with pLSD1wt and pLSD1-Ala, were lysated with Lysis Buffer and lysates were incubated with anti-FLAG antibodies (Sigma A4596) in the presence of 1mM $CaCl_2$ for 2h at 4°C. Beads were washed two times with lysis buffer and then with kinase buffer (Hepes 50mM, $MgCl_2$ 1mM, DTT 1mM). For each sample the reaction was carried out in 50μl of kinase reaction mixture containing or not 50ng of PKA catalytic subunit (P2645, Sigma) and 25μM ATP with γP^{32}-ATP (3mC_i/mM), in the presence or in the absence of 1μM PKI (P0300, Sigma), and in the presence or in the absence of 25μM PKC inhibitor (476480 Calbiochem). After 5 minutes of incubation at 30°C, the immunoprecipitated proteins were eluited first with a buffer containing 2mM EDTA, 400mM $NaCl_2$, 200mM Tris-HCl pH 7.4, and then with glycine 0,1M pH 3. After that, sample buffer (2x) was added to the eluates followed by boiling for 5 min. The samples were subjected to SDS-PAGE and Western-Blot and finally the nitrocellulose membrane were exposed to light sensitive film.

RNA isolation and qRT-PCR.

Total RNA, from MCF7 cells transfected with LSD1wt and mutant, was extracted using TRIzol Reagent (Gibco/Invitrogen) following the manufacturer's instructions. cDNA was prepared from 1μg of total RNA with 100 U of Superscript III Reverse Transcriptase (Invitrogen) and 2 μl random

hexamer (20 ng/µl) (Invitrogen), the reverse-transcribed was carried out for 1 h at 50 °C, and the reaction was heat inactivated for 15 min at 70 °C. The cDNA products were stored at -20 °C until use. Each sample was assayed in triplicate.

Quantitative reverse Transcription Polymerase Chain Reaction (qRT-PCR) and Quantitative Polymerase Chain Reaction (qPCR) were performed three times in six replicates on a 7500 Real Time PCR System (Applied Biosystems) using the SYBR Green-detection system (FS Universal SYBR Green MasterRox/Roche Applied Science). All reactions were normalized to 18S mRNA. The complete list of oligonucleotides used is reported in Table-1.

Chromatin immunoprecipitation

2.5 x 10^6 cells for each antibody, transfected and/or treated as indicated in the legends of the figures, were fixed with 1% formaldehyde for 10 minutes at room temperature. The reaction was quenched by the addition of glycine to a final concentration of 125 mM. Fixed cells were harvested and the pellet was resuspended in 1 ml of Lysis Buffer (10 mM Tris-HCl pH 8.0, 10 mM NaCl, 0.2 % NP40) containing 1X protease inhibitor cocktail (Roche Applied Science). The lysates were sonicated in order to have DNA fragments from 300 to 600 bp. Sonicated samples were centrifuged and supernatants diluted 2 fold in the ChIP Buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl pH 8.0). An aliquot (1/10) of sheared chromatin was further treated with proteinase K (4U every 1 x 106 nuclei), extracted with 1 volume of phenol/chloroform/isoamyl alcohol (25:24:1) and precipitated in LiCl 0,4 M/ ethanol 75% to determine DNA concentration and shearing efficiency (input DNA). The ChIP reaction was set up according to the manufacturer's instructions. Briefly, the sheared chromatin was precleared for 2 h with 1 µg of non-immune IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and 20 µl of Protein A/G PLUS-Agarose (Santa Cruz Biotechnology) saturated with salmon sperm (1 mg/ml). Precleared chromatin was divided in aliquots and incubated at 4 °C for 16 h with 1 µg of the specific antibody (anti-FLAG M1 and anti-H3K9me2) and non-immune IgG respectively. The immuno-

complexes were recovered by incubation for 3 h at 4°C with 20 µl of protein-A/G PLUS agarose, beads were washed with wash buffers according to the manufacturer's instructions and immunoprecipitated DNA was recovered through phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation and redissolved in TE buffer (10 mM Tris-HCl, 1mM EDTA, pH 8,0). Samples were subjected to qPCR using the primers indicated in the legend of the specific figures, primers sequences are reported in Table 1. Real Time-qPCRs were performed using FastStart Universal SYBR Green Master (Rox) (Roche Applied Science) with cycle conditions as follows:

Caveolina 1 ERE: 95 °C 10'; 5x (95 °C 45'', 65 °C 30'', 72 °C 30''); 40x (95 °C 45'', 62 °C 30'', 72 °C 30''); 72 °C 10 min.

TSHR exone 10: 95 °C 10'; 40x (95 °C 45'', 52 °C 30'', 72 °C 35''); 72 °C 10 min.

Data analysis.

All data are presented as mean ± standard error of at least three experiments in triplicate (n≥9). Differences between treatments were tested for statistical significance using Student's matched pairs t test. Statistical analysis was performed using the JMP 6.0.3 software (SAS Institute Inc., USA - http://www.sas.com).

Table 1

	PRIMERS for mRNA	
locus	**sequence**	
pS2	5'-CCCTCCCAGTGTGCAAATA-3'	Fw
	5'-GATCCCTGCAGAAGTGTCTAAAA-3	Rev
BCL2	5'-AGTACCTGAACCGGCACCT-3'	Fw
	5'-GGCCGTACAGTTCCACAAA-3'	Rev
Cav1	5'-AAACGTTCTCACTCGCTCTC-3'	Fw
	5'-CAAAGGTTTGTTCTGCTCGC-3'	Rev
S100p	5'-GGGAGCTCAAGGTGCTGAT-3'	Fw
	5'-AGCAATTTATCCACGGCATC-3'	Rev
18S	5’-TCCCCATGAACGAGGAATTC-3’	Fw
	5’-GGCCTCACTAAACCATCCAA-3’	Rev
	PRIMERS for ChIP	
locus	**sequence**	
Cav1- ERE	5’-TAAAGCTGGAAGGGATTACCG-’3	Fw
	5’-CTTCTCCCGGACTCCCTAAG-’3	Rev
TSHR-Ex 10	5’-ACCGAGACCCCTCTTGCTCT-3’	Fw
	5’-AGTTGCTAACAGTGATGAGAGGCT-3’	Rev

Results

Results

Estrogens and cAMP-PKA stimulate the formation of the complex: LSD1, the estrogen receptor α and RNA Polymerase II.

Estrogens, in addition to canonical genomic pathway, activate several non-canonical transduction systems (Beato M. et al., 1989; Kelly M. J. and Levin E. R., 2001). Estrogen has been shown to activate cAMP-PKA signaling that cooperates by propagating the estrogen signals to several cellular cytoplasmic and nuclear effectors (Aronica S. M. et al, 1994). PKA has been involved in ERα-mediated rapid effects in many different processes. For example, in the basal forebrain cholinergic neurons *in vivo* PKA inhibition reduces the estrogen-induced CREB phosphorylation (Szego E'va M. et al., 2006). PKA inhibition reduces estrogen induction of breast cancer cell proliferation (Houslay M. D. and Kolch W., 2000) and the transcription activation (Liu D. et al., 2009).

To confirm that estrogens induce the cAMP-PKA pathway in our cellular model, MCF-7 cells were pretreated or not with H89, a permeable PKA inhibitor, and stimulated for 5' and 30' with E_2 or for 15' with Forskolin, a cell-permeable adenylate cyclase activator. Total cell extracts were resolved on SDS-PAGE and blotted with antibodies against anti-P (Ser/Thr) PKA substrate to detect bands corresponding to substrates phosphorylated by PKA. We find that E_2 is able to rapidly induce (5') phosphorylation of many PKA substrates, (Figure 9A and B) and that prolonged exposure to E_2 (30') does not increase the number of the bands, suggesting the effect of E2 on cAMP-PKA signal is rapid and transient. Inhibition of PKA with H89 affects this early PKA-dependent phosphorylation reducing and delaying the phosphorylation profile at 30' of E_2 stimulation (Figure 9, compare lane 3 to 6). Furthermore, the phosphorylation of CREB, a well-known PKA substrate, confirms that E_2 induces the activation of PKA pathway in our cellular model. Forskolin induces the same pattern of phosphorylated bands seen with E_2 (Figure 9, lane 8).

Although many studies have pointed out the importance of LSD1 during carcinogenesis or development (Rotili D. and Mai A. 2011; Shi Y. et al., 2005; Lynch J.T. et al., 2012; Cohen I. et al., 2011), its role in regulating gene expression is emerging only recently. LSD1 is involved as co-repressor in several molecular complexes, including CoREST and NuRD and as co-activator in androgen or estrogen receptors (Shi Y. et al., 2003; Perillo B. et al., 2008). Consistent with its role in transcriptional repression, LSD1 demethylates mono-methyl and di-methyl histone H3 lysine 4 (H3K4me1 and H3K4me2), which mark active chromatin. Nevertheless, the precise mechanism of activation of the enzyme *in vivo* remains still elusive.

To dissect the mechanism of LSD1 activation/recruitment to ERE/chromatin by E_2, we performed immunoprecipitation experiments in protein extracts from MCF-7 cells exposed to E_2 and/or cAMP. To this end, MCF-7 cells were starved from the hormone with 10% Charcoal Stripped Serum (CSS) for 3 days and exposed to E_2 and/or cAMP in the presence and in the absence of the classical anti-estrogen or anti–PKA drugs, ICI-18278 and/or H89, respectively. To detect the proteins interacting with LSD1 during the estrogen-stimulation, total protein extract was immunoprecipitated with anti-LSD1 antibody and tested for the presence of the estrogen receptor alpha (ERα) and the RNA polymerase II (RNA Pol II) by immunoblotting. Figures 9 C and D show that the binding of LSD1 to the ERα and to RNA Pol II is stimulated by E_2 and cAMP and prevented by H89 and ICI-18278. The simultaneous treatment with cAMP and E_2 does not improve the efficiency of binding compared to single treatments, suggesting that the two pathways are not synergic. Moreover, ICI and H89 prevent the interaction of LSD1 with ERα and RNA Pol II, induced by E_2 and cAMP (Figure 9C and D). These data demonstrate that the binding of LSD1 to ER-α and RNA Pol II occurs *via* cAMP/PKA pathway stimulated by E_2.

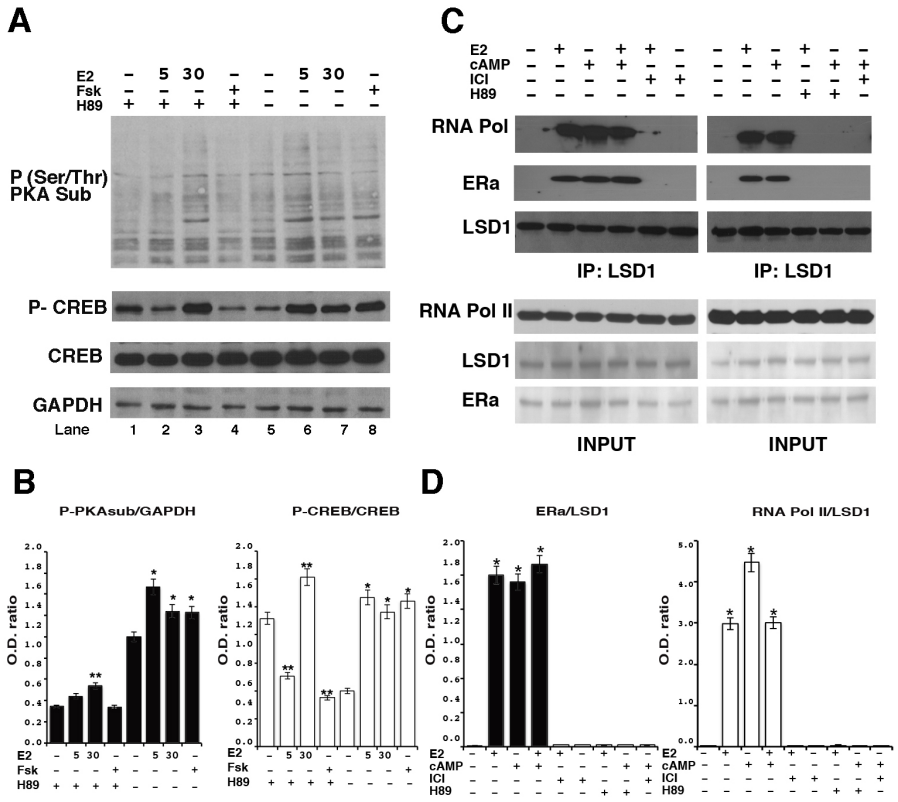

Figure 9. Estrogens and cAMP-PKA stimulate the binding of LSD1 to ERα and RNA Pol II. A: MCF-7 cells, starved for 3 days, were stimulated with E_2 for 5' and 30' and with Foskolin for 15' as indicated in the figure, with or without 30' of H89 pre-treatment. Total protein extracts were tested with the indicated antibodies. C: MCF-7 starved, were stimulated with E_2 for 30' or 8-Br-cAMP for 15' in the presence or in the absence of the molecules indicated on the top of the lanes (see Materials and Methods). The co-treatment of E_2 and 8-Br-cAMP was for 30'. Cell extracts were immunoprecipitated with antibodies against LSD1. The immunoprecipitates were analysed by blotting with antibodies against ERα or the large subunit of RNA polymerase II (upper panel). The lower panel shows in the total protein extracts (input). B & D: The histogram shows O. D. ratio of densitometric analysis indicated in the single panels, normalized to the untreated control. The statistical analysis derived from at least 3 experiments in triplicate (Student's t test) are shown * $p<0.001$ compared to the untreated control; ** $p<0.01$ compared to the H89-treated control.

LSD1 interacts with the N-terminal domain of ERα and co-localizes in the nucleus after estrogen stimulation.

To determine which domain of the estrogen receptor is involved in the interaction with LSD1, we have performed an "*in vitro*" pull-down assay (Figure 10A) using different GST-ER fusion proteins (see material and methods and Figure 10B, upper panel). MCF-7 cell extracts were incubated for 3h at 4°C in the presence or in the absence of E_2 with different Sepharose-bound GST-ER fusion proteins and then subjected to western blot analysis with anti-LSD1 antibodies. We find that LSD1 binds only the ERα recombinant proteins that contain the N-terminus fragment (Heg0, Heg15), indicating that LSD1 does not interact with C-terminal domain of ERα. The interaction with the full-length receptor form is dependent on estrogens; in contrast, Heg15-LSD1 interaction is not induced by estrogen, because this protein does not contain E_2 binding domain (Figure 10B, lower panel).

To visualize the cellular compartment where the formation of ERα-LSD1 complex occurs, we have stained MCF-7 cells, treated for 15', 30', 45' with E_2, with anti-LSD1 and anti-ERα antibodies and analyzed the localization of the proteins by confocal microscopy. We find that LSD1 is essentially localized in the nucleus and this localization is stimulated by exposure to E_2. In particular, between 15 and 30 min, the two signals (LSD1, red; ERα, green) increase and overlap, indicating that upon E2 stimulation, the receptor rapidly accumulates into nucleus, where it binds and targets LSD1 to the estrogen-specific sites of chromatin (Figure 10C).

Collectively, these data indicate that LSD1 is able to bind ERα in the nucleus both "*in vivo*" and "*in vitro*" in estrogen-dependent manner.

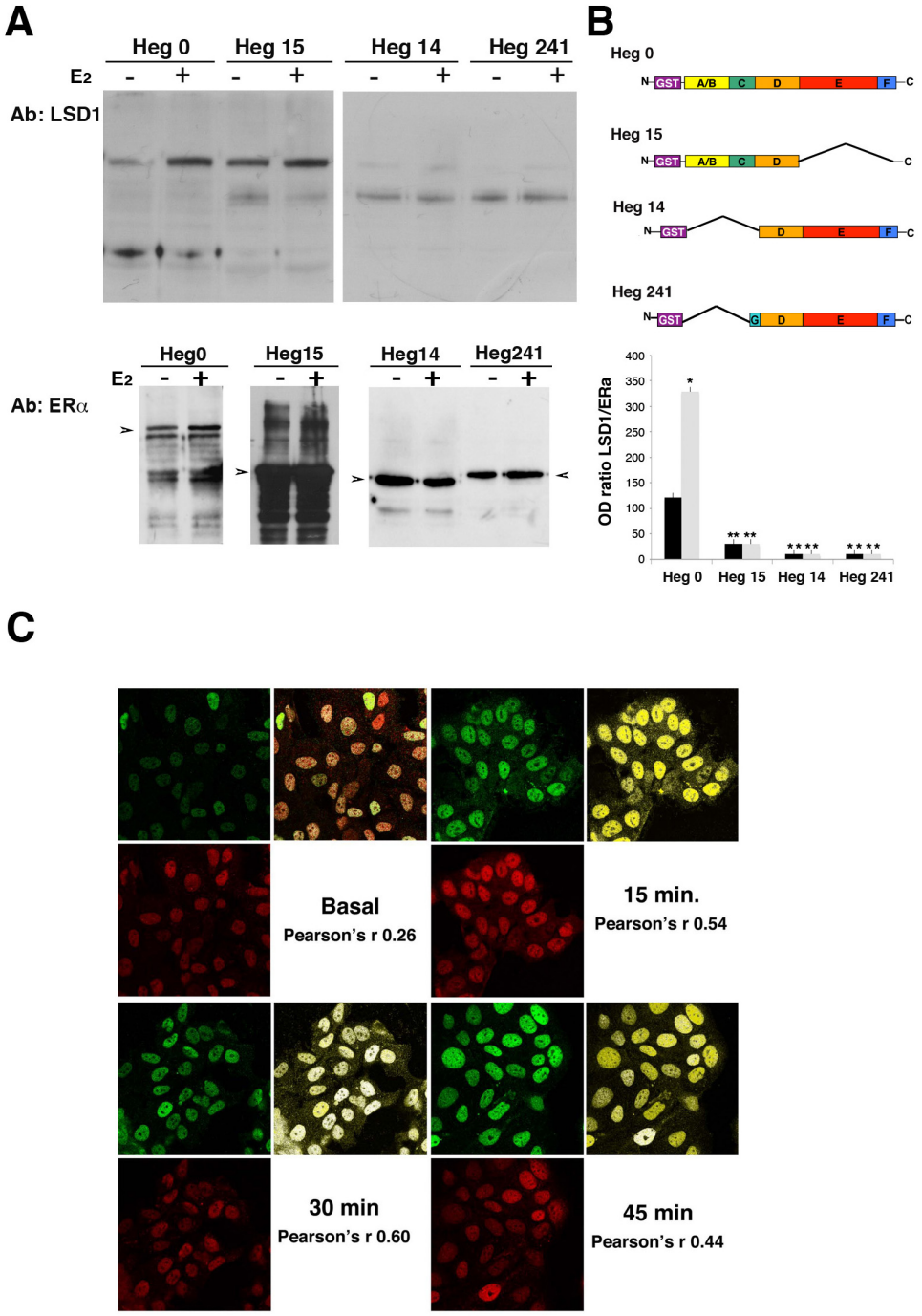

Figure 10. LSD1 binds the N-terminal domain of ERα and co-localizes with it in the nucleus following estrogen stimulation. A: The total protein extract from MCF-7 was subjected to "in vitro" pull down-assay with the sepharose-GST-ER fusion proteins indicated in the figure (see panel B) with or without E_2. All the samples were subjected to SDS-PAGE and tested for the presence of LSD1 (upper panel) and ERα (lower panel). B: It is shown a schematic representation of the estrogen receptor α-deletion forms fused to the GST. The histogram shows the LSD1/ERα ratio O. D. The statistical analysis derived from at least 3 experiments in triplicate (Student's t test) are shown *p<0.01 compared to the untreated control; **p<0.01 compared to the untreated Heg0. C: Confocal localization analysis of MCF-7 cells stimulated with E_2 for 15', 30' and 45' (see Materials and Methods). Cells were fixed, stained with anti-LSD1 (red) and anti-ERα receptor (green) antibodies. The red-green merged images are shown. The microphotographs were analyzed with ImageJ software and the Pearson's coefficient was calculated for each experimental point.

LSD1 Threonine 110 is a specific PKA phosphorylation target both "*in vivo*" and "*in vitro*".

Recently, many research groups focused their attention on the role of different phosphorylation sites in the N-terminal region of LSD1 and their involvement in the regulation of histone methylation. Costa R. et al. have found Ser131, Ser137 and Ser166 residues in LSD1 as new substrates of protein kinase CK2 and demonstrate that the phosphorylation of these sites modulate the interaction with partners involved in formation of repression and activation complexes (Costa R. et al., 2014). Moreover, several data suggest that the LSD1 is a target of PKA. Perillo et al. demonstrated that LSD1 recruitment to BCL-2 ERE site is stimulated by cAMP-PKA, because H89 prevents the H3K9me2 demethylation and the recruitment of LSD1 to BCL-2 chromatin in E_2-stimulated cells (Perillo B. et al., 2013). In addition, a LSD1 threonine mutant (T110A) inhibits cAMP and Myc-induced gene expression compared to the LSD1 wild type (Amente S. et al., 2011). However, to date the direct proof that T110 in LSD1 is a PKA site is lacking. Here we demonstrate that LSD1-T 110 is a specific target of PKA by performing experiments *in vivo* and *in vitro*".

First, we co-transfected MCF-7 cells with an expression vector encoding tagged (FLAG)-LSD1 variants: the wild type and a mutant in T110 (LSD1-Ala) in the presence and in the absence of pRSV-PKI that expresses the synthetic protein kinase inhibitor peptide (Day R. N. et al., 1989). Transfected cells were hormone starved and treated with E_2. Total proteins were extracted and immunoprecipitated with anti-FLAG antibodies. LSD1 phosphorylation was assayed by Western blot analysis with antibodies against the phosphorylated Ser/ Thr PKA substrates. A specific PKA phosphorylated protein band migrating with an apparent M.W. of 100 KDa was visible **only** in the immunoprecipitates from MCF-7 cells transfected with LSD1wt and stimulated with estrogens, indicating that estrogens induce PKA-dependent phosphorylation of LSD1 (Figure 11A).

To confirm that LSD1 is directly phosphorylated by cAMP-PKA, the expressed protein was submitted to an "in vitro" phosphorylation assay. To this end, LSD-wt and LSD-Ala were expressed in MCF-7 and the relative proteins were purified by immune-precipitation with anti-FLAG antibodies, eluted and incubated "*in vitro*" with the purified catalytic subunit of PKA and γ-P^{32}-ATP, with or without the specific PKA inhibitor, PKI, and in the absence or in presence of a PKC inhibitor (PKCi) to test the kinase specificity. Figure 11B shows that LSD1wt is phosphorylated by PKA, because the phosphorylation is significantly reduced by PKI. However, the mutated protein also in the presence of PKI shows a residual and significant phosphorylation. Differently from LSD1wt, the phosphorylation of LSD1ala is totally abrogated by PKCi. We conclude that PKC can also phosphorylate LSD1, but at another site and that Threonine 110 of LSD1 is a specific site phosphorylated by PKA.

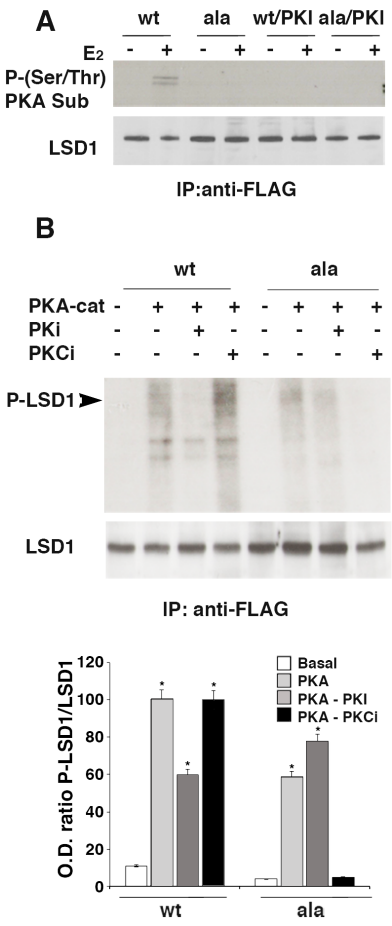

Figure 11. PKA phosphorylates LSD1 both "in vivo" and "in vitro" at T110. A: MCF7 cells were transiently co-transfected with LSD1-wt or LSD1-ala and PKI (see Material and Methods). 48 h after transfection the cells were stimulated or not with E_2 for 30', lysed and processed by immunoprecipitation with anti-FLAG antibodies. The immunoprecipitates were probed with antibodies against P-(Ser/Thr) PKA substrate and LSD1. B: MCF7 cells were transiently co-transfected with LSD1-wt or LSD1-ala, lysates immunoprecipitated with anti-FLAG antibodies. The immunocomplexes were subjected to a PKA "in vitro" kinase assay in the presence of PKA and PKC inhibitor. It is shown the P-LSD1 (upper panel) and the total LSD1 (lower panel). The histogram shows the O.D. ratio normalized to the untreated control. The statistical analysis derived from at least 3 experiments in triplicate (Student's t test) are shown *p<0.01 compared to the each untreated control.

Phosphorylation of LSD1 at Threonine 110 regulates its interaction with estrogen-induced initiation transcriptional complex.

Since the alanine substitution in LSD1 does not affect the enzymatic activity of LSD1 (Zuchegna C. et al, 2014), we hypothesize that phosphorylation of T 110 residue modulates the interaction with different partners that regulates estrogen-dependent transcription. To test the binding ability of wild type and alanine mutant LSD1 with other nuclear proteins involved in E2-induced transcription, we probed the anti-FLAG immunoprecipitates with the antibodies targeting several proteins, as shown in Figure 4A. The data demonstrate that the treatment with E_2 or cAMP induces the interaction between LSD1-wt and ER-α, RNA polymerase II, PKA catalytic sub-unit and the single strand binding protein, RPA. Under the same conditions, LSD1-Ala mutant weakly interacts with ER-α, RNA polymerase II, PKA catalytic subunit compared to the wild type form, and does not bind RPA. It is worth noting that LSD1-Ala binds more efficiently N-CoR and histone H1 to than LSD1-wt upon E_2 and cAMP treatments. These data suggest that LSD1 mutant interacts with histone H1 and N-CoR, which are associated with compacted chromatin and repression of transcription, respectively, suggesting that non-phosporylated LSD1 acts as a negative dominant regulator of estrogen-induced gene transcription (Figure 12 A&B).

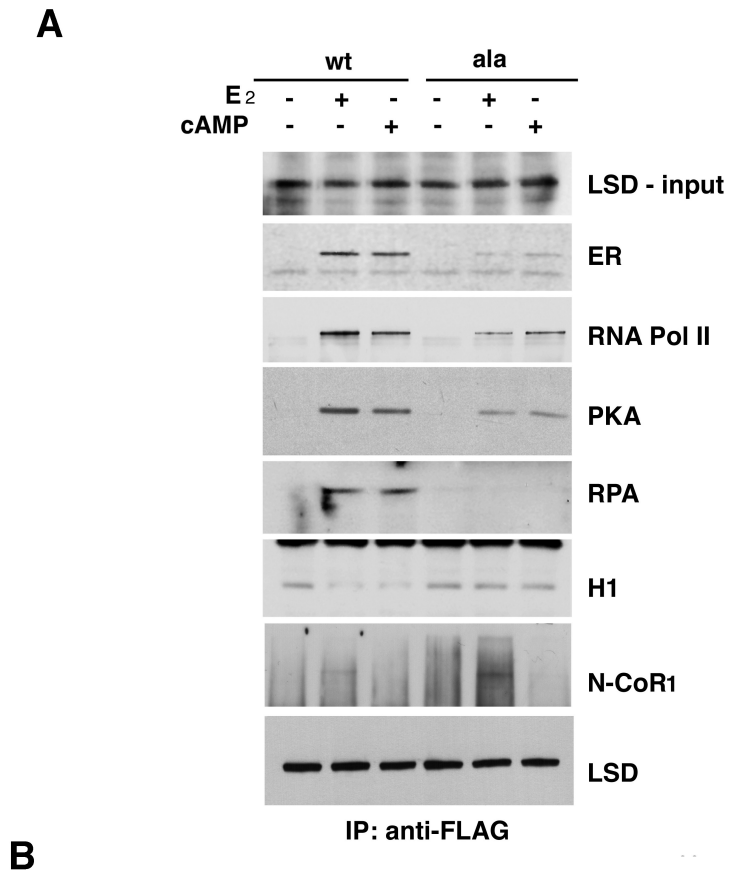

Figure 12. The phosphorylation of LSD1 interferes with the transcription initiation complex. A: MCF7 cells were transiently co-transfected with LSD1-wt or LSD1-Ala (see Material and Methods). 48 h after transfection the cells were stimulated or not with E_2 for 30' or 8-Br-cAMP for 15', lysed and processed by immunoprecipitation with anti-FLAG antibodies. The immunoprecipitates were probed with antibodies against LSD1, ERα, large subunit of RNA polymerase II, PKA, RPA, N-CoR1, and H1. B: The histograms show the quantification of the protein bands immuno-precipitated with anti-LSD1 antibodies derived from at least 3 experiments in triplicate. * $p<0.01$ compared to the each un-stimulated control (Student's t test).

LSD1-T110 phosphorylation is required for estrogen-dependent transcription.

LSD1 mediates inter-chromosomal interactions necessary for estrogen-dependent transcription (Hu Q. et al., 2008). In particular, the physical presence of LSD1 both as a scaffolding protein, and as demethylase enzyme at the ERE region was important for ERα-regulated transcription. In fact E_2 treatment induces the recruitment of ERα and LSD1 to ERE of pS2, and influences the methylation status of histones at the pS2 ERE chromatin (Pollock Julie A. et al., 2012). To determine whether LSD1-Ala alters the methylation of H3K9 or H3K4me2 and to find out the consequences on transcription induced by estrogens, chromatin immunoprecipitation assay and gene-expression analysis were performed in cells exposed to E_2. Figures 13A and B show that LSD1 wild type is recruited to the ERE upon estrogen challenge. The recruitment of wild type LSD1 to the ERE-containing chromatin is associated with a strong reduction of H3K9me2 levels (Figures 13A and B). LSD1-Ala, on the other hand, is present at several chromatin regions, but its recruitment is not stimulated by estrogens. Over-expression LSD1-Ala is associated with reduction of basal H3K9me2 levels, which are not dependent on estrogens (Figures 13 A and B). Lower H3K9me2 levels are non specific of chromatin containing ERE consensus, since other sites, non induced by estrogens, display reduced levels of H3K9me2, indicating that LSD1 is recruited to chromatin by other factors (Figure 13B). To determine the consequences of LSD1-Ala expression on estrogen-induced transcription, we have analyzed the expression of several genes (pS2 or TFF1, BCL-2, CAV1 and S100p) induced by estrogens in cells expressing LSD1-Ala. Figure 13C shows that the LSD1-Ala mutant significantly inhibits estrogen-induced transcription of all genes analyzed. We notice that LSD1-Ala also, increased the basal transcription, hormone-independent, of some estrogens-induced genes, such as pS2 and S100p, indicating that LSD1-Ala renders the expression of these genes constitutive and estrogen independent.

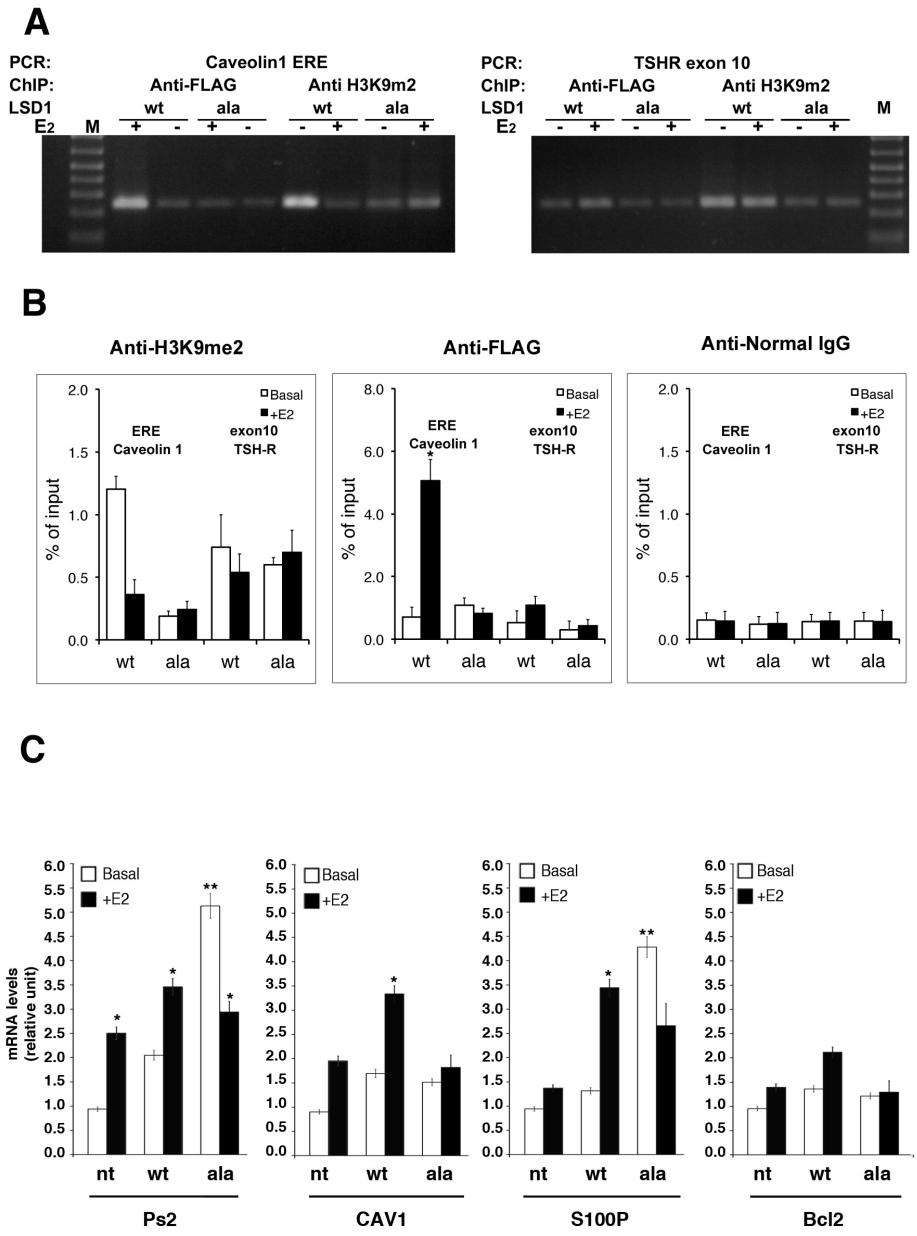

Figure 13. Demethylase activity and binding to ERE-containing chromatin of wild type and threonine 110 LSD1-ala mutant. A: Chromatin immunoprecipitation with anti-FLAG or anti-H3K9me2 antibodies in cells expressing wild type or LSD1-Ala mutant in the absence or presence of E_2; qPCRs were carried out on the ERE sites of Caveolin1 and exon 10 of TSH receptor. B. RT-PCR of the same experiment in A. The values reported were calculated as fold percentage of the amount of immunoprecipitated DNA relative to that present in total input chromatin. qChIP data are presented as mean of 3 independent experiments. * $p<0.01$ (matched pairs t test) compared to untreated control (t test). Exon 10 TSH receptor-gene was used as control of chromatin without ERE sequences.

C: LSD1 wt and LSD1-Ala were transiently transfected in MCF7 cells. 48 h later, total RNA was prepared from cells starved or E_2 induced for 45 min and analyzed by qPCR with specific primers to BCL2, pS2, CAV1, S100p. Statistical analysis: * $p<0.01$ (matched pairs t test) compared to untreated sample; ** $p<0.01$ (t test) compared to the basal of cells transfected with control plasmid in the absence of estrogens for each gene.

Discussion

cAMP and Estrogens cooperate at multiple levels to stimulate transcription.

PKA cooperates with many transduction pathways. The cooperation with estrogens is not confined to the nucleus, but it is also operating in the membrane-cytosolic compartments, where cAMP-PKA amplify estrogen induction of PI3K (Cosentino C. et al., 2007). The major estrogen receptor anchor protein in the membrane-cytoplasmic compartments is p85-PI3K, while in the nucleus the receptor is mainly associated with LSD1 (Figure 10C). Both p85-PI3K and LSD1 bind the E2 receptor only when they are phosphorylated by PKA at specific sites (Cosentino C. et al., 2007 and Figure 12). Inhibition of cAMP-PKA signaling severely impairs genomic and non-genomic estrogen signaling and recent evidence indicates that sustained PKA activation leads to phosphorylation and binding of the receptor to a coactivator associated arginine methyl transferase, but is not able alone, to activate ERα (Carascossa S. et al., 2010). LSD1, bound to the receptor complex, is tightly dependent on ligated receptor and may sense nuclear PKA oscillations to finely tune nuclear responses to estrogen passage from the cytoplasm to the nucleus.

LSD1 as repressor and activator of transcription

LSD1 is also a repressor of transcription, because it has been found associated to Rb and p53 in a cell cycle dependent manner (Chau C. M. et al., 2008; Huang J. et al., 2007), to repress telomerase reverse transcriptase (hTert) expression (Zhu Q. et al., 2008), and to induce and maintain the silenced state of the zinc finger transcription factor Snail1 target genes in invasive cancer cells. (Lin T. et al., 2010). LSD1 has been initially isolated as repressor of transcription, mainly because it erases the activation mark H3K4me2 (Shi Y. et al., 2004). However, there is evidence that LSD1 can also activate transcription by removing the repressor mark H3-K9me2 (Perillo B. et al., 2008). The mono aminoxidase activity of LSD1 is essential both for transcription activation induced by androgens, estrogens and Myc and transcription repression. During these processes, LSD1 changes its demethylating activity from H3K4me2 to

H3K9me2, favoring the demethylation of H3K9me2 "*in vivo*" (Metzger E. et al., 2005; Perillo B. et al. 2008; Amente S. et al., 2010). One interesting hypothesis that combines these apparent contradictory results is the following: many genes contain at the transcription start site nucleosomes carrying negative (H3K9me2) and positive (H3K4me2) marks. These genes are poised in an intermediate state, since they can be activated or inhibited depending on the stimulus. LSD1 can mediate both effects in dependence on the type of activator; removal of H3K4me2 leads to repression, whereas, removal of H3K9me2 activates transcription.

LSD1 forms a platform for the assembly of the transcription initiation complex

The data presented here show that LSD1 recruits multiple components of the transcription initiation complex. The binding of LSD1 to ER-α and the RNA Polymerase II is induced by estrogens and requires a short cAMP-PKA signal. This is shown by the experiments with ICI. ICI interferes with the estrogen receptor shuttling from the cytoplasm to the nucleus, preventing the access of activated receptors to the chromatin. Treatment with this anti-estrogen molecule prevents the interaction of estrogen receptor with LSD1 and many nuclear components. The same process is also inhibited by H89, indicating that cAMP and PKA are essential to complete the assembly of the transcription initiation complex. The very rapid PKA induction (5') shows the convergence on LSD1 of the early nuclear and cytoplasmic signaling of estrogens and cAMP.

We suggest that PKA serves as a general nutrient sensing mechanism that amplifies the action of many transcription factors and LSD1 represents the structural and enzymatic platform (achieved by demethylating histone H3K9 or K4 and DNA oxidation) that assembles the transcription initiation complex.

As to the specific mechanism governing the ordered assembly of the initiation complex, our data indicate that phosphorylation at threonine 110 of LSD1 influences the choice of the chromatin partners. For example, LSD1-Ala binds H1 and NCoR, not RNA polymerase II and this complex mediates the repression of transcription in the absence of PKA and estrogens.

In conclusion, we propose that the choice of partners by LSD1 is dependent on the phosphorylation of threonine 110 induced by cAMP-PKA and this represents a simple and elegant strategy linking estrogen signaling to metabolism and nutrient sensing.

Acknowledgements

I would like to thank Alessandro for having always been close and supported in all my choice, for helping me to overcome any obstacle, making it less difficult.

I would like to thank Antonella, Mara, Tina, Angelo, Simona, Alessandra, Cinzia, Clorinda, Maddalena, Marina and especially Candida because in you I have found not only wonderful traveling companions, but also precious friends, always willing to help me, listen to me, advise me on several occasions in the life. A thank goes to the whole laboratory of Molecular Medicine and Medical Biotechnology, University of Naples "Federico II" too.

In Addition, I would like to thank Prof. Vittorio Enrico Avvedimento, Prof. Antonio Porcellini, Prof. Marina Di Domenico, Prof. Antimo Migliaccio, and Prof. Vincent Dion for giving me the opportunity to make this wonderful experience.

This study was partly supported by Microtech S.r.l. and P.O.R. Creme 2007/2013.

References

Abbondanza C, Rossi V, Roscigno A, Gallo L, Belsito A, Piluso G, Medici N, Nigro V, Molinari A M, Moncharmont B, Puca G A (1998) Interaction of Vault Particles with Estrogen Receptor in the MCF-7 Breast Cancer Cell J. Cell Biol 141 (6): 1301–10.

Adamo A, Sese B, Boue S, Castano J, Paramonov I, Barrero MJ, Izpisua Belmonte JC (2011) LSD1 regulates the balance between self-renewal and differentiation in human embryonic stem cells, Nat. Cell Biol. 13 652–659.

Agalioti T, Lomvardas S, Parekh B, Yie J, Maniatis T, Thanos D (2000) Ordered recruitment of chromatin modifying and general transcription factors to the IFN-beta promoter, Cell 103, 667 – 678.

Amente S, Bertoni A, Morano A, Lania L, Avvedimento EV, Majello B (2010) LSD1-mediated demethylation of histone H3 lysine 4 triggers Myc-induced transcription, Oncogene 29, 3691-3702.

Amente S, Lania L, Majello B (2011) Epigenetic reprogramming of Myc target genes, Am J Cancer Res 3, 413-418.

Amente S, Lania L, Majello B (2013) The histone LSD1 demethylase in stemness and cancer transcription programs, Biochim Biophys Acta 1829(10):981-6.

Aronica SM, Kraus WL, Katzenellenbogen BS (1994) Estrogen Action via the cAMP Signaling Pathway: Stimulation of Adenylate Cyclase and cAMP-Regulated Gene Transcription, PNAS, 91(18), 8517-8521.

Bai W, Rowan BG, Allgood VE, O'Malley BW, Weigel NL (1997) Differential Phosphorylation of Chicken Progesterone Receptor in Hormone-dependent and Ligand- independent Activation, J Biol Chem 272(16), 10457- 10463.

Bannister AJ, Kouzarides T (2004) Histone methylation: recognizing the methyl mark, Methods Enzymol. 376:269-88.

Beato M, Chalepakis G, Schauer M, Slater EP (1989) DNA regulatory elements for steroid hormones, J Steroid Biochem 32 (5): 737-47.

Bedford MT1, Richard S (2005) Arginine methylation an emerging regulator of protein function, Mol Cell. 18(3):263-72.

Berger SL (2007) The complex language of chromatin regulation during transcription, Nature 447, 407–412.

Binda C, Mattevi A, Edmondson DE (2002) Structure-function relationships in flavoenzyme-dependent amine oxidations: a comparison of polyamine oxidase and monoamine oxidase, J Biol Chem 277,

23973–23976.

Carascossa S, Dudek P, Cenni B, Briand PA, Picard D (2010) CARM1 mediates the ligand-independent and tamoxifenresistant activation of the estrogen receptor α by cAMP. Genes Dev; 24: 708-719.

Castoria G, Migliaccio A, D'Amato L, Di Stasio R, Ciociola A, Lombardi M, Bilancio A, Di Domenico M, de Falco A, Auricchio F (2008) Integrating signals between cAMP and MAPK pathways in breast cancer, Front Biosci 13:1318-27.

Chau CM, Deng Z, Kang H, Lieberman PM (2008) Cell cycle association of the retinoblastoma protein Rb and the histone demethylase LSD1 with the Epstein-Barr virus latency promoter Cp. J Virol; 82: 3428-3437.

Chen JD, Evans RM (1995) A transcriptional co-repressor that interacts with nuclear hormone receptors, Nature 377(6548):454-7.

Cohen I, Poręba E, Kamieniarz K, Schneider R (2011) Histone modifiers in cancer: friends or foes?, Genes Cancer 2(6):631-47.

Cosentino C, Di Domenico M, Porcellini A, Cuozzo C, De Gregorio G (2007) p85 regulatory subunit of PI3K mediates cAMP-PKA and estrogens biological effects on growth and survival. Oncogene; 26: 2095-2103.

Costa R, Arrigoni G, Cozza G, Lolli G, Battistutta R, Izpisua Belmonte JC, Pinna LA, Sarno S (2014) The lysine-specific demethylase 1 is a novel substrate of protein kinase CK2, Biochim Biophys Acta 1844(4):722-9.

Couse JF1 and Korach KS (1999) Reproductive phenotypes in the estrogen receptor-alpha knockout mouse, Ann Endocrinol 60(2):143-8.

Da G, Lenkart J, Zhao K, Shiekhattar R, Cairns BR, Marmorstein R (2006) Structure and function of the SWIRM domain, a conserved protein module found in chromatin regulatory complexes, Proc Natl Acad Sci 103, 2057–2062.

Dauvois S, Danielian PS, White R, Parker MG (1992) Antiestrogen ICI 164,384 reduces cellular estrogen receptor content by increasing its turnover, Proc Natl Acad Sci 89:4037–4041.

Dauvois S, White R, Parker MG (1993) The antiestrogen ICI 182780 disrupts estrogen receptor nucleocytoplasmic shuttling, J Cell Sci 106:1377–1388.

Day RN, Walder JA, Maurer RA (1989) A protein kinase inhibitor gene reduces both basal and multihormone-stimulated prolactin gene transcription, J Biol Chem 264(1):431-6.

DeCherney A (1993) Physiologic and pharmacologic effects of estrogen and progestins on bone, J Reprod Med 1007-14.

Deroo BJ and Korach KS (2006) Estrogen receptors and human disease, J Clin Invest 116(3):561-70.

Flemming, W. (1882). Zellsubstanz, Kern und Zelltheilung (Leipzig, Germany: F.C.W Vogel).

Forneris F, Binda C, Vanoni MA, Battaglioli E, Mattevi A (2005) Human histone demethylase LSD1 reads the histone code, J Biol Chem 280, 41360-41365.

Forneris F, Binda C, Vanoni MA, Mattevi A, Battaglioli E (2005) Histone demethylation catalysed by LSD1 is a flavin-dependent oxidative process, FEBS Lett 579, 2203.

Foster CT, Dovey OM, Lezina L, Luo JL, Gant TW, Barlev N, Bradley A, Cowley SM (2010) Cowley, Lysine-specific demethylase 1 regulates the embryonic transcriptome and CoREST stability, Mol. Cell. Biol. 30 4851–4863.

Foster KR and Ratnieks FL (2001) The effect of sex-allocation biasing on the evolution of worker policing in hymenopteran societies, Am Nat 158(6):615-23

Garcia-Bassets I, Kwon YS, Telese F, Prefontaine GG, Hutt KR, Cheng CS, Ju BG, Ohgi KA, Wang J, Escoubet-Lozach (2007) Histone methylation-dependent mechanisms impose ligand dependency for gene activation by nuclear receptors, Cell 128, 505–518.

Gerits N, Kostenko S, Shiryaev A, Johannessen M, Moens U (2008) Relations between the mitogen-activated protein kinase and the cAMP-dependent protein kinase pathways: Comradeship and hostility, Cell. Signal 20, 1592–1607.

Heinzel T1, Lavinsky RM, Mullen TM, Söderstrom M, Laherty CD, Torchia J, Yang WM, Brard G, Ngo SD, Davie JR, Seto E, Eisenman RN, Rose DW, Glass CK, Rosenfeld MG (1997) A complex containing N-CoR, mSin3 and histone deacetylase mediates transcriptional repression, Nature 387(6628):43-8

Hörlein AJ1, Näär AM, Heinzel T, Torchia J, Gloss B, Kurokawa R, Ryan A, Kamei Y, Söderström M, Glass CK (1995) Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor, Nature 377(6548):397-404.

Houslay MD and Kolch W (2000) Cell-type specific integration of cross-talk between extracellular signal-regulated kinase and cAMP signaling, Mol Pharmacol 58(4):659-68.

Hu Q, Kwon YS, Nunez E, Cardamone MD, Hutt KR, Ohgi KA, Garcia-Bassets I, Rose DW, Glass CK, Rosenfeld MG, Fu XD (2008) Enhancing nuclear receptor-induced transcription requires nuclear motor and LSD1-dependent gene networking in interchromatin granules, Proc Natl Acad Sci 105(49):19199-204.

Huang J, Sengupta R, Espejo AB, Lee MG, Dorsey JA, et al. (2007) p53 is regulated by the lysine demethylase LSD1. Nature; 449: 105-108.

Human histone demethylase LSD1 reads the histone code, J Biol Chem 280(50):41360-5.

Imhof A, Patel D, Kahl P, Hoffmeyer K, Friedrichs N, Müller JM, Greschik H, Kirfel J, Ji S, Kunowska N, Beisenherz-Huss C, Günther T, Buettner R, Schüle R (2010) Phosphorylation of histone H3T6 by PKCbeta(I) controls demethylation at histone H3K4, Nature 464(7289):792-6.

Insel PA, Zhang L, Murray F, Yokouchi H, Zambon AC (2012) Cyclic AMP is both a pro-apoptotic and anti-apoptotic second messenger, Acta Physiol 204, 277–287.

Kelly MJ, Levin ER (2001) Rapid actions of plasma membrane estrogen receptors, Trends Endocrinol Metab 12(4):152-6.

Kouzarides T (2007) Chromatin modifications and their function, Cell 128, 693–705.

Latham JA and Dent SY (2007) Cross-regulation of histone modifications, Nat Struct Mol Biol 14, 1017–1024.

Lee MG, Wynder C, Cooch N, Shiekhattar R (2005) An essential role for CoREST in nucleosomal histone 3 lysine 4 demethylation, Nature 437, 432–435.

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

Liu D, Benlhabib H, Mendelson CR (2009) cAMP enhances estrogen-related receptor alpha (ERRalpha) transcriptional activity at the SP-A promoter by increasing its interaction with protein kinase A and steroid receptor coactivator 2 (SRC-2), Mol Endocrinol 23(6):772-83.

Lynch JT, Harris WJ, Somervaille TC (2012) LSD1 inhibition: a therapeutic strategy in cancer?, Expert Opin Ther Targets 16(12):1239-49.

Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schütz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P, Evans RM (1995)

The nuclear receptor superfamily: the second decade, Cell 83(6):835-9.

Margueron R, Trojer P, Reinberg D (2005) The key to development: interpreting the histone code?, Curr Opin Genet Dev. 15(2):163-76.

Martin C, Zhang Y (2005) The diverse functions of histone lysine methylation, Nat Rev Mol Cell Biol 6(11):838-49.

Mayr B and Montminy M (2001) Transcriptional regulation by the phosphorylation-dependent factor CREB, Nat Rev Mol Cell Biol 2, 599–609.

Metzger E, Wissmann M, Yin N, Müller JM, Schneider R, Peters AH, Günther T, Buettner R, Schüle R (2005) LSD1 demethylates repressive histone marks to promote androgen-receptor-dependent transcription, Nature 437(7057):436-9.

Moras D and Gronemeyer H (1998) The nuclear receptor ligand-binding domain: Structure and function, Curr Opin Cell Biol 10:384–391.

Nakhla A, Khan M, Romas N, Rosner W (1994) Estradiol causes the rapid accumulation of cAMP in human prostate, Proc Natl Acad Sci 91(12), 5402-5405.

Nef S and Parada LF (2000) Hormones in male sexual development, Genes Dev 14(24):3075-86.

Nielsen SJ, Schneider R, Bauer UM, Bannister AJ, Morrison A, O'Carroll D, Firestein R, Cleary M, Jenuwein T, Herrera RE (2001) Rb targets histone H3 methylation and HP1 to promoters, Nature 412, 561-565.

Paik WK, Kim S (1971) Protein methylation, Science 174(4005):114-9.

Perillo B, Ombra MN, Bertoni A, Cuozzo C, Sacchetti S, Sasso A, Chiariotti L, Malorni A, Abbondanza C, Avvedimento EV (2008) DNA oxidation as triggered by H3K9me2 demethylation drives estrogen-induced gene expression, Science 319(5860):202-6.

Pollock JA, Larrea MD, Jasper JS, McDonnell DP, McCafferty DG (2012) Lysine-specific histone demethylase 1 inhibitors control breast cancer proliferation in ERα-dependent and -independent manners, ACS Chem Biol 7(7):1221-31.

Prall OW, Rogan EM, Sutherland RL (1998) Estrogen regulation of cell cycle progression in breast cancer cells, J Steroid Biochem Mol Biol 65(1-6):169-74.

Qian C, Zhang Q, Li S, Zeng L, Walsh MJ, Zhou MM (2005) Structure and chromosomal DNA binding of the SWIRM domain, Nat Struct Mol Biol 12, 1078–1085.

Rehmann H, Wittinghofer A, Bos JL (2007) Capturing cyclic nucleotides in action: Snapshots from crystallographic studies, Nat Rev Mol Cell Biol 8, 63–73.

Rotili D and Mai A (2011) Targeting Histone Demethylases: A New Avenue for the Fight against Cancer, Genes Cancer 2(6):663-79.

Rowan BG and O'Malley BW (2000) Progesterone receptor coactivators, Steroids 65(10-11), 545-549.

Ruthenburg AJ, Allis CD, Wysocka J (2007) Methylation of lysine 4 on histone H3: intricacy of writing and reading a single epigenetic mark, Mol Cell. 12;25(1):15-30.

Sassone-Corsi P (1995) Transcription factors responsive to cAMP, Annu Rev Cell Dev Biol 11: 355–377.

Schwabe JWR, Chapman L, Finch JT, Rhodes D (1993) The crystal structure of the estrogen receptor DNA- binding domain bound to DNA: How receptors discriminate between their response elements, Cell 70:567–578.

Shi Y, Lan F, Matson C, Mulligan P, Whetstine JR, Cole PA, Casero RA (2004) Histone demethylation mediated by the nuclear amine oxidase homolog LSD1, Cell 119, 941–953.

Shi Y. and Whetstine J. R (2007) Dynamic Regulation of Histone Lysine Methylation by Demethylases, Molecular Cell 25(1):1-14.

Shi YJ, Matson C, Lan F, Iwase S, Baba T, Shi Y (2005) Regulation of LSD1 histone demethylase activity by its associated factors, Mol Cell 19(6):857-64.

Starzec AB, Spanakis E, Nehme A, Salle V, Veber N, Mainguene C, Planchon P, Valette A, Prevost G, Israel L (1994) Proliferative responses of epithelial-cells to 8-bromo-cyclic AMP and to a phorbol ester change during breast pathogenesis, J Cell Physiol 161:31-38.

Stavropoulos P, Blobel G, and Hoelz A (2006) Crystal structure and mechanism of human lysine-specific demethylase-1, Nat Struct Mol Biol 13, 626–632.

Szego C and Davis J (1967) Adenosine 3', 5'-monophosphate in rat uterus: acute elevation by estrogen, Proc Natl Acad Sci 58(4), 1711-1718.

Szego EM1, Barabás K, Balog J, Szilágyi N, Korach KS, Juhász G, Abrahám IM (2006) Estrogen induces estrogen receptor alpha-dependent cAMP response element-binding protein phosphorylation via mitogen activated protein kinase pathway in basal forebrain cholinergic neurons in vivo, J Neurosci 26(15):4104-10.

Tanenbaum DM, Wang Y, Williams SP, Sigler PB (1998) Crystallographic comparison of the estrogen and progesterone receptor's ligand binding domains, Proc Natl Acad Sci 95:5998–6003.

Tasken K, Skalhegg BS, Tasken KA, Solberg R, Knutsen HK, Levy FO, Sandberg M, Orstavik S, Larsen T, Johansen AK (1997) Structure, function and regulation of human cAMP-dependent protein kinases, Adv Second Messenger Phosphoprotein Res 31: 191–203.

Taverna SD, Li H, Ruthenburg AJ, Allis CD Patel DJ (2007) How chromatin-binding modules interpret histone modifications: lessons from professional pocket pickers, Nat Struct Mol Biol 14(11):1025-40.

Tierney T and Robinson ICAF (2002) Increased lactotrophs despite decreased somatotrophs in the dwarf (dw/dw) rat: a defect in the regulation of lactotroph/somatotroph cell fate?, J Endocrinol 175:435-446.

Tsai MJ and O'Malley BW (1994) Molecular mechanisms of action of steroid/thyroid receptor superfamily members, Ann. Rev. Biochem 63:451–486.

Väänänen HK and Härkönen PL (1996) Estrogen and bone metabolism, Maturitas S65-9.

Vakoc CR, Mandat SA, Olenchock BA, Blobel GA (2005) Histone H3 lysine 9 methylation and HP1gamma are associated with transcription elongation through mammalian chromatin, Mol Cell 19(3):381-91.

Watters JJ, Dorsa DM 1998 Transcriptional effects of estrogen on neuronal neurotensin gene expression involve cAMP/protein kinase A-dependent sig- naling mechanisms. J Neurosci 18:6672–6680.

Wielinga PR, Heijden I, Reid G, Beijnen JH, Wijnholds J, Borst P (2003) Characterization of the MRP4- and MRP5-mediated transport of cyclic nucleotides from intact cells, J Biol Chem 278, 17664–17671.

Wissmann M, Yin N, Müller JM, Greschik H, Fodor BD, Jenuwein T, Vogler C, Schneider R, Günther T, Buettner R, Metzger E, Schüle R (2014) Cooperative demethylation by JMJD2C and LSD1 promotes androgen receptor-dependent gene expression, Nucleic Acids Res 42(17):11040-55.

Wong W, Scott JD (2004) AKAP signaling complexes: Focal points in space and time, Nat Rev Mol Cell Biol 5: 959–970.

Yang M, Gocke CB, Luo X, Borek D, Tomchick DR, Machius M, Otwinowski Z, Yu H (2006) Structural basis for CoREST-dependent demethylation of nucleosomes by the human LSD1 histone

demethylase, Mol Cell 23(3):377-87.

Yoneyama M, Tochio N, Umehara T, Koshiba S, Inoue M, Yabuki T, Aoki M, Seki E, Matsuda T, Watanabe S, Tomo Y, Nishimura Y, Harada T, Terada T, Shirouzu M, Hayashizaki Y, Ohara O, Tanaka A, Kigawa T, Yokoyama (2007) Structural and functional differences of SWIRM domain subtypes, J Mol Biol 369, 222–238.

Yu SE and Jang YK (2012) The histone demethylase LSD1 is required for estrogen-dependent S100A7 gene expression in human breast cancer cells, Biochem Biophys Res Commun 427(2):336-42.

Zhang Y and Reinberg D. (2001) Transcription regulation by histone methylation: interplay between different covalent modifications of the core histone tails, Genes Dev. 15(18):2343-60

Zhu Q, Huang Y, Marton LJ, Woster PM, Davidson NE, Casero RA Jr (2012) Polyamine analogs modulate gene expression by inhibiting lysine-specific demethylase 1 (LSD1) and altering chromatin structure in human breast cancer cells, Amino Acids 42(2-3):887-98.

Zhu Q, Liu C, Ge Z, Fang X, Zhang X, et al. (2008) Lysine-specific demethylase 1 (LSD1) Is required for the transcriptional repression of the telomerase reverse transcriptase (hTERT) gene. PLoS One; 3:e1446.

Zuchegna C, Aceto F, Bertoni A, Romano A, Perillo B, Laccetti P, Gottesman ME, Avvedimento EV, Porcellini A (2014) Mechanism of retinoic acid-induced transcription: histone code, DNA oxidation and formation of chromatin loops, Nat Cell Biol 9(3):347-53.