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New insights in the epigenetic control of EMT

Memòria presentada per en **Nicolás Herranz Martín** per optar al Grau de Doctor

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Als meus pares

Men love to wonder, and that is the seed of science.

Ralph Waldo Emerson

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ABSTRACT

The epithelial to mesenchymal transition (EMT) is a highly conserved cellular program that allows well-differentiated epithelial cells to convert to motile mesenchymal cells. EMT is critical for appropriate embryogenesis and plays a crucial role in tumorigenesis and cancer progression. At this regard, it has become increasingly evident that, in addition to genetic alterations, tumour development involves the alteration of gene expression patterns owing to epigenetic changes. Taking this into account, this thesis mainly addresses the description of new molecular epigenetic mechanisms underlying one of the hallmark processes governing EMT, the Snail1-mediated E-cadherin repression. Indeed, our results demonstrate that both Polycomb group (PcG) proteins and the LOXL2 protein are involved in this process. Apart from providing novel insights into the significance of these proteins in tumor progression, our work uncovers the characterization of a new epigenetic modification carried out by LOXL2; H3K4 deamination.

RESUM

La transició epiteli-mesènquima (EMT) és un programa cel·lular molt conservat que permet a les cèl·lules epitelials convertir-se en cèl·lules mesenquimals indiferenciades. La EMT és un procés crucial pel desenvolupament embrionari i per la progressió tumoral. A aquest respecte, ha esdevingut cada cop més evident que el desenvolupament tumoral no només està associat a alteracions genètiques, sinó també a l'alteració de l'expressió gènica causada per canvis epigenètics. Tenint això en compte, aquesta tesi es centra en la descripció de nous mecanismes moleculars en l'àmbit de l'epigenètica associats a un dels processos clau en la EMT, la repressió de la E-cadherina mitjançada pel factor de transcripció Snail1. De fet, els nostres resultats demostren que tant les proteïnes del grup Polycomb (PcG) com la proteïna LOXL2 estan implicades en aquest procés. A part de proporcionar nova informació respecte la importància d'aquestes proteïnes en la progressió tumoral, la nostra feina ha permès la caracterització d'una nova modificació epigenètica duta a terme per la proteïna LOXL2; la deaminació de H3K4.

ABBREVIATIONS

5-aza-dC: 5-aza-20-deoxycytidine

BMI1: BMI polycomb ring finger oncogene

BSA: Bovine Serum Albumin

CAF-1: Chromatin assembly factor 1

CDH1: E-cadherin Gene

CDKN2A: cyclin-dependent kinase inhibitor 2A

cDNA: complementary DNA

CFTF: Cell fate transcription factor

ChIP: Chromatin IP

CoREST: Corepressor for REST

CpG: Cytosine Phosphate Guanine

CSC: cancer stem cells

DMEM: Dulbecco's Modified Eagle's

DNA: Deoxyribonucleic acid

DNMT1: DNA methyltransferase 1

ECM: Extracellular Matrix

EED: Embryonic Ectoderm Development

EMT: Epithelial-to-Mesenchymal

Ezh ½: Enhancer of zeste 1/2

FBS: Fetal Bovine Serum

GFP: Green Fluorescent Protein

GSK-3β: Glycogen Synthase Kinase-3 **β**

HAT: histone acetyltransferases

HDAC: Hystone Deacetylase

HEK293: Human Embryonic Kidney 293 cells

HOX: Homeobox

HP1: heterochromatin protein 1

HPRT: Hypoxanthine Guanine Phosphoribosyl Transferase

IF: immunofluorescence

IgG: Immunoglobulin

IP: Immunoprecipitation

JMJ: jumonji

KO: Knock Out

LOX: Lysil oxidase

LOXL2: Lysyl Oxidase-Like 2

Lys: Lysine

MCS: migrating cancer stem cells

MET: Mesenchymal-to-Epithelial Transition

miRNA: microRNA

MLL: mixed-lineage leukaemia

MMP: matrix metalloproteinase

mRNA: Messenger RNA

Muc-1: Mucin1

ncRNA: non-coding RNA

NES: Nuclear Export Signal

PBS: Phosphate Buffered Saline

PcG: Polycomb Group of proteins

PRC1/2: polycomb repressive complex 1/2

PRMT: protein arginine methytransferase

PTEN: phosphatase and tensin homolog

qRT-PCR: quantitative RT-PCR

Ring1B: ring finger protein 2

RNA: Ribonucleic acid

SAM: S-adenosyl methionine

SD: Standard Deviation

SDS: sodium dodecy sulfate

shRNA: short hairpin RNA

SUZ12: supressor of zeste 12

SWI/SNF: mating type SWItch/sucrose non fermentable

TF: Transcription factor

TGFβ: Transforming Growth Factor beta

TSS: Transcription Start Site

UTR: Untranslated Region

WB: Western Blot

WT: Wild type

ZEB1/2: Zinc finger E-box binding homeobox ½

Introduction

1.CANCER and EMT

Today, cancer is a leading cause of death worldwide. Although tumors show marked heterogeneity in their cellular morphology, proliferative index, genetic lesions and therapeutic response, almost all of them shared some of the following essential traits; self-sufficient growth, unlimited replicative potential, evasion of apoptosis and immune response, and ability for sustained angiogenesis, tissue invasion and metastasis.[1,2]

Within cancer pathogenesis, metastasis is responsible for as much as 90% of cancer-associated mortality, and at the same time is the most poorly understood component.

During metastatic dissemination, a cancer cell from a primary tumor executes the following sequence of steps: It locally invades the surrounding tissue, enters the microvasculature of the lymph and blood systems (intravasation), survives and go through the bloodstream to microvessels of distant tissues, exits from the bloodstream (extravasation), survives in the microenvironment of distant tissues, and finally adapts to the foreign microenvironment of these tissues in ways that facilitate cell proliferation and the formation of a macroscopic secondary tumor (colonization)[3] (Fig. I1)

1.1 Carcinoma, general insights

Carcinomas arise in epithelial tissues. Normally, the cells forming the epithelial layers in these tissues are tightly bound to neighboring cells and to basement membranes by adherens junctions, tight junctions, desmosomes and hemi-desmosomes. These tight physical constraints are found not only in normal epithelial cells, but also in those within many benign carcinomas. However, as a tumor progresses, carcinoma cells liberate themselves from these associations, first by dissolving underlying basement membranes and then invading adjacent stromal compartments. This invasiveness seems to give power to carcinoma cells to both intravasate and subsequently extravasate[4].

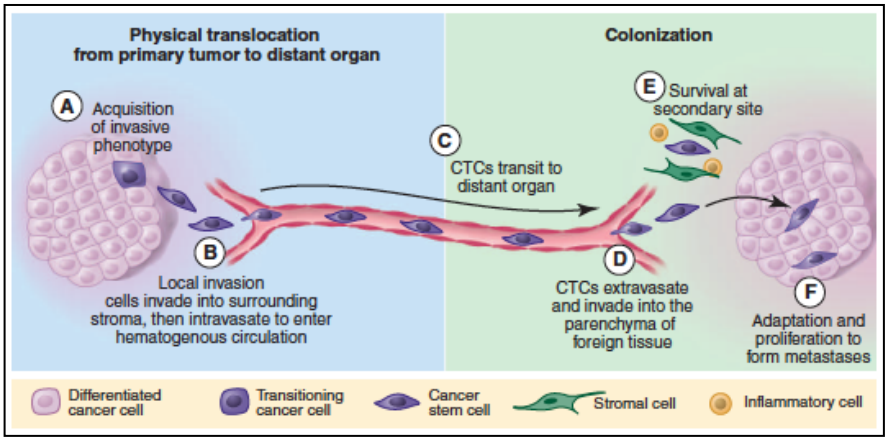


Figure I1. A general model for the metastasis process. Metastasis can be understood as a process that occurs in two major phases: (i) physical translocation of cancer cells from the primary tumor to a distant organ and (ii) colonization of the translocated cells within that organ. Summary of the different steps (A-F) can be found in the figure itself. CTCs refer to cancer cells travelling through circulation. *From Chaffer and Weinberg, 2011*[5]

The cellular origins of most carcinomas are largely unknown, but it has been speculated that different subtypes reflect distinct cells of origin at the time of tumor initiation. In addition to the acquisition of genetic and epigenetic mutations, interactions between tumor cells and their microenvironment (stroma, inflammatory cells and recruited vasculature) have a very important influence on the tumorigenic process.

In addition to different tumor subtypes, cells within the tumor population itself often exhibit functional heterogeneity, with cells exhibiting distinct proliferative and differentiative capacities[6]. To date, at least two models have been proposed to explain heterogeneity and observed differences in tumor-regenerating capacity: the cancer stem cell (CSC)[7,8]and clonal evolution models[9,10].

CSCs refer to a subset of tumor cells that has the ability to self-renew and generate the diverse cells that comprise the tumor[7,8,11]. One feature of the CSC model is that cancers are hierarchically arranged with CSCs lying at the top of the hierarchy[7].

The clonal evolution model postulates that tumor cells containing genetic and epigenetic mutations acquire a growth advantage and are selected and expanded. Both paradigms of tumor propagation are likely to exist in human cancer and are not mutually exclusive, as for example CSCs themselves, undergo clonal evolution. In fact, a second, more dominant CSC may emerge if a mutation confers more aggressive self-renewal or growth properties.

It is important to note that CSCs are distinct from the so-called cell of origin. The cell of origin specifically refers to the cell type that receives the first oncogenic hit(s). Moreover, CSCs do not necessarily

originate from the transformation of normal stem cells, CSCs may arise from restricted progenitors or more differentiated cells that have acquired self-renewing capacity.

Many of the malignant traits found in carcinomas have been associated with subpopulations of CSCs[12-14]. At this regard, CSCs have shown the ability to generate new tumors when implanted into appropriate animal hosts[15,16]. Thus, it is clear that CSCs and disseminated cancer cells share self-renewal and tumor-initiating abilities. However, other typical metastasis traits as motility, invasiveness and resistance to apoptosis have not been that clearly associated with CSCs[12-14]. This implies that whichever is the cancer origin cell population, a coordinated series of cellular and molecular processes are required to enable cancer cells within primary tumors to execute the multiple steps of the invasion-metastasis cascade. At this regard, one of the processes that have been more intensively investigated is the epithelial-to-mesenchymal transition (EMT). EMT triggers a change of phenotype that provides epithelial cells with carcinoma features, being enhanced invasiveness one of the most remarkable ones.

1.2 Epithelial-to-mesenchymal transition (EMT)

The term EMT describes a series of events during which epithelial cells lose many of their epithelial characteristics and take on properties that are typical of mesenchymal cells. This mechanism was originally identified during specific stages of embryonic development, in which epithelial cells migrate and colonize different embryonic territories in order to form new structures[17,18]. The reverse process,

known as mesenchymal–to-epithelial transition (MET) has also been reported.

Although EMT does not necessarily refer to a lineage switch, it involves profound phenotypic changes that include; the loss of cell-cell adhesion structures, modulation of cell polarity and cytoskeleton rearrangement, and the acquisition of migratory, invasive and anti-apoptotic properties.

Epithelial and mesenchymal cells differ in various functional and phenotypic characteristics (Fig.I2).

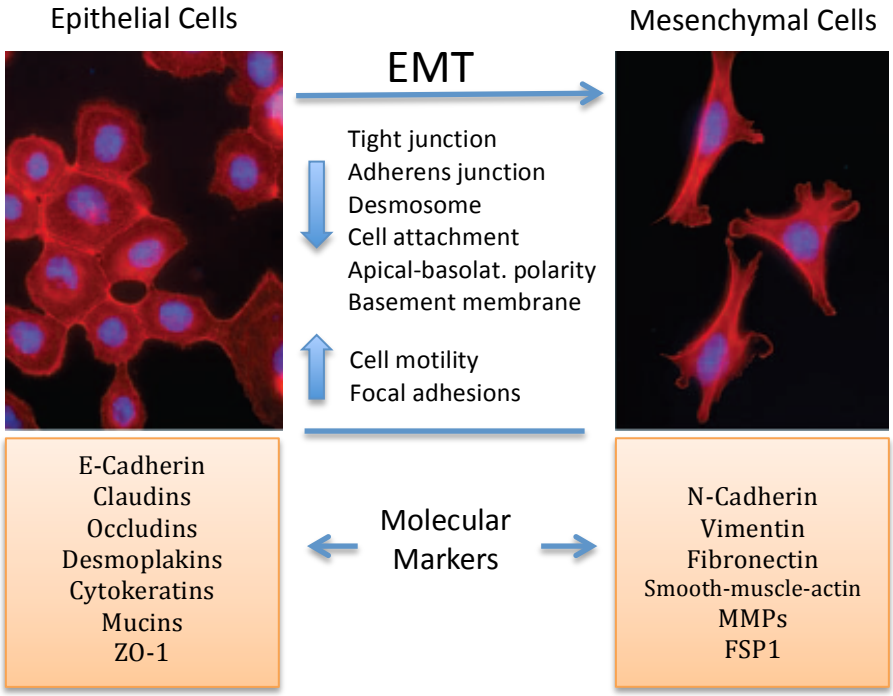


Figure I2. Epithelial and mesenchymal cell traits. The figure summarizes the morphological and physical differences between epithelial and mesenchymal cells.

As mentioned before, epithelial cells form layers of tightly bound cells through the sequential arrangement of adherent junctions, tight junctions, desmosomes and hemi-desmosomes. One of the best-characterized components of the adherens junctions, which form a continuous belt below the apical surface, is the transmembrane protein E-cadherin. Its extracellular domain mediates calcium-dependent homotypic interactions with adjacent cells while the intracellular domain connects with the actin cytoskeleton indirectly via catenins. Early contacts between two cells are also mediated by E-cadherin molecules, which cluster into small complexes expanding afterwards to form stable adherens junctions and promote the formation of desmosomes below them[19,20].

Tight junctions are situated just above the adherens junctions, at the apical side of the lateral membrane. Claudins and occludins are the transmembrane proteins typical of this type of junctions. Together with the adherens junctions, tight junctions seal intercellular spaces between cells and form permeability barriers. This localized distribution of the adhesion molecules together with polarized organization of the actin cytoskeleton and the presence of a basal lamina at the basal surface establish the apical–basolateral polarization observed in epithelial cells, which, under normal conditions, do not detach and move away from the epithelial layer.

In contrast to well-differentiated epithelial cells, mesenchymal cells do not form an organized cell layer, nor do they have the same apical–basolateral organization and polarization of the cell-surface molecules and the actin cytoskeleton as epithelial cells. They contact neighboring mesenchymal cells only focally, and are not typically associated with a basal lamina. In culture, whereas epithelial cells

grow as clusters of cells, mesenchymal cells have a spindle- shaped, fibroblast-like morphology. Other important features of these cells include; front-back end asymmetry that facilitates motility and locomotion[21] , filopodia, formed at the leading edge and enriched with integrin receptors that interact with the extracellular matrix, matrix metalloproteinases (MMP) that digest basement membranes[22] and invasive motility. Intermediate filaments, such as vimentin, and extracellular matrix components, such as fibronectin and collagen precursors, are increased in mesenchymal cells[23].

1.2.1Physiological EMT

- EMT in development

As mentioned before, the transition of epithelial to mesenchymal cells is not irreversible. Indeed, several rounds of EMT and MET are necessary for the final differentiation of specialized cell types and the acquisition of the complex three-dimensional structure of internal organs. Accordingly, these sequential rounds are referred to as primary, secondary, and tertiary EMT. Examples of primary EMT include gastrulation and neural crest delamination.

Gastrulation is the universal process by which the body plan is established. Among the required processes for proper gastrulation, EMT is involved in the formation of the mesoderm (also known as the third germ layer) from the primitive ectoderm. Following invagination of epithelial cells around the primitive streak, the basal membrane breaches locally and cells lose their cell-cell adhesion, remaining attached to the neighboring cells only by disperse focal contacts. The completion of EMT during gastrulation and in consequence the formation of the mesoderm occurs when referred

cells migrate along the extracellular space underneath the ectoderm[24]. (Fig. I3)

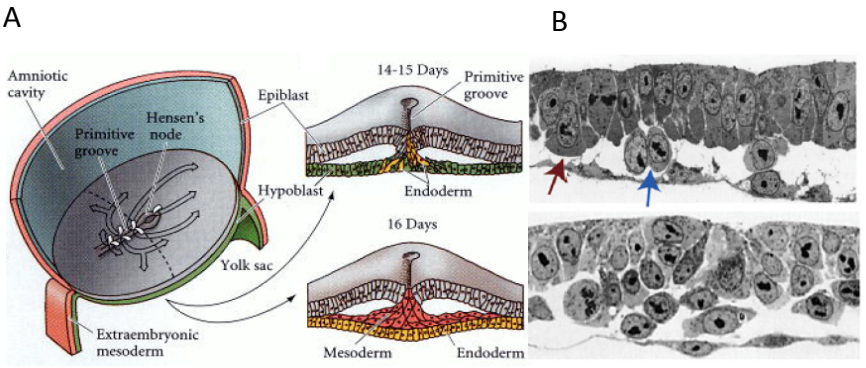


Figure I3. Cell movements during human gastrulation. A) Movements of the epiblast cells through the primitive streak and Hensen's node and underneath the epiblast are superimposed on the dorsal surface view. At days 14 and 15, the ingressing epiblast cells are thought to replace the hypoblast cells, while at day 16, the ingressing cells fan out to form the mesodermal layer[25]. **B)** Posterior third of a midsagittal 1 mm section from rabbit embryo at 6.6 days with three definitive mesoderm cells (blue arrow) between epiblast and hypoblast and a bottle-shaped epiblast cell (red arrow), which is about to ingress to become a definitive mesoderm cell.[24]

Another example of EMT is provided by the neural crest delamination. After gastrulation, neural crest is developed at the boundary between the neural plate and the epidermal ectoderm. An specific subpopulation of cells acquire a rounded and pleiomorphic shape, very different from that observed in the polarized cells of the neural tube, and proceed to lose cell-cell adhesion, thus becoming excluded from the neural epithelium[26] and actively invading through the basal lamina to migrate away from the neural tube (Fig.I4). These cells will finally differentiate into bone, smooth muscle, peripheral neurons, glia and melanocytes[27-29].

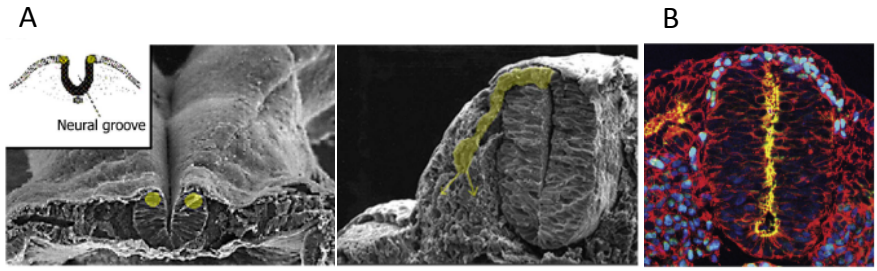


Figure I4. Neural crest delamination. A) The cells at the tips of he neural folds, lying between the neural tube and the overlying epidermis, become the neural crest cells (left). Following the closure of the trunk neural folds, the neural crest cells leave the dorsal aspect of the neural tube (right).[30] **B)** Immunofluorescence image showing that neural crest cells (light blue) emerge from the neural epithelium (yellow).[24]

- EMT in adult tissues

Physiological response to injury is another example of EMT, in this case, in adult tissues. During wound healing, keratinocytes at the border of the wound run through part of the EMT process. In contrast to cells on-going a complete EMT, these keratynocites appear to acquire an intermediate phenotype known as the "metastable" state, which allows them to spread and migrate actively but maintain some cell-cell cohesiveness allowing the so-called cohort migration. Cell-cell adhesion structures such as adherens junctions and desmosomes are reorganized with a sparse distribution[31].

1.2.2 Pathological EMT

EMT not only occurs during embryonic development or as a physiological response to injury, but, as previously indicated, is also an important element in cancer progression, as well as in other pathologies that involve organ degeneration, such as fibrosis. At the cellular level, pathological EMTs are quite similar to physiological

EMTs, as similar signalling pathways, regulators, and effector molecules govern them. Here we focus on EMT role as a metastasis-promoting process.

During the past years, many studies of in vitro cancer models have evidenced that EMT triggers dissemination of single carcinoma cells from the site of the primary tumors to distant sites. Lately it has also become more evident that the activation of an EMT program during tumorigenesis often requires signalling between cancer cells and neighbouring stromal cells[24].

It is thought that islands of cancer cells in advanced primary carcinomas recruit a variety of cell types into the surrounding stroma, such as fibroblasts, myofibroblasts, granulocytes, macrophages, mesenchymal stem cells, and lymphocytes. These recruited cells create an inflammatory microenvironment known as "reactive" stroma, which releases EMT-inducing signals. The carcinoma cells respond to these contextual signals by activating expression of certain transcription factors that proceed to orchestrate EMT programs within these cells.

Although the great number of emerging evidences, EMT relevance in human cancer tissues has remained a matter of debate until very recently. This resistance was mainly due to the lack of convincing evidence of EMT in clinical samples, in part because of the technical difficulties of capturing this transitory process in human cancer patients. At this regard, EMT may be a focal event that is easily overlooked, as may only operate in a small fraction of cancer cells that are in intimate contact with adjacent stroma. At a more practical level, individual mesenchymal cells after EMT are very difficult to distinguish from stromal cells or other tumor-associated fibroblasts.

Another point of discussion comes from the observation that metastases appear histologically similar to the primary tumor[32]. At this regard, some have suggested that carcinoma cells would activate the EMT program to achieve invasion and dissemination to different organs yet, once they have reached those organs, these mesenchymal cells may revert via a MET to an epithelial identity and regain proliferative ability. It is plausible that after extravasating, these cells will not encounter an activated stroma and may well convert back to an epithelial state.

Importantly, it has been proposed that is likely that transformed cancer cells do not undergo a complete EMT. This hypothesis is supported by the fact that they express both epithelial and mesenchymal markers. These intermediate phenotype, referred by Savagner as "metastable phenotype"[33] might be very similar to that described for keratinocytes during physiological wound healing, and surely would facilitate invasive cells to revert its phenotype again when arriving to distant sites (MET).

As indicated before, EMT has been related with CSCs-metastatic potential. In pancreatic carcinoma, it has been reported the existence of two different stem cell-like populations: one that maintains the growth of the primary tumor and another that produces metastatic growth[34]. This hypothesis was previously formulated by Brabletz and colleagues[35] from an analysis of the progression of colon primary tumors and liver metastases. They proposed that CSCs could be divided in two types, stationary cancer cells (SCS) and migratory cancer cells (MCS). MCS can disseminate, and disseminating cancer cells that retain stem-cell functionality can reverse to a differentiated phenotype through MET and generate an epithelial-like structure on

the secondary tumor mass. (Fig.I5) Big similarities can be found between these MCS and the migratory embryonic cells with a mesenchymal phenotype that generate multiple differentiated cell types once they reach their destination.

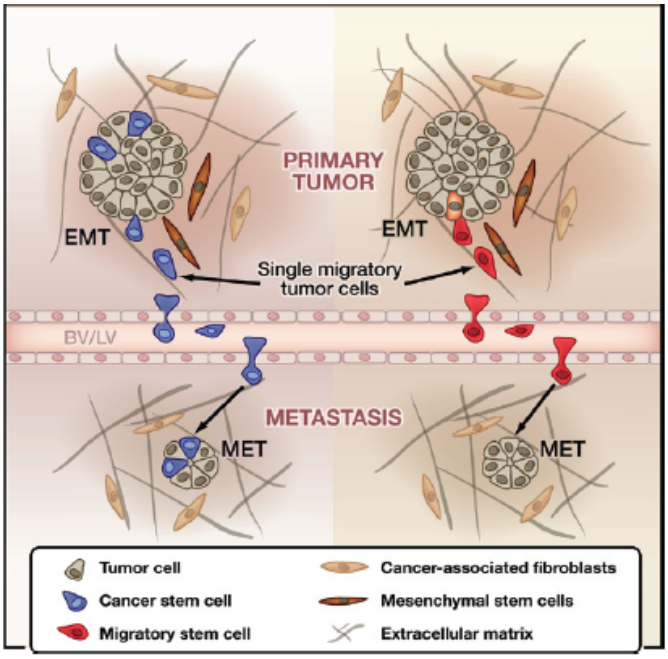


Figure I5. EMT role in tumor progression. EMT is thought to play a fundamental role in tumor progression and metastasis formation. Individual cells delaminate from primary tumors and migrate following the extracellular matrix network. Current challenges focus on understand whether malignant migratory cells are cancer stem cells acting as tumor-initiating cells in the primary tumor (blue cells), if they are derived from somatic epithelial tumor cells that have undergone EMT to acquire stem cell-like properties (red cells), or some combination of these two possibilities. BV/LV, blood vessels/lymphatic vessels.

Interestingly, recent evidences have demonstrated that EMT by itself can induce non-CSCs to enter into a CSC-like state[36,37]. These observations raise the interest in determining whether the invasive

cells disseminating from the primary tumor originate from resident stem cells or if they derive from somatic tumor cells that have undergone EMT. (Fig.I5). In any case, regardless of their origin, such cells must undergo a full mesenchymal transition to become motile and invasive.

Apart from providing cancer cells with migratory and/or stem-like properties, EMT has also been demonstrated to strongly enhance resistance to apoptosis[38,39], which is surely critical to the ability of carcinoma cells to survive the hostile environment from primary tumors to sites of dissemination.

1.3 EMT Molecular Network

A huge number of signaling pathways and agents have been described to induce EMT in numerous cellular contexts, both during development and in normal and transformed cell line. The complexity is so extensive that is preferred to talk about the so-called EMT regulatory network. The signaling pathways include those triggered by different members of the TGFβ superfamily, Wnts, Notch, Receptor tyrosine kinases (EGF, HGF, FGF), HIF, and many others[24,40]. The vast majority of them converge at the induction of the E-cadherin repressors, and in particular, of the Snail1 genes. (Fig. I.6)

1.3.1 E-cadherin (CDH1)

One of the hallmarks of EMT is the functional loss of E-cadherin (encoded by CDH1). E-cadherin downregulation has been associated with carcinoma progression and poor prognosis in various human and mouse tumors[41]. Accordingly, ectopic expression of CDH1 in

certain carcinoma cultured cells deficient for this protein, induces a more epithelial phenotype and decreases their ability to migrate and metastasize[42-44].

The disappearance of E-cadherin from adherens junctions has been associated with the activation of signalling pathways promoting EMT, more precisely, the β-catenin -mediated transcription program. . When CDH1 is disrupted, its mediated sequestering of β-catenin in the cytoplasm is abolished. Then, b-catenin is released and can translocate into the nucleus where it can activate LEF/TCF-mediated transcription[4].

Although E-cadherin functional inactivation has been related with somatic mutations and promoter methylation, in general, CDH1 is not expressed in mesenchymal cells as a consequence of the action of transcriptional repressors. Apart from this transcriptional control, E-cadherin protein stability is also finely tuned: functional E- cadherin, which is present in mature adherens junctions, is highly stable, whereas when not associated to the cytoskeleton the protein is much more labile[45,46]. Thus, only epithelial cells where the adhesive function has been altered ("primed" epithelial cells) will be suitable for down-regulate E-cadherin and undergo an EMT.

Much effort has been dedicated to understand how E-cadherin is regulated at a transcriptional level. Today, CDH1 repressors can be classified into two groups depending on their effects on the *E-cadherin* promoter. Snail1/Slug, Zeb1/2, E47 and KLF8 factors bind to and repress the activity of the E-cadherin promoter[47-52], whereas factors such as Twist, goosecoid, E2.2 and FoxC2 repress it indirectly[24,53].

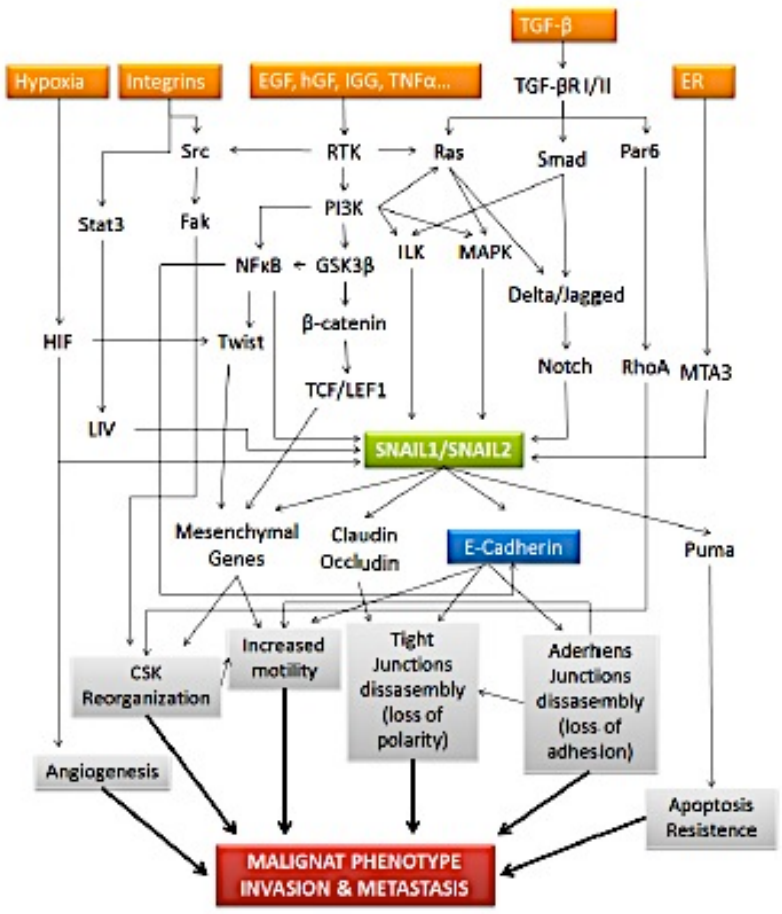


Figure I6. Scheme of the EMT signalling network. In the orange boxes the different stimuli than can promote EMT. In the green box SNAIL1/SNAIL12, the intersection where main pathways cross. In the blue box E-cadherin, the protein which downregulation marks EMT. In grey and red boxes the physiological function regulated, and the final consequence**.**

Here we focus on Snail1 and Zeb family of proteins. These transcription factors binds to specific sequences in the CDH1 promoter, these short base elements are denominated E-boxes (5'-CACCTG-3' or 5'-CAGGTG-3')[54,55]. In the tumor cell lines lacking CDH1, mutation of these elements stimulates CDH1 gene expression by

interfering with the binding of these specific transcriptional repressors[47].

1.3.2 Snail1 protein (SNA1)

Snail1 transcriptional repressors, and particularly Snail1, are the most widely studied effectors of EMT and E-cadherin repression. Snail1 family members belong to the Snail1 superfamily of transcription factors, composed by the SNAIL1 and the SCRATCH family[55]. The three vertebrate members belonging to the SNAIL1 family are known as SNAIL1 (SNAIL1), SLUG (SNAIL12) and the less characterized SMUC (SNAIL13). All the family members encode transcription factors of the zinc-finger type and all share a similar organization with a highly conserved C-terminal domain, which contains from four to six C_2H_2 type zinc fingers responsible for DNA binding through the E-boxes referred before. (Fig.I7)

The N-terminal half of Snail1 is responsible for the fine control of this transcriptional factor activity. (Fig.I7) This part of the protein (in human or murine Snail1 comprising amino acids 1–150) is much more divergent and holds several motifs, being the SNAG domain the most functionally relevant to date. This domain, placed at the N-terminus of all vertebrate Snail1 proteins[55], is required for the binding of co-repressors and epigenetic modifiers. Indeed, SNAG domain integrity is crucial to achieve Snail1-mediated repression. (Detailed information can be found in the following section of this chapter)

The central part of the Snail1 proteins is involved in the regulation of protein stability and localization. (Fig.I7) Different phosphorylation motifs have been defined in this domain. For instance, GSK3β-

mediated phosphorylation of Snail1 has been proposed to facilitate Snail1 nuclear exit by unmasking a nuclear export sequence. Once in the cytosol, further phosphorylation on different residues by the same protein kinase[56] induces Snail1 binding to β-TrCP1 ubiquitin ligase, leading to its ubiquitination and degradation[57]. Snail1 is an unstable protein with a half-life from 20 to 45 min, therefore is likely that other molecules actively regulate its stability, indeed, our lab have recently described another E3 ubiquitin ligase, FBXL14, which interacts with Snail1 through its central domain and promotes its ubiquitination and proteasome degradation[58].

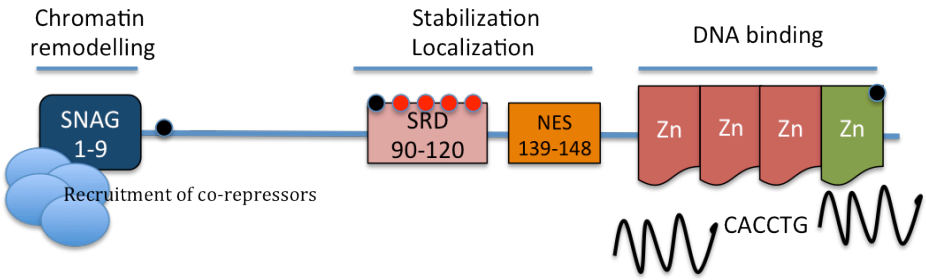


Figure I7. Structure of Snail1 protein. The figure shows a diagram depicting the different domains of Snail1 and their function. Phosphorylation sites are indicated as dots: black, if they stimulate Snail1 action; red, if they inhibit it.

Snail1 pro-metastatic function was strongly highlighted when our lab and others reported that transfection of Snail1 in epithelial cells promotes down regulation of E-cadherin and was sufficient to trigger EMT[47,49]. Accordingly, Snail1 was also pointed as key factor in developmental EMT, as Snail1 KO mouse shows defects in mesoderm formation due to impaired E-cadherin down regulation[59].

As a general rule, CDH1 and Snail1 expression are contrary in cancer cells. When Snail1 expression is depleted, E-cadherin levels are restored and mesenchymal cells revert to a more epithelial phenotype.

E-cadherin is not the only known target of Snail1. Snail1 prevents the expression of epithelium-specific genes such as PTEN, Muc1, Claudin, Occludin, cytokeratin18, desmoplakin as well as some nuclear factor receptors (Vitamin D receptor, HNF-1α)[54]. To exert its repression function, Snail1 may require associating with co-repressors through the SNAG domain, as well as to other proteins (Smad2/3 complex)[60]

Interestingly, Snail1 is also able to stimulate mesenchymal genes transcription. Although the precise mechanism remains unclear it has been proposed that Snail1 activation function depends on E-cadherin repression and, as explained before, the subsequent release of TFs retained by this protein. However, stimulation of gene transcription should not be exclusively explained by E-cadherin down regulation, since Snail1 effects on mesenchymal genes are detected even in cells defective for E-cadherin expression. At this regard, our lab and others, have suggested that, at least in certain conditions, Snail1 might work as direct activator trough interaction with other proteins as β-catenin or NF-κB[61].

Another important point of interest is how Snail1 is regulated at a transcriptional level. Although up-regulated Snail1 gene expression has been detected in cultured cells treated with cytokines such as TGF-β or by over-stimulation of receptor tyrosine kinases, only some downstream factors have been proved to control Snail1 promoter (HGF-Egr1/MAPK1 and TGF-β-HMGA2/Smads)[62,63].

Importantly, it has been observed that Snail1 gene can be regulated by its product, evidencing the existence of a fine-tuning feedback mechanism of regulation of Snail1 transcription[64]. It is worth noting that this kind of self-regulation is particularly relevant in cellular pathways involved in embryonic development[65].

1.3.2.1 Mechanisms underlying Snail1-mediated repression

Till few years ago, the molecular mechanisms underlying E-cadherin repression by factors belonging to the Snail1 family remained unknown. Although there was no evidence for the interaction of Snail1 with co-repressors[66], some previous works had provided insights into the function of the N-terminal region. The repressor effect of Snail1 was associated with a motif found in a short amino-terminal sequence, the SNAG domain (1-9 amino acids)[47,67](Fig. I.15). On the other hand, the repressor domain of human Slug had been attributed to a 32-amino-acid N-terminal domain (containing the SNAG motif) and proposed to require association with HDACs at sites of active transcription[68].

In 2004, Peinado and colleagues reported the first Snail1-containing co-repressor complex, since they identified a functional association of mouse Snail1 (mSnail1), HDAC1/2 and the co-repressor mSin3A[69]. These elements interact to repress E-cadherin expression through the SNAG motif of Snail1. Their results highlight that Snail1-mediated E-cadherin repression is impaired in the absence of the referred co-repressors and that SNAG domain integrity is crucial for the complex assembly. HDACs and Sin3A connection has previously been reported, indeed, the major multiprotein repressive complexes containing class I HDACs(1/2) are Sin3, NuRD, and CoREST[70]. Interestingly, in this work is also suggested that E-cadherin

repression could involve other epigenetic modifiers, as Snail1 expression is related with a decrease of activation methyl marks (K4) and, in some cell lines, DNA hypermethylation of the proximal E-cadherin promoter.

1.3.3 The Zeb family of transcription factors

The Zeb family of transcription factors consists of two members: Zeb1 (also known as TCF8 and δEF1) and Zeb2 (ZFXH1B and SIP1)[71]. Their protein structure is characterized by the presence of two zinc finger domains at both ends of the protein and a homeodomain located in the central part. The zinc finger domains contain from three to four zinc fingers of the C_2H_2 and C_3H type. The members of this family interact with the DNA through the simultaneous binding of the two zinc-finger domains to E-boxes[71]. They have been associated with invasiveness and aggressive behaviour[72]; for instance, Zeb1 expression is important during colon cancer progression, whereas Zeb2 has been studied in ovarian, gastric, and pancreatic tumors[54].

In the context of EMT, Zeb proteins are not as potent as Snail1 in the induction of EMT or in the repression of CDH1 in vitro assays[73], however, their silencing, especially that of Zeb1, has a higher impact on CDH1 expression than Snail1[52,71,74]. Taking into account these observations as well as the fact that Zeb1 activation occurs frequently upon Snail1 activation[75], it has been suggested that whereas Snail1 role could be more important for initiating EMT and E-cadherin repression, Zeb1 downstream activation could be more relevant for the maintenance of this repression. However, it is worth to remark that Zeb genes are active in some tumors that lack Snail1 expression[76]. Thus, the regulation of Zeb1/2 expression might also be independent of Snail1 depending on the cellular context.

Although the list of Zeb1 target genes is growing fast, its mechanism of action remains much more unclear than in the case of Snail1. The structural complexity of ZEB proteins, combining several binding sites for co-repressors with potential posttranslational modifications, points to complex modes of action[71]. Zeb1 and Zeb2-mediated E-cadherin repression has only been associated to their binding elements for CtBP, a ubiquitous co-repressor.

2. EPIGENETICS

Over the past years, it has become increasingly evident that, in addition to genetic alterations, tumor development involves the alteration of gene expression patterns owing to epigenetic changes. Understanding how altered chromatin dynamics leads to malignancy will be essential for designing therapies that reverse the epigenetic state of a tumor cell. Taking this into account, this thesis have been focused on the characterization of the epigenetic mechanisms underlying one of the hallmark processes governing EMT, the Snail1-mediated CDH1 repression. The introduction that follows addresses general topics of the epigenetic field as well as detailed information about epigenetics modifiers involved in histone methylation dynamics, such as Polycomb group of Proteins (PcGs) and histone demethylases. These proteins, especially PcGs, are well-defined developmental regulators; thereby it is plausible that they can have a relevant role in the regulation of both physiological and pathological EMT.

Epigenetics term is used to classify 'those processes that ensure the inheritance of variation (-genetics) above and beyond (epi-) changes in the DNA sequence'[77]. This includes; the study of how patterns of gene expression are passed from one cell to its descendants, how gene expression changes during the differentiation of one cell type into another, and how environmental factors can change the way genes are expressed.

Epigenetic modifications are constituted, principally, by DNA methylation and histone covalent marks. Non-covalent mechanisms,

such as chromatin remodeling and topological replacement of histone variants have also been considered to participate in epigenetic regulation. Indeed, ATP-dependent chromatin remodeling complexes can modify chromatin structure by altering histone-DNA interactions and histone variants are exchanged in specific chromatin contexts. More recently, RNA, in particular, noncoding RNA (ncRNA), has also emerged as an important and intriguing epigenetic mechanism as has been exemplified by X-chromosomal gene-loci silencing by Xist RNA[78]. In fact, this last process is a great example of crosstalk between epigenetic mechanisms (DNA methylation and an specific pattern of histone modifications are also required), but it is not the only one, evidences indicating that a given pattern of different epigenetic events will result in a specific transcriptional output have been accumulating over the past decade. Increasing interest in the field has been accompanied by technological breakthroughs that now make possible to undertake large-scale epigenomic studies. These allow the mapping of epigenetic marks, such as DNA methylation, histone modifications and nucleosome positioning. Integration of such a large amount of data and a better understanding of the underlying mechanisms of the different epigenetics events will facilitate a much better understanding of gene regulation in health and disease.

2.1. Chromatin structure

The DNA within our cells exists in the form of chromatin. The basic building block of chromatin is the nucleosome. In each nucleosome, approximately 147 bp of DNA are wrapped twice around an octamer of the four core histone proteins: an H3-H4 tetramer and two H2A-H2B dimers[79-81]. (Fig.I8)

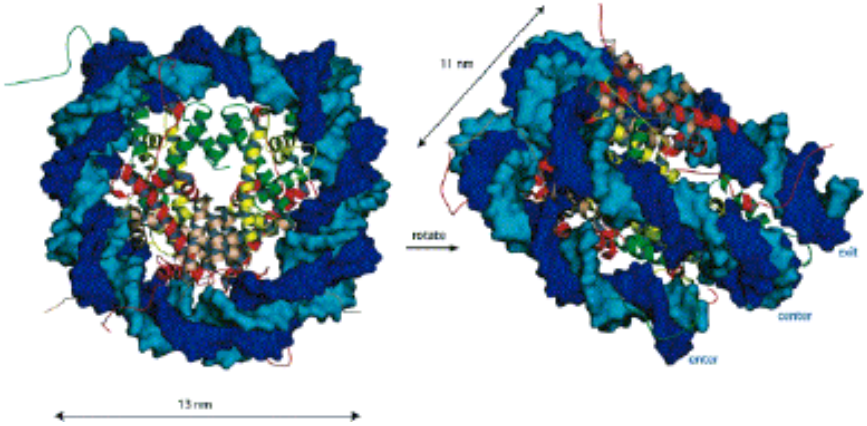


Figure I8. Nucleosome crystal structure. DNA strands are in different colours. DNA makes 1.7 turns around the histone octamer to form a disk-like structure. Atomic structure of the nucleosome core is composed by the H3 (green)-H4 (yellow) tetramer and the H2A (red)-H2B (pink) dimer[82]

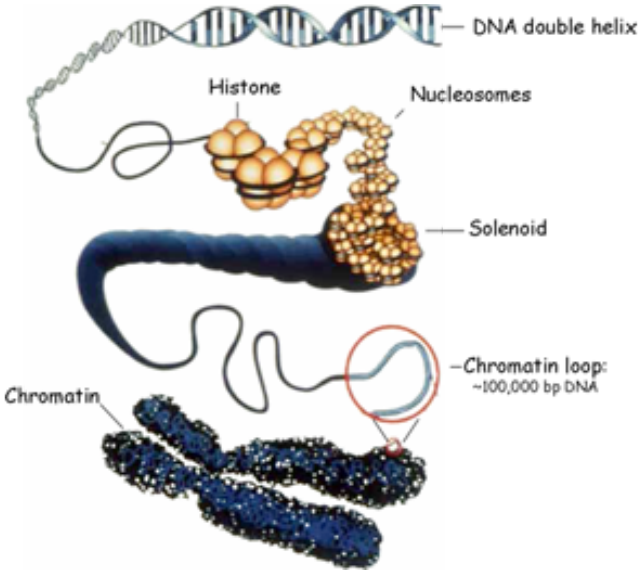


Figure I9. Simplified scheme of chromatin structure. It could be observed how the progressive coiling of nucleosomes leads to the formation of high-oder chromatin structures. (Image from Strahl's lab webpage)

In chromatin's simplest form, the repeating nucleosomal units adopt a decondensed beads-on-a-string configuration. Internucleosomal, intrafiber and interfiber interactions result in the progressive condensation of chromatin[83]. (Fig.I9). Adjacent nucleosomes are connected by linker DNA, where multiple proteins can bind, as for example, the linker histone H1, which completes the so-called chromatosome by protecting internucleosomal linker DNA near the nucleosome entry-exit point[84].

2.2. Histone modifications

The ability of chromatin to condense can be regulated in part by post-translational modification (PTM) of the N-terminal tails of the histones. The four core histones are predominantly globular except for their unstructured N-terminal "tails". These tail domains overhang from the DNA and are, in this way, exposed to chromatin modifying enzymes. These enzymes transducing histone tail modifications are normally highly specific for particular amino acid positions.

At least eight distinct types of modifications on over 60 different residues have been identified in core histones. These modifications include: acetylation, methylation, phosporilation, ubiquitylation, sumoylation, proline isomerization, deimination and ADP ribosylation. (Fig.I10)

The repeated occurrence of certain marks in defined chromatin environments led to the postulation of the so-called "histone code" hypothesis[85,86]. This hypothesis predicted that distinct histone modifications, on one or more tails, act sequentially, or in

combination, to form a `histone code' that is read by other proteins to orchestrate chromatin dynamics and gene expression. Following to this postulate, strong efforts have been directed toward relating histone PTMs with clear transcriptional outcomes and identifying the effector proteins that recognize specific marks. Although knowledge of histones, their effectors, and the influence they carry have advanced tremendously in light of this hypothesis, comprehensive analyses suggest that rather than constituting a general code, the covalent modifications of proteins (including histones) provide surfaces that are recognized by effectors that can give rise to intricate interactions and downstream events. In fact, although often correlative, PTMs have yet to be shown to generally translate into a predictable encrypted outcome.

Chromatin Modification	**Residues**	**Relevant Sites**	**Transcriptional Role**
Acetylation	K-Ac	H3(9,14,18,56)	Activation
		H4(5,8,13,16) H2A/B	Activation
Methylation lysines	K-me1/2/3	H3(4,36,79)	Activation
		H3(9,27) H4(20)	Repression
Methylation arginines	R-me1 R-me2a/s	H3 (17,23) H4 (3)	Activation
Phosphorilation	S-ph T-ph	H3(3,10,28) H2A/B	Activation
Ubiquitylation	K-ub	H2B (120)	Activation
		H2A (119)	Repression
Sumoylation	K-su	H2B(6/7) H2A(126)	Repression
Proline Isomerization	R > Cit	H3(30-38)	Act./Repr.
Deimination	P-cis > P trans	H3(R2,8,17,26) H4(R3)	Repression
Poly-ADP ribosylation	E-ar	H1	Activation

Figure I10. Chromatin modifications. Adapted from Kouzarides, 2007[87,88] and Berger, 2007[13]

2.2.1 Histone methylation

In the 1960s, evidence was accumulating that histone proteins were modified at the posttranslational level by acetylation and methylation. Methylation was shown to occur on the ε-amino group of lysine (K)[89] and the guanidino group of arginine (R)[90,91] and was catalyzed by enzymes using S-adenosyl-L-methionine (SAM) as the methyl group donor[92,93]. In 2000 the first histone methyltransferase was discovered[94].

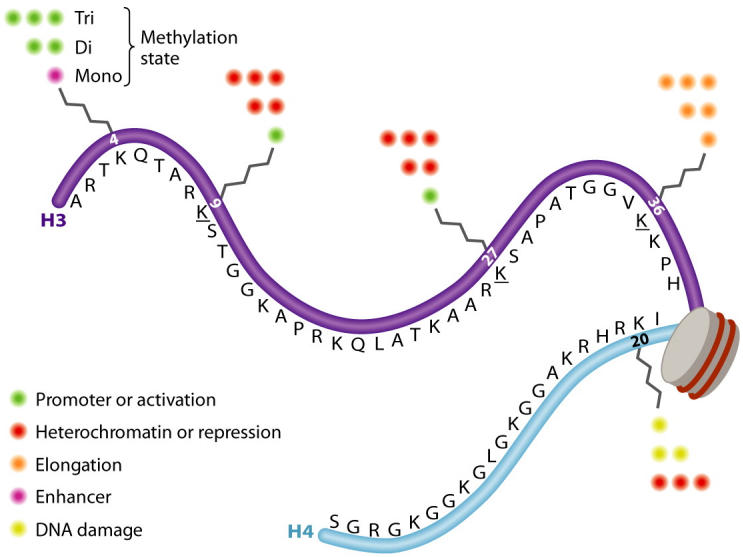


Figure I11. Histone lysine methylation. Major lysine methylation marks on the amino-termini of histones H3 (purple) and H4 (blue). The embedded numbers refer to the methylated amino acid residue on each histone. The general function of each mono-, di-, and trimethylation state is depicted in dots of distinct colors as shown in the figure key. *From Mosammaparast and Shi, 2010*[95]

Histone methylation is now recognized as an important modification linked to both transcriptional activation and repression[96]. Histones

contain numerous lysine and arginine residues, of which many are methylated in vivo[96-98]. Six of the lysine residues, including histones H3K4, -9, -27, -36, and -79 as well as histone H4K20, have been studied extensively and linked to chromatin and transcriptional regulation as well as DNA damage response[96,99] (Fig. I11). Lysine can be mono-, di-, and trimethylated[100], while arginine can be both monomethylated and symmetrically or asymmetrically dimethylated[101]. The numerous lysine and arginine residues on the histone tails, in conjunction with the various methylation levels that can be generated at each of these sites, provide tremendous regulatory potentials for histone modifications.

2.2.1.1. Polycomb group of Proteins (PcGs)

One of the most-well characterized histone modifications is the trimethylation of Lysine 27 in histone H3 (H3K27me3). This modification is carried out by EZH2, one of the components of the Polycomb group of proteins (PcG).

PcG proteins, together with Trithorax group of proteins (TrxGs), were first identified in *Drosophila melanogaster* through their roles as regulators of homeotic (Hox) genes[102-104]. The term Polycomb (Pc) initially referred to a Drosophila mutant that displayed improper body segmentation[105]. The Polycomb group (PcG) now defines a set of genes characterized by mutations that result in similar phenotypes to those of Polycomb. The crucial role of PcG proteins during development is highlighted by early embryonic lethality in mice after the deletion of genes encoding some of these proteins (Eed, Ezh2, Suz12 and Ring1B).

The antagonistic activities of the PcG (repressors) and the trithorax (activators) families of proteins constitute a global regulatory system with key roles not only in development, but also in adult somatic cell differentiation[106], stem cell biology[107] (maintaining correct identities of stem, progenitor and differentiated cells) and cancer[108].

2.2.1.2 Polycomb repressive complexes (PRCs)

At the molecular level, PcG proteins are found in multiprotein repressive complexes, called Polycomb repressive complexes (PRCs). Two main protein complexes have been defined, Polycomb repressive complex 1 (PRC1) and 2 (PRC2). (Fig.I12)

- PRC2

The core PRC2 complex, which is conserved from Drosophila to mammals, comprises four components: EZH1/2, SUZ12, EED and RbAp46/48 (also known as RBBP7/4). PRC2 complex is responsible for the methylation (di- and tri-) of Lys 27 of histone H3 (H3K27me2/3)[109,110] through the HMT activity of its SET domain-containing enzymatic subunits, EZH1 and EZH2. EED and SUZ12 do not possess HMT activity, but are required for the HMT activity of, at least, EZH2[111]. Other PRC2 components; JARID2, PCL and AEBP2, have been recently discovered by different laboratories that were reanalysing the composition of PRC2 with the aim of finding new interaction partners[112-118]. However, these new subunits seem to be present in substoichiometric amounts and interact with the PcG complexes in a cell-context-dependent manner[119]. Further studies are required to elucidate if they are integral PRC2 components as well as to characterize their functions within the complex.

HMTs of PRC2, EZH1 and EZH2, target the same genes and are expected to contribute to the same silencing pathway, however, recent studies point out that EZH1 and EZH2 subunits may not have interchangeable functions[120]. It has been suggested that EZH2 would be responsible for the establishment of the mark, while Ezh1 would be involved in its maintenance.

- PRC1

The composition of PRC1 complexes is much more variable, with only two core common components, the E3 ubiquitin ligases, RING1A and RING1B. RING proteins can be found together with BMI1, MEL18 (PCGF2) or NSPC1 (PCGF1)[110,121]. Both BMI1 and RING proteins have RING finger domains, motifs that are crucial for nuclear localization. PRC1 complexes in humans also include human polyhomeotic homologues (HPH1, HPH2 and HPH3), and Pc components (known as CBX in mammals; CBX2, CBX4, CBX6, CBX7 and CBX8). While HPH proteins have Zinc-finger domains, Chromobox (CBX) proteins contain a chromatin organization domain (chromodomain), some of them specifically recognize and bind to the product of PRC2 catalysis, H3K27me3.

PRC1 components are not as highly evolutionary conserved as PRC2 ones, as for example; they are absent in nematodes and plants. Accordingly, knock-out mice for each core PRC2 components are embryonic lethal, while homozygous null mutant mice for PRC1 genes, except for Ring1B, survive to birth. These could be explained due to functional redundancy and compensation by the wide range of PRC1 genes that are found in vertebrates. Increasing evidences indicates that PRC1 exact composition can determine not only tissue-

specific expression pattern but also specific functions in pathological situations[109,110]

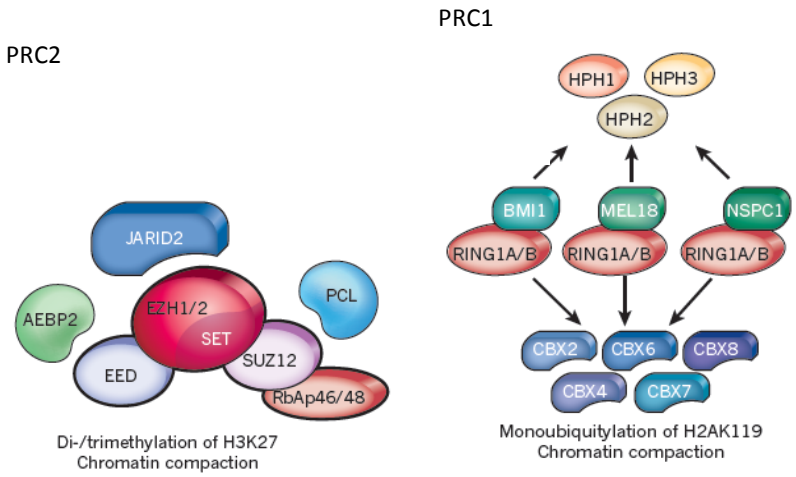


Figure I12. Polycomb repressive complexes (PRCs). Diagrams representing the composition of PRC2 and PRC1 are shown. As it could be observed, the composition of PRC1 complexes is much more variable, with only two core common components, the E3 ubiquitin ligases, RING1A/B. The 'pocket' shape of the CBX proteins represents the chromodomain that specifically recognized H3K9/27me3. *Adapted from Margueron and Reinberg, 2011*[122]

2.2.1.3 PcGs transcriptional repression mechanism

A working model for how PcG complexes modulate chromatin is that transcription factors and their associated machinery dictate loci destined for silencing, and PRC2 tags these target loci by methylating lysine 27 of histone H3.

Though H3K27me3 mark strongly correlates with gene silencing[123], it has not yet been reported that this mark can alter chromatin structure by itself, hence, it has been proposed that H3K27me3 mark is recognized and bound by the chromodomain of CBX proteins within PRC1, which mediates transcriptional silencing by binding

methylated histones in the vicinity and by monoubiquitylating Lys 119 of histone H2A (H2AK119ub) via RING1A/B[124,125]. (Fig.I13)

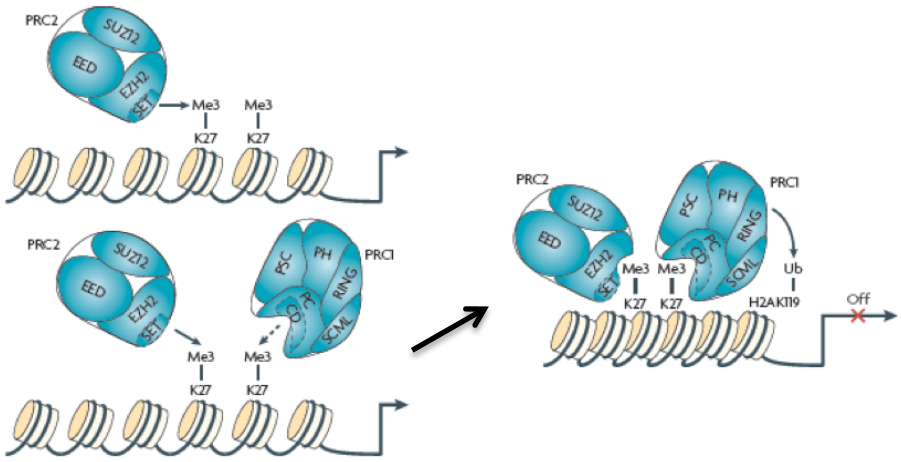


Figure I13. Model for the coordinated action of Polycomb repressive complexes. The PRC2 catalyses the trimethylation of histone H3 at lysine K27 (H3K27me3). The EZH2 SET domain confers this activity. Some results have suggested that PRC1 complexes are recruited by the affinity of chromodomains in chromobox (Cbx) proteins to the H3K27me3 mark. PRC1 recruitment results in the RING1A and RING1B-mediated ubiquitylation of histone H2A on lysine 119, which is thought to be important for transcriptional repression. *From Bracken and Helin, 2009*[126]

Regardless of the proposed hierarchical recruitment of PRC1 and PRC2, there is not a clear description of how PcG complexes impede transcription. At this regard, it has been suggested that PRCs could prevent ATP-dependent nucleosome remodelling by the SWI/SNF complex as well as directly block the transcription machinery. At this respect, although in vitro assays have suggested that PRCs mediate silencing by physically blocking the binding of transcriptional machinery at promoters[127], PRC complexes are more likely to function by impeding RNA polymerase II (RNA Pol II) initiation or

elongation. Indeed, several groups have shown that RNA Pol II and basal transcription factors are present at PcG-silenced loci[128-130].

In addition to methylation, PcG complexes either directly or indirectly recruit proteins that facilitate other covalent modifications, such as histone acetylation and DNA methylation. Histone acetylation status is regulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs). Acetylation of histones near promoters is associated with transcriptional activation; therefore, HATs and HDACs favor transcriptional activation and repression, respectively. As expected, Polycomb function have been linked with HDAC activity; HDACs sirtuin 1 (SIRT1) and HDAC2 have been purified in PRC complexes[131].

PcG complexes can also recruit DNA methyltransferases (DNMTs). More precisely, EZH2, as part of the PRC2 complex, recruits DNMTs to selected target genes[132]. DNA methylation is one of the best-characterized covalent modifications of chromatin. In eukaryotes, DNA methylation involves mostly cytosine bases in the context of a CpG dinucleotide and is associated with a repressive state of chromatin[133]. This dinucleotide tends to cluster in regions called CpG islands[134] which are found in promoters and in the 5' end of many genes (nearly 60% of all genes) and are unmethylated in normal cells. Indeed, DNA hypermethylation of gene promoter regions is an event frequently associated with the suppression of tumour-suppressor genes in cancer cells[135].

2.2.1.4 PcGs recruitment

Exactly how mammalian PRCs are recruited to chromatin is not clear. The effect of H3K27me3 on histone binding by the chromodomains of

Cbx proteins does not explain how PcG proteins are selectively targeted to specific genes in the first place.

In Drosophila, DNA sequences known as Polycomb response elements (PREs) have been identified as targets for PcG protein recruitment based on their ability to confer PcG silencing on reporter genes[109,136,137]. The best-characterized PREs have been delimited to several hundred base pairs, but there is no simple consensus sequence that can supply PRE function. Several TFs have been reported to recruit PcG proteins to PREs; as zinc finger DNA-binding proteins, PHO and PHO-like, and other DNA-binding proteins[136-140]; however, genome-wide analysis showed that any one of these transacting factors only partially overlaps with PcG target genes[122]. It is thought, then, that PREs are composed of multiple binding sites and that a combination of these and other proteins might be responsible for the PcG recruitment.

Mammalian PREs are not yet defined. PRCs-targeted sequences are highly enriched in C+G, most of them being classified as CpG islands, but these sequences alone do not indicate a consensus response element[141]. PRC1 and PRC2 tend to be broadly distributed across many kilobases of mammalian developmental genes[141-143], which, in one hand, confuses identification of PREs as sites of PcG complex accumulation, and in the other, strongly suggest that many different mammalian TFs might contribute to PcG recruitment.

2.1.1.5 PRC2 and cancer

Over past years, there have been lots of evidences that link deregulation of PcG proteins with cancer. The first link between cancer and Polycomb was reported when Bmi1 was found to promote

cancer by inhibiting the Cdkn2a locus[144,145], which encodes the tumor-suppressor proteins $p16^{Ink4a}$, $p15^{Ink4b}$ and $p19^{Arf}$.

Several studies that link Polycomb with cancer, show that upregulation of PcG proteins is related with poor prognosis, indeed, expression of PRC2 components is upregulated in various cancers such as melanoma, lymphoma, and breast and prostate cancer. For example, EZH2 has been reported to be a marker of the aggressive stages of prostate and breast malignancies[146,147], and its overexpression promotes neoplastic transformation of normal prostatic cells[148] and hyperplasia in breast epithelium[147,149]. Contexts were PRC2 components are associated with cancer are not limited to overexpression, but also to PRC2 missense mutations[150-152] and chromosomal translocations. For example, PRC2 proteins are known to target genes by physically interacting with chimeric fusion proteins involved in leukemia, such as PML-RARalpha and TMPRSS2-ERG[153,154].

Taken together, the literature clearly points to a key role of PRC2 proteins in cancer, though, it is unclear if these proteins could be oncogenes per se. At this regard, it has been suggested that their aberrant expression could partly be a consequence of the high proportion of proliferating and/or 'stem-like' cells found in tumors. Accordingly, PRC2 components have been shown to act downstream of the retinoblastoma protein (pRB)-E2F pathway, essential for proliferation in cancer[155]. In fact, it has been observed that deletion of PRC2 components in somatic cells leads to a marked reduction in cell proliferation[146,155].

But which is the precise role of PRC2 from a more mechanistic point of view? One potential scenario is that PcG proteins aberrant

upregulation leads to a progressive recruitment of DNMTs to PcG target genes[156-158] (as indicated before, EZH2 was found to physically associate with DNMTs[132]). Accordingly, PcG target genes are as much as 12 times more likely to be aberrantly silenced by DNA methylation in cancer than non-PcG target genes. Moreover, poorly differentiated and aggressive human tumors show preferential repression of PcG target genes[159].

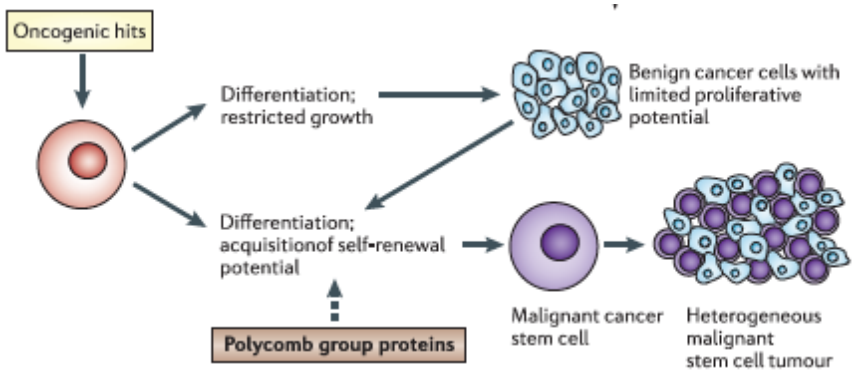


Figure I14. Potential functions of Polycomb proteins during tumour development. PcG-mediated regulation of the stem cell fate might also contribute to the oncogenic properties of these proteins. Tumor cells that have not gained stem cell characteristics have limited proliferative potential and will undergo restricted benign growth. Acquisition of self-renewal properties, possibly through inappropriate expression of PcGs, promotes the formation of malignant tumors with a cancer stem cell compartment driving neoplastic growth and progression. *Adapted from Sparmann and Van Lohuizen, 2006*[106]

PRC2 function has been intensively studied in stem cells. Interestingly, it has been observed that PRC2 is required (i) for the acquisition of stem cells pluripotency[160], and (ii) for the silencing of important genes within cellular differentiation[142,143,161]. With such a

PRC2 pivotal role, some have suggested[106,122] that if similar mechanisms operate during reprogramming of somatic cells or EMT, the overexpression of PRC2 proteins, through gradual Polycomb-mediated de novo DNA methylation, or other mechanisms that remain elusive, could convey self-renewal properties to the cells, induce them to an undifferentiated state and predispose them to malignant transformation. (Fig. I14)

2.2.2 Histone demethylation

Many of the covalent modifications that take place on the histone tails are enzymatically reversible. This enzyme-based reversibility makes sense because it provides the cell with the ability to respond quickly to changes through rapid alteration in its gene expression programs. Unlike phosphorylation or acetylation, methylation on histones tails was thought to be irreversible; in part because of the more stable nature of the C-N bond, but principally based on experiments demonstrating that half-life of histone methyl marks was quite similar to that of the histone itself[162,163]. Some indirect mechanisms, as active histone exchange[164] or proteolitic removal of histone N-terminal[165] were then suggested to explain histone demethylation. In 2004 a study of a corepressor led to the identification of the first histone demethylase[166], thus changing the understanding of histone methylation regulation. Since then, many other demethylases have been identified. (Fig. I8)

2.2.2.1 Lysine-Specific Demethylase 1 (LSD1)

Human LSD1 was originally identified as a component of the BRAF-HDAC (or BHC) transcriptional corepressor complex containing the

REST corepressor, CoREST[167-169]. LSD1 itself could serve as a transcriptional repressor, and this function was reported to be dependent on its amine oxidase domain[166]. Therefore, it was further hypothesized that because methylation of K4 on histone H3 was associated with transcriptional activation, LSD1 may function as an H3K4 demethylase. Indeed it was, revealing for the first time that histone methylation was a dynamic, reversible process[166]. LSD1 demethylation is limited to mono- or dimethylated H3K4 peptides.

The mechanism of histone demethylation by LSD1 appears to be highly conserved among most eukaryotes. In all cases, each organism contains at least two, if not more, LSD1 homologs. Homologs of LSD1 have also been characterized in S. pombe, but surprisingly; demethylate H3K9 instead of H3K4[170,171], suggesting that this mechanism of demethylation may also be used to target other sites of methylation. In fact, human LSD1 was found to function as an H3K9 demethylase and a transcriptional activator in the presence of the androgen receptor (AR)[172]. Although it is likely that protein-protein interaction could regulate substrate specificity, structural basis for this observation remains to be investigated.

In addition to the roles as a histone demethylase, a recent study reported that LSD1 demethylates a methylated form of Lys 370 of the transcription factor p53, thus, modulating p53 action[173].

2.2.2.2 Jumonji C (JmjC) domain-containing proteins

LSD1 discovery suggested that nuclear proteins with the ability to oxidate N-methylated lysines would be excellent demethylases candidates. Zhang and colleagues first reported experimental evidence for an alternative oxidation-reduction mechanism for

histone demethylation[174]. First JmjC domain-containing histone demethylase (JHDM) was discovered using a biochemical assay coupled with chromatography, which leads to the identification of FBXL11 (also known as JHDMI1 and KDM2A). KDM2A contains a Fe(II) dioxygenase Jumonji C (JmjC) domain critical for specific demethylation of mono- and di- but not trimethylated lysine 36 at histone H3 (H3-K36)[174]. Shortly after KDM2A discovery, several reports showed that other JmjC domain-containing proteins can demethylate H3K9me3/me2 and H3K36me3/2, formally demonstrating the reversibility of trimethylated lysine marks[175-178] .

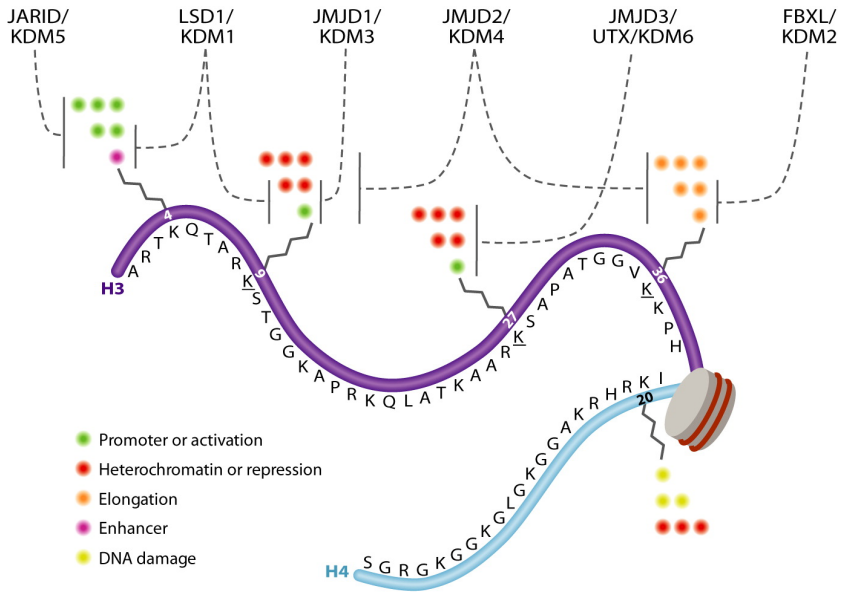


Figure I15. Histone lysine demethylases. Substrate specificity of histone demethylases described to date. Dashed lines point to the methylated residue(s) that are demethylated by the indicated enzymes. The embedded numbers refer to the methylated amino acid residue on each histone. The general function of each mono-, di-, and trimethylation state is depicted in dots of distinct colors as shown in the figure key. . *From Mosammaparast and Shi, 2010*[95]

Today, 27 different JmjC domain proteins have been identified within the human genome, of which 15 have been published to demethylate specific lysines or arginines in the H3 tail. To date, many of the key methylated histone lysine marks have a corresponding JmjC domain-containing histone demethylase. (Fig. I15)

2.2.2.3 Indirect demethylation mechanisms

Demethylation has not been the only reported mechanism to counteract histone methylation. As referred above, in 1980 Allis and colleagues already suggested that protease-mediated histone tail clipping could affect transcriptional regulation[165]. Recently two other articles have described the existence of yeast[179] and mammalian[180] endopeptidases that process the N-terminal H3 tail at specific cleavage sites and exert a transcriptional effect in different physiological contexts. In both studies is suggested that H3 cleavage may be regulated by covalent modifications present on the histone tail itself.

On the other hand, demethylation has also been associated with enzymes shown to convert either non- or mono-methylated arginines into citrulline, therefore effectively eliminating arginine from histone tails[181,182]

Although all these enzymes showed functional consequences in vitro and in vivo, a big question remains unclear; by which mechanisms histones are revert to the original unmethylated state?

3. LOXL2 protein

Lysyl oxidase-like 2 (LOXL2) protein is a member of the LOX family of proteins. This family of proteins has been generally defined as extracellular matrix (ECM) enzymes that catalyze the cross-linking of collagens or elastin in the extracellular compartment, thereby regulating the tensile strength of tissues. However, recent reports have demonstrated novel roles for them, including the ability to regulate gene transcription, motility/migration, and cell adhesion. These diverse functions have led researchers to hypothesize that LOX family of proteins may have multiple roles affecting both extra- and intracellular cell functions. Particularly noteworthy, is the reported aberrant expression of these proteins in various tumor tissues and cancer cell lines.

Here, we focus on the LOXL2 protein. Taken together, the literature indicates that LOXL2 may be an important player in regulating tumor progression and metastasis. Interestingly, it has been reported that LOXL2 binds to Snail1 and is involved in E-cadherin repression and EMT induction; however, the specific LOXL2 mechanism of action in this context remains elusive. In this thesis this matter will be further studied.

The introduction that follows addresses the most relevant observations about LOX family with particular interest in LOXL2 described effects on the context of tumor progression and EMT.

3.1. LOX family of proteins

Five LOX family genes have been identified so far in mammalian genomes encoding the prototypic LOX and LOX-like proteins 1 to 4 (LOXL1, LOXL2, LOXL3, and LOXL4)[183-187](Fig.I16)

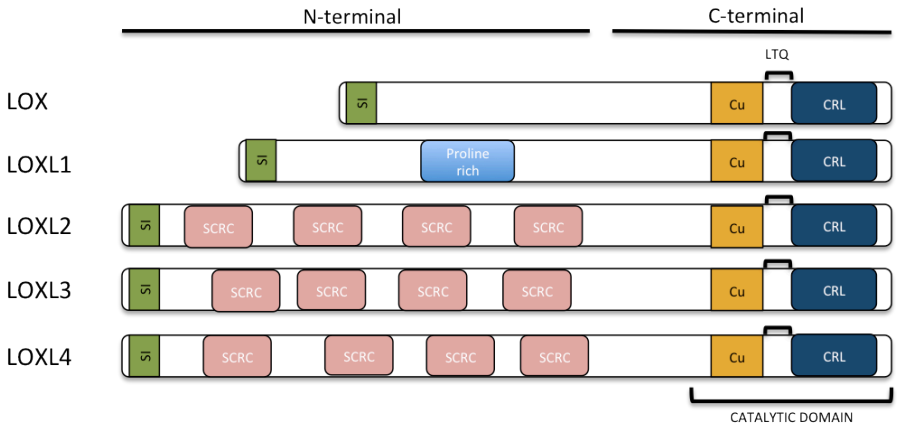


Figure I16. LOX family of proteins. Schematic representation of the five members of LOX family of proteins. si, signal peptide; Cu, copper binding domain; LTQ, LTQ cofactor binding site; CRL, cytokine receptor-like domain; SRCR, scavenger-receptor cysteine-rich domain

The prototypic Lysyl oxidase (LOX) is a copper-dependent amine oxidase that regulates formation of lysine-derived cross-links in elastin and collagen fibers. A variety of chemical, kinetic and spectroscopic studies have detailed a likely mechanism of action of LOX[188-191]. Briefly, LOX oxidatively deaminates a substrate amine of peptidyl lysine residues to an aldehyde product leaving the enzyme in a reduced state. (Fig.I17) Subsequently, molecular oxygen reduces the enzyme back to a catalytically active state and in the process generates ammonium and hydrogen peroxide[192].

Each LOX family protein contains a copper-binding motif, lysyl-tyrosyl-quinone (LTQ) residues, and a cytokine receptor-like (CRL) domain in its highly conserved carboxyl (C)-terminus. In contrast to the characteristic C-terminal domains, the LOX family members show strong sequence divergence in their amino (N)-terminal regions, which indeed, are thought to determine the individual role and tissue distribution of each enzyme. In particular, LOXL2, LOXL3, and LOXL4 contain four scavenger receptor cysteine-rich (SRCR) domains in their N-terminal regions[184-186]. The functional role of the SRCR domains in these three LOX paralogues has not yet been characterized; however, SRCR domains are involved in protein–protein interactions in several secreted or receptor proteins[193].

Lysine crosslinking mediated by at least LOX and LOXL1, is known to be involved in physiological events such as proper elastic fiber homeostasis and cardiovascular system development[194,195]. To promote crosslinking, these proteins must undergo sequential cleavages to be secreted and catalytically active. LOX and LOXL1 are synthesized as preproLOX/LOX1. Following intracellular signal peptide cleavage, proLOX/LOXL1 are secreted, but still as catalytically inactive proteins[196]. Final active enzymes are obtained by sequence-specific proteolytic cleavage[197], which results in the release of the N-terminal propeptide regions that immediately precede the catalytic domains[183,198]. LOXL2, 3, and 4 are also thought to be secreted as proenzymes. However, as they don't share specific-cleavage residues with LOX and LOXL1, it remains unclear from which propeptides are proteolytically released, an, in consequence their role within connective tissue biogenesis. At this regard, it has been recently reported that LOXL2 functions as an amine oxidase toward collagen and elastin in vitro[199].

Over the past years, lysyl oxidases have been related with additional functions beyond their biological role in normal connective tissue regulation. These new functions are partly explained by the fact that LOX proteins expression is not only restricted to the extracellular compartment; but is also detected in the cytoplasm and in the nucleus[192,198,200-203].

Among new roles identified for LOX family of proteins, the most intensively studied to date is their connection to carcinogenesis and metastasis[204]. Both down and up regulation of LOX proteins has been described in tumor tissues and cancer cell lines, suggesting a dual role for these proteins, both as tumor suppressors and metastasis promoter genes. Yet, regardless their contribution to cancer, the relationship between their catalytic activity and their pathologic function is poorly understood. The most appealing mechanisms have been attributed to LOX and LOXL2.

2. LOXL2 protein

LOXL2 was first isolated as an overexpressed gene in senescent fibroblast[186]. Although in early studies, LOXL2 down regulation was found in RAS-transformed rat fibroblasts[205] as well as in some tumors[206-208], the majority of the recent studies have strongly related LOXL2 with tumor invasion and metastasis. In fact LOXL2 overexpression promotes the invasiveness of tumor cells in vivo and in vitro[209,210] and its upregulation has been reported in breast, prostatic[210], esophageal, colon[211], and head-and-neck and oral squamous cell carcinomas[212,213]. LOXL2 is also one of the most highly and specifically upregulated genes in pancreatic cancer compared

with normal pancreatic tissues[214]. In general, LOXL2 upregulation has been related with poor prognosis, in fact, it has been reported more often in invasive metastatic tumors than in non-invasive ones.[211,215]

At a more functional level, it has been proposed that secreted LOXL2 promotes gastric cancer metastasis via the Src/FAK pathway[216] and enhances breast cancer progression[217] through regulation of extracellular proteins like TIMP1 and MMP9. Whether these studies do not provide a specific mechanism of action for LOXL2, they keep on reinforcing LOXL2 key role in metastasis, since they demonstrate that genetic, chemical or antibody-mediated inhibition of LOXL2 results in decreased metastasis.

Regarding anti-LOXL2 therapeutics for metastatic cancers, a recent report from Arresto BioSciences[218] claims that allosteric inhibition of LOXL2 by a monoclonal antibody impedes the development of a pathological microenvironment, and thus, reduces metastatic potential. Inhibition of LOXL2 results in a marked reduction in activated fibroblasts, desmoplasia and endothelial cells. Accordingly, a decreased production of growth factors, cytokines and TGF-B pathway signalling in vivo is also observed.

2.1 LOXL2 and EMT

As previously indicated, LOXL2 expression and function have also been detected inside the cell. The most important study concerning intracellular LOXL2 was reported by Peinado and colleagues[219]. They focused on LOXL2 since they found it interacts with Snail1, which, as explained in detail earlier, is a key factor in the EMT process. They show that LOXL2 represses E-cadherin at a transcriptional level and that its function is essential for Snail1-mediated repression.

Remarkably, they also observe that LOXL2 overexpression by itself can induce EMT.

Depletion of LOXL2 in cells defective for E-cadherin has no effect on proliferation in culture, but it strongly reduces tumor growth and progression when the indicated cells are injected into nude mice. Analysis of these xenografts at different times shows a reactivation of E-cadherin. Although this study strongly demonstrates LOXL2 nexus with EMT, it does not provide a specific functional mechanism. At this regard, the authors hypothesize that LOXL2 could stabilize Snail1 by oxidizing specific lysine residues, which they point out to be essential for Snail1 stability.

Finally, it is worth noting to indicate that, in this same study, LOXL3 is also identified as a Snail1 partner. Whether this protein is also involved in E-cadherin repression, its effects on EMT induction are not as strong as in the case of LOXL2.

Concerning LOXL2-mediated E-cadherin repression, a recent study demonstrates that LOX and LOXL2 are required and sufficient for repression of E-cadherin under hypoxic conditions[220]. In this work is shown that LOXL2, as well as LOX, are strongly induced by hypoxia. Several studies had previously reported that hypoxic incubation was able to suppress E-cadherin protein expression through HIF-1[221-223]. Accordingly, LOXL2 is presented as a direct transcriptional target of HIF-1.

Taken together, the literature strongly suggests that LOXL2 have both extra- and intracellular functions. However, it is not clear how this lysine oxidase exerts its function at a molecular level, nor if the different functions that have been reported are somehow connected.

Interestingly, Peng and colleagues[216] observed that, whereas RNAi of LOXL2 increase E-cadherin expression levels, antibody-mediated inhibition of secreted LOXL2 do not, thus suggesting that E-cadherin repression is independent of extracellular LOXL2 and reinforcing the scenario of a location-dependent role for LOXL2.

Objectives

The objective of this PhD thesis is to further characterize the epigenetic mechanisms underlying the Snail1-mediated CDH1 repression in the context of EMT.

The detailed objectives are:

- To elucidate the importance of Polycomb proteins in the EMT process, both in ES cells and in tumor cell lines. (Results I)
- To further characterize the mechanisms by which LOXL2 controls CDH1 repression at a transcriptional level (Results II)

Results-Part I

1.PRC2 is required for Snail1-mediated E-cadherin repression

1.1 The PRC2 component Suz12 is required for the correct expression of E-cadherin in murine embryos and ES cells.

In order to check whether expression of the E-cadherin gene is controlled by PRC2, we first analysed the E-cadherin expression pattern in murine embryos deficient for one of the essential components of this complex, Suz12. Previous reports have indicated that depletion of Suz12 prevents embryo progression beyond E8.5 due to compromised gastrulation[224]. The Snail1 and E-cadherin expression levels in control and Suz12 knockout murine embryos were analysed by immunohistochemistry.

As shown in figure R1.1, control embryos ($Suz12^{+/-}$) display alternative and inverse distributions of E-cadherin (Fig.R1A) and Snail1 (Fig. R1B). Whereas E-cadherin expression is found both in the ectoderm and in the endoderm, Snail1 expression is restricted to the mesoderm. In contrast, Suz12 mutants ($Suz12^{-/-}$), show a high proportion of cells where the expressions of both proteins are simultaneously detected (Fig. R1C and D). In these cells, Snail1 is detected in the nucleus (Fig. R1D, detail), thus ruling out the possibility that impaired repression of E-cadherin is a consequence of the altered Snail1 subcellular localization. This result suggests that, in the absence of PRC2 activity, E-cadherin cannot be properly repressed, even in the presence of Snail1.

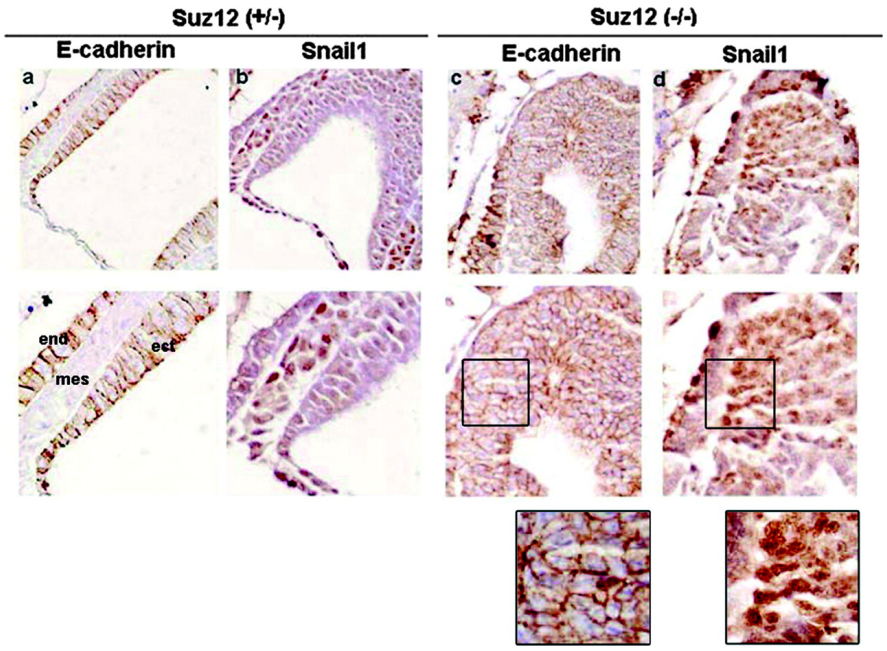


Figure R1. Expression of E-cadherin is altered in Suz12$^{-/-}$ E7.5 embryos. Sagittal sections from E7.5 Suz12$^{+/-}$ or Suz12$^{-/-}$ embryos were immunostained with antibodies against E-cadherin (a and c) or Snail1 (b and d). The lower panels show a higher magnification of the same section. Magnifications: upper panels, 200; lower panels, 400. Details of the lower images in panels c and d are also shown. mes, mesoderm; ect, ectoderm; end, endoderm.

Similar results were obtained using ES cells. ES cells were cultured in the absence of leukemia inhibitory factor (LIF) in order to induce differentiation to embryonic bodies. After 6 or 9 days of LIF removal, CDH1 mRNA levels in Suz12$^{+/-}$ ES cells were completely downregulated, in contrast, Snail1 mRNA was increased, with a maximum at day 6, as we determined by quantitative RT-PCR (qRT-PCR) (Fig.R2). In Suz12$^{-/-}$ ES cells, CDH1 mRNA levels were lower at day 0, and they were only partially downregulated during differentiation (approximately 50% of the initial value in these cells). As a consequence, CDH1 mRNA levels were substantially higher at

day 9 of differentiation in Suz12$^{-/-}$ cells than in control ES cells. These higher levels of CDH1 were not a consequence of impaired Snail1 synthesis in Suz12$^{-/-}$ cells, since Snail1 mRNA was upregulated during differentiation even to a higher extent in these cells than in control cells. Therefore, these results suggest again that Snail1 cannot properly repress CDH1 in the absence of PRC2 activity.

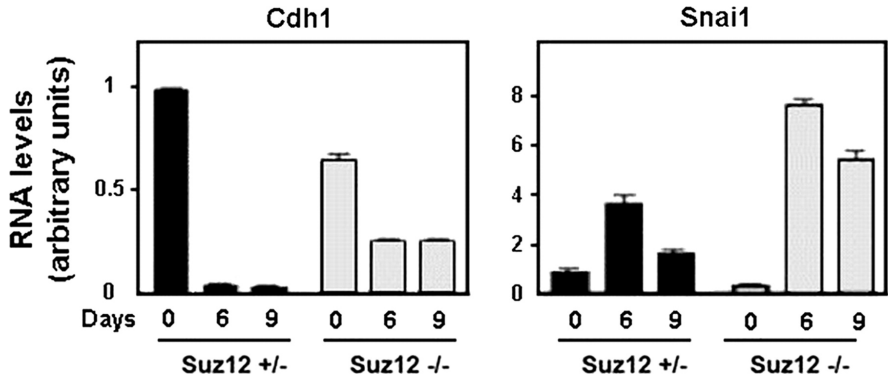


Figure R2. The E-cadherin expression pattern is altered in Suz12$^{-/-}$ ES cells. Embryonic bodies from Suz12$^{+/-}$ or Suz12$^{-/-}$ ES cells were allowed to form hanging drops for 2 days in the absence of LIF and collected at the indicated time points. E-cadherin (CDH1) and Snail1 (Sna1) mRNA levels were determined by qRT-PCR.

1.2 Depletion of PRC2 components affects CDH1 repression by Snail1 in tumor cells.

The involvement of PRC2 in the repressive activity of Snail1 on CDH1 expression was also investigated with tumor cell lines. PRC2 activity was blocked by ectopic expression of an shRNA against Suz12 that significantly decreased the endogenous levels of this protein (see Fig. S1 in the supplemental material). Suz12 knockdown totally prevented the ability of Snail1 to downregulate CDH1 mRNA levels in RWP-1 cells (Fig.R3A, left panel). The effect of this shRNA on the repressive

effect of Snail1 on the CDH1 promoter was studied. As shown in figure R3B, overexpression of Snail1 in epithelial cells decreased the activity of the CDH1 promoter, an effect that was dependent on the integrity of the three E boxes present in this promoter[47]. Simultaneous expression of Suz12 shRNA diminished the effect of Snail1 on this promoter, although it did not totally abolish it (Fig.R3B).

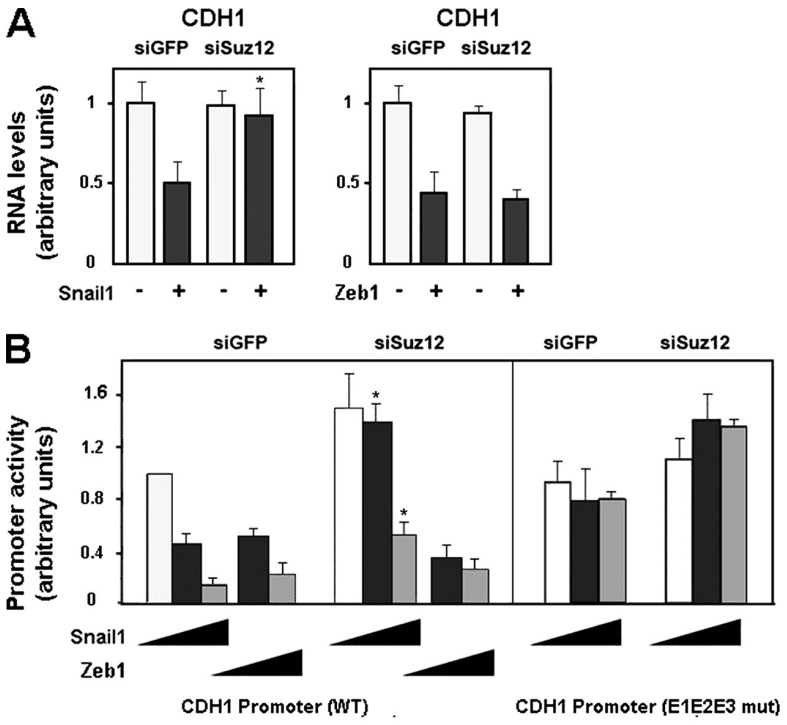


Figure R3. PRC2 downregulation affects CDH1 repression by Snail1 in tumor cell lines. A. CDH1 mRNA levels were determined by qRT-PCR after cotransfecting the shRNA for Suz12 (siSuz12) and Snail1 or Zeb1. An shRNA for GFP (siGFP) was used as a control. Cells were selected for 48 h with puromycin after transfection. **B.** The activities of the indicated forms of the CDH1 promoter (wild type [WT] and mutated [E1E2E3 mut]) in RWP-1 cells were determined by transient transfection of the pGL3-E-cad (178/ 92) CDH1 promoter, pcDNA-3-Snail1, or pcDNA-3-Zeb1 in the presence of an shRNA against Suz12 or GFP. This and all figures show the Averages ± SD (experiments performed in triplicate, if not, indicated). The asterisks indicate P values of < 0.01.

As mentioned in the introduction, the Zeb1 repressor also controls CDH1 expression. Thus, we decided to check whether the action of this repressor was also controlled by PRC2. As shown in figure R3A (right panel), downregulation of CDH1 mRNA levels caused by Zeb1 overexpression was not affected by Suz12 depletion. Similar results were obtained when the Zeb1 effect on the CDH1 promoter activity was determined by a reporter assay: absence of Suz12 did not significantly alter the CDH1 promoter repression by Zeb1 (Fig.R3B). Taken together, these results suggest that Snail1 and Zeb1 use different mechanisms to directly inhibit CDH1.

We also analysed the effect of PRC2 interference on CDH1mRNA levels in cells of SW-620, a cell line presenting low E-cadherin and high Snail1 and Zeb1 expression levels[47]. As shown in figure R4, disruption of PRC2 activity derepresses the CDH1 gene. Similar results were obtained using shRNA against Ezh2 or Suz12 or overexpressing a dominant-negative Ezh2 mutant (H694L)[225] .A comparable upregulation of CDH1 mRNA levels was observed after depletion of Suz12 in HT-29 M6 and in RWP-1 cells stably transfected with Snai1 (data not shown).

Snail1 protein also represses the expression of the PTEN gene[39]. Then, we also analysed the effect of PRC2 inactivation on the mRNA levels of this gene. As shown in figure R4, Suz12 or Ezh2 knockdown in SW-620 cells also upregulates PTEN mRNA, indicating that the requirement of PRC2 for Snail1 repression is not limited to the CDH1 gene.

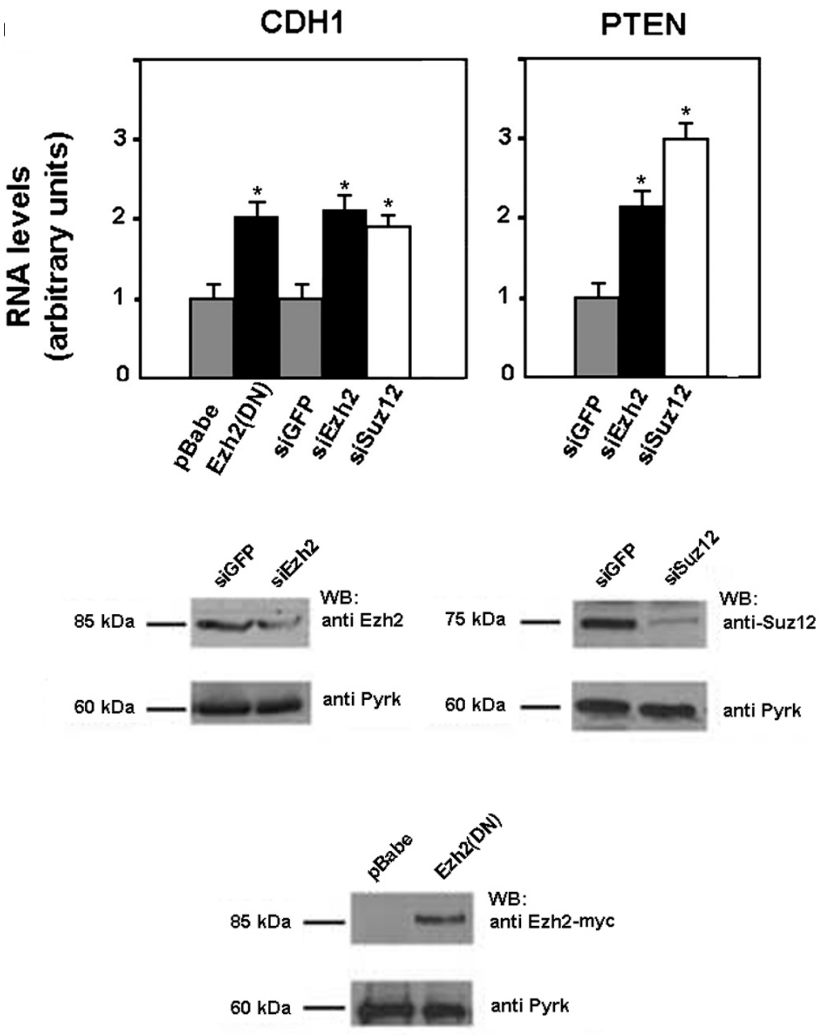


Figure R4. PRC2 proteins depletion causes upregulation of Snail1 targets. **(Top panel)** SW-620 cells were infected with a mutant of Ezh2 (H649L), a control plasmid (pBabe), or a retrovirus containing an shRNA specific for Suz12, Ezh2 or GFP as a control. CDH1 and PTEN mRNA levels were determined by qRT-PCR analysis as described above. **(Lower panels)** Ectopic Ezh2 mutant (H649L)-myc or endogenous Ezh2 and Suz12 were determined in cellular extracts by Western Blot.

1.3 Snail1 recruits PRC2 complex to the CDH1 promoter.

In order to determine if PRC2 was recruited to the CDH1 gene, we performed ChIP assays. CDH1 promoter sequences were detected bound to Snail1 and Suz12 only after differentiation of control ES cells (Fig.R5A), when the expression of this gene is repressed (Fig.R2). Similar results were observed when we determined the levels of H3K27me3 in the CDH1 promoter.

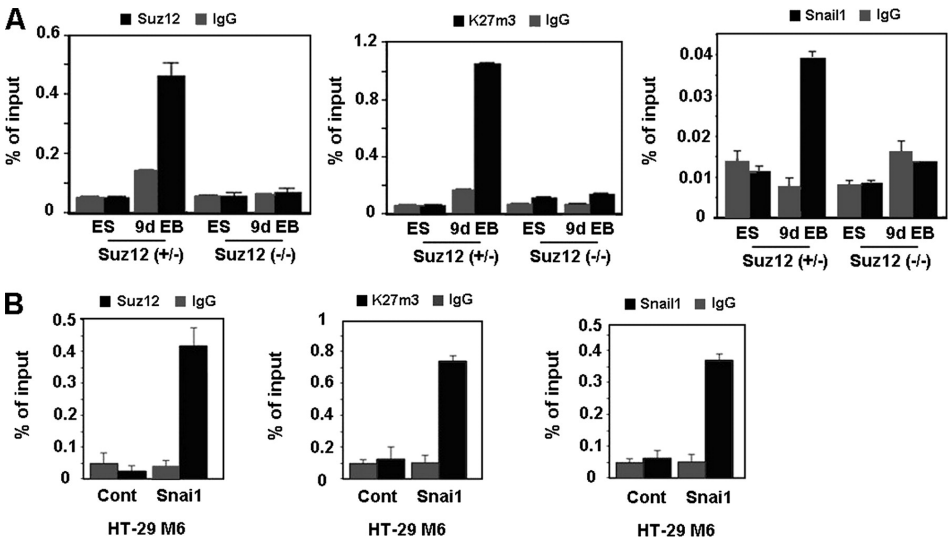


Figure R5. Suz12 binding to the CDH1 promoter is Snail1 dependent. A. Embryonic bodies from Suz12$^{+/-}$ or Suz12$^{-/-}$ were derived from ES cells that were allowed to form hanging drops for 2 days in the absence of LIF and collected at day 9. Binding of Snail1 and Suz12 and levels of H3K27me3 in the CDH1 promoter were determined by a ChIP assay at the undifferentiated (ES) and differentiated (9d EB) states. **B**. Binding of Snai1-HA and Suz12 and levels of H3K27me3 in the CDH1 promoter in HT-29 M6 cells stably transfected with Snai1-HA were determined by a ChIP assay.

As expected, association of Suz12 and trimethylation of K27 in H3 in the CDH1 promoter were not detected in Suz12-null cells. Surprisingly, Snail1 was not detected bound to the CDH1 promoter in these cells either, suggesting that the presence of PRC2 is necessary to stabilize the Snail1 repressive complex at the promoter. Chip assays performed with cell lines also confirmed that the recruitment of Suz12 to the CDH1 promoter is Snail1 dependent. As shown in Fig.R5B, binding of Snail1 and Suz12 and levels of H3K27me3 in the CDH1 promoter were detected in HT-29 M6 clones stably expressing Snai1 but not in the control clones.

We also analysed whether Suz12 occupancy at the CDH1 promoter was dependent on the integrity of the E boxes located in the CDH1 promoter. We transfected stable RWP-1- Snai1 cells with either the wild-type CDH1 promoter or a promoter containing the mutated E boxes (E1E2E3 CDH1 promoter). ChIP assays were performed to analyse the association of Snail1, Suz12, or H3K27me3 with ectopic promoters. As shown in Fig.R6A, the amount of the CDH1 promoter immunoprecipitated with Suz12 or H3K27me3 was substantially lower when binding of Snail1 was prevented by mutation of the three E boxes. We also used this experimental approach to investigate if the Snai1 P2A mutant, a SNAG domain mutant unable to repress transcription[47], was capable to recruit the PRC2 complex to the CDH1 promoter. As shown in Fig.R6B, in contrast to what was observed with the wild-type form, the Snai1-P2A mutant did not induce Suz12 binding to the CDH1 promoter. As a consequence, the levels of H3K27me3 associated with this promoter were lower than those in cells expressing wild-type Snai1 (Fig.R6B).

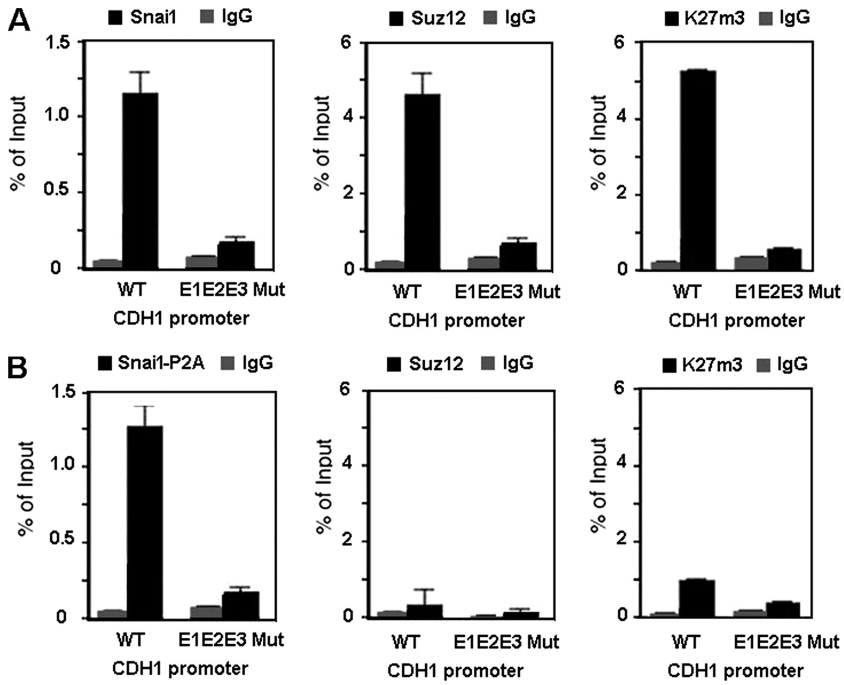


Figure R6. PRC2 binding and activity in the CDH1 promoter require the integrity of the Snail1-binding sites and the SNAG domain of Snail1. RWP-1 cells stably transfected with Snai1 **(A)** or Snai1-P2A mutant **(B)** were transiently transfected with an exogenous CDH1 promoter, either the wild type (WT) or a form with the three E boxes mutated (E1E2E3 Mut). The transfection efficiencies were the same for the two promoters. Binding of Snai1-HA, Snai1-P2A-HA, and Suz12 and levels of H3K27me3 in the exogenous CDH1 promoter were determined by ChIP.

At this point, our data pointed to Snail1-dependent PRC2 recruitment to the CDH1 promoter. To further validate our results, we knockdown Snail1 in SW-620 cells and we studied PRC2 recruitment to the endogenous CDH1 promoter by ChIP. As shown in Fig.R7A, Snail1 depletion compromised not only the binding of the Snail1 protein to but that of Suz12 as well. As expected, the enrichment of H3K27me3 in the CDH1 promoter was also decreased in the absence of Snail1.

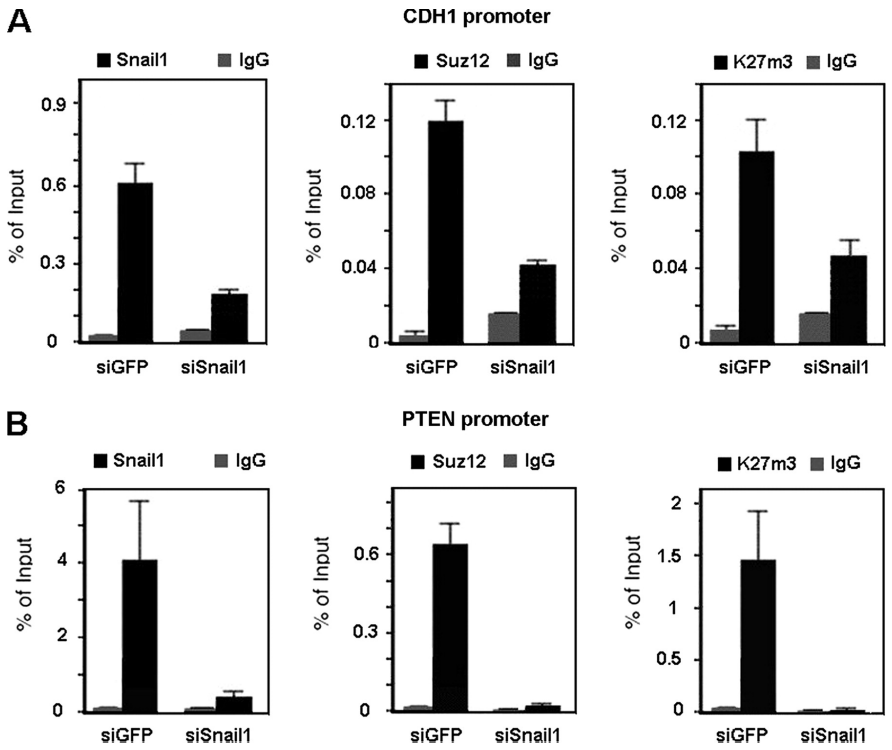


Figure R7. PRC2 recruitment is Snail1 dependent. ChIP assays were carried out as described in Materials and Methods, immunoprecipitating cross-linked nuclear extracts from SW-620 cells infected with a retroviral construct generating SNA1-specific small hairpin RNA (siSnail1) or with an shRNA specific for GFP (siGFP) as a control and selected with puromycin for 48 h. Specific binding for Snail1, Suz12, and K27me3 in the CDH1 promoter **(A)** and in the PTEN promoter **(B)** was analyzed.

In addition, we also checked whether Snail1 knockdown in SW-620 cells would also affect binding of Suz12 to the other Snail1 targets such as PTEN promoter. As shown in Fig.R7.B, whereas association of Snail1 and Suz12 and levels of H3K27me3 were observed in the promoter region in control cells, downregulation of Snail1 expression drastically decreased the association of both proteins and, consequently, the H3K27me3 levels. Analysis of other Snail1 target genes, such as the MUC1 promoter, revealed the same results (not shown). Therefore, recruitment of PRC2 by Snail1 is not restricted to the CDH1 promoter.

1.4 Snail1 associates with PRC2 components.

Since recruitment of Suz12 to the CDH1 promoter is Snail1 dependent, we investigated whether Snail1 can interact with components of the PRC2 complex. As shown in Fig.R8, the presence of HA-tagged Snail1 was observed in the immunoprecipitates obtained with an antibody against Suz12 in RWP-1 (Fig.R8A) and in HT 29 M6 stably transfected with Snai1-HA (Fig. R8B). Association of Snail1 with other PRC2 components was also detected, since Ezh2 was coimmunoprecipitated with Snail1-HA in HT 29 M6-Snai1 cells (Fig. R8C). Moreover, this association was also observed between the endogenous proteins in SW-620 cells; Ezh2 protein was detected in the immunocomplex obtained with a monoclonal antibody against Snail1[226] (Fig. R8D).

We also performed a coimmunoprecipitation assay with RWP-1 cells stably transfected with the Snail1 P2A mutant or with the wild-type form of this protein. As shown in Fig.R8E, both transfectants express

similar amounts of Snail1 protein. However, whereas wild-type Snail1 was observed in Suz12 immunocomplexes, Snail1 P2A was not, suggesting that this mutant was unable to interact with Suz12 and indicating that the same Snail1-SNAG domain is required for repression, PRC2 binding, and PRC2 recruitment to the CDH1 promoter.

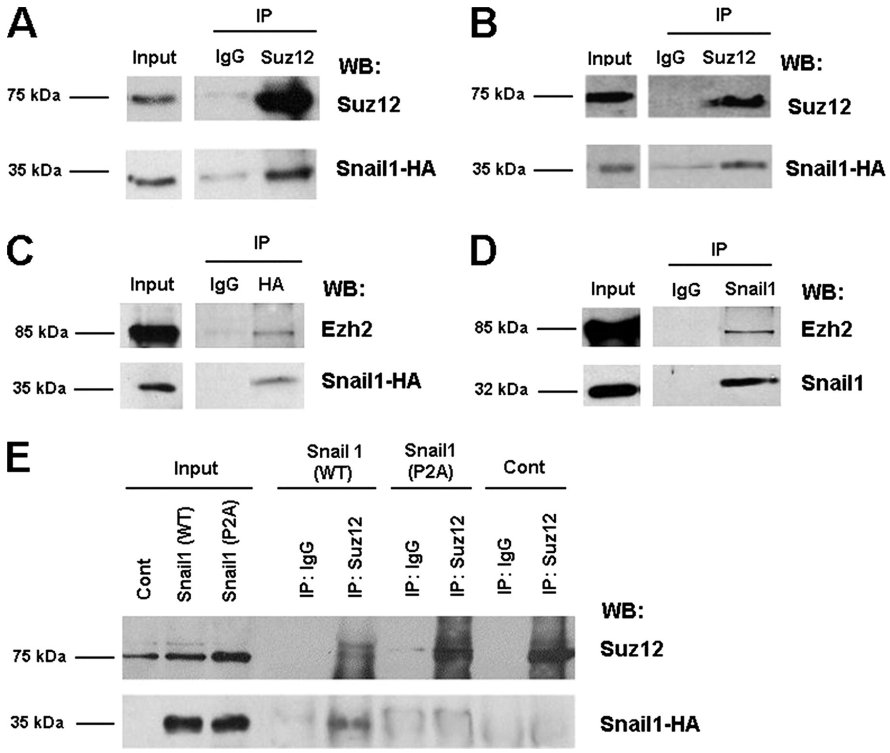


Figure R8. PRC2 components and Snail1 interact in vivo. RWP-1 cells **(A)** and HT-29 M6 **(B and C)** stably transfected with Snai1-HA were lysed and immunoprecipitated (IP) with anti-Suz12 (A and B) or anti-HA (C). The immunocomplex was analysed by Western blotting (WB), using antibodies against HA (A and B), Suz12 (A and B), or Ezh2 (C). **D.** SW-620 cells were lysed and IP with a monoclonal antibody specific for Snail1. Interaction of Ezh2 with Snail1 was analysed by WB. **E.** RWP-1 cells stably transfected with Snai1-HA or with the Snai1-P2A mutant were IP with anti-Suz12. The presence of Snail1-HA and the Snail1-P2A-HA mutant was analysed by WB RWP-1 cells stably transfected with pcDNA-3 were used as a control.

Finally, we analysed Snail1 and Suz12 expression during mouse embryo development in order to characterize whether Snail1-positive cells were also expressing Suz12.

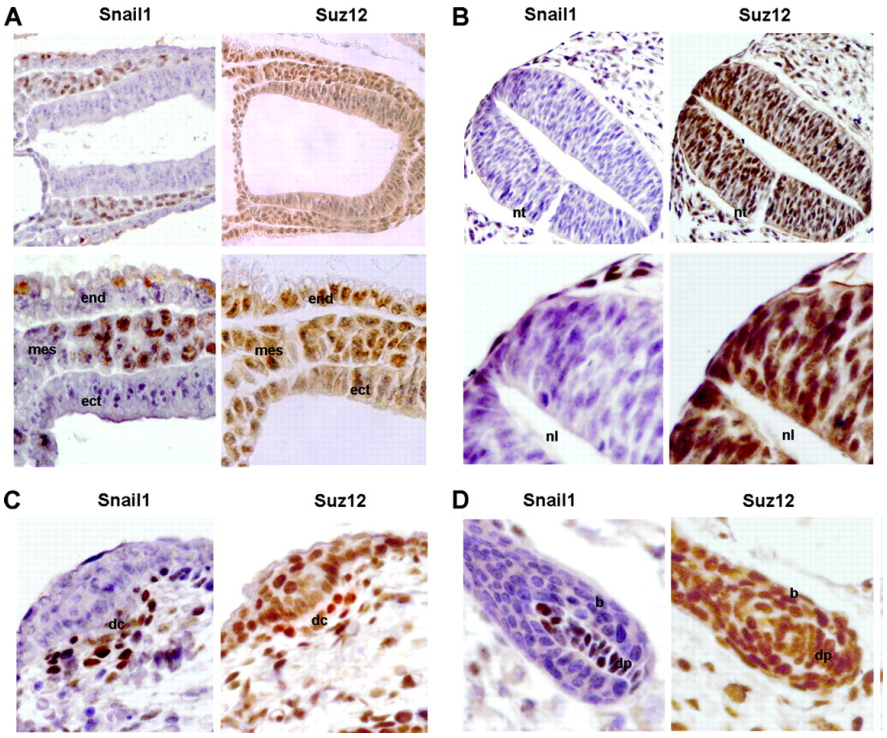


Figure R9. Snail1 and Suz12 are co-expressed in mesenchymal cells from murine embryos. Sagittal sections obtained from murine embryos were analyzed by immunohistochemical analysis of Snail1 and Suz12. **A.** Sagittal section from an E7.5 embryo. Magnifications: upper panel, x200; lower panel, x400. mes, mesoderm; ect, ectoderm; end, endoderm. **B.** E9.5. Magnifications: upper panel, x200; lower panel, x400. nt, neural tube; nl, neural lumen. **C.** E15. Magnification, x400. dc, dermal condensate. **D.** E18. Magnification, x400. dp, dermal papilla; b, bulb.

As observed in Fig.R9 and according to previous reports[224], Suz12 showed a broader expression than Snail1 and was present in all cells with Snail1 immunoreactivity. More specifically, at E7.5 Snail1 is present only in the mesoderm (Fig.R9A, left panels), with a contrary

distribution to E-cadherin (Fig. R1). Suz12 was detected in the nucleus in this mesenchymal embryonic layer (Fig.R9A, right panels). At E9.5, we analysed the mesenchymal cells that are migrating from the neural crest. As we can observe in Fig.R9B (left panels) and as expected by RNA analyses[227], Snail1 is present in these cells, which also show expression of Suz12 (Fig.R9B, right panels). At later stages of development, we focused on hair follicle morphogenesis, a process in which Snail1 is involved[228]. As previously reported[226,228], Snail1 is expressed in the dermal condensate at E15 (Fig.R9C, left panel) and in the dermal papilla at E18 (Fig.R9D, left panel), whereas it is absent from the adjacent epidermal cells and the bulb. Suz12 was present in all the cells expressing Snail1, although it showed a more general distribution since it was also detected in the epithelial cells (Fig.R9C and D, right panels).

1.5. PRC1 and DNMT1 bind silent E-cadherin gene.

As mentioned in the introduction, it has been suggested that PRC1 functions downstream PRC2 and it is required to achieve Polycomb-mediated repression. Thus, we decided to check by ChIP assays if PRC1 proteins were also bound to silent E-cadherin gene. As shown in Fig.R10A, CBX8, one of the components of PRC1 involved in the recognition of the H3K27me3 mark, was found to bind the E-cadherin promoter only after differentiation of control ES cells, when the expression of this gene is repressed (Fig.R2). As expected, this binding was abolished in Suz12-null ES cells, where PRC2-associated H3K27 trimethylation is disrupted (Fig.R5A).

Next, ChIP assays were also performed with cell lines. Binding of BMI-1, a PRC1-containing protein was detected in HT-29 M6 clones stably

expressing Snai1 but not in the control clones (Fig.R10B). Considering that Bmi-1 binding to the CDH1 promoter nicely correlates with those of Snail1 and Suz12 as well as with the enrichment of H3K27me3 mark (Fig.R5B), it could be suggested that PRC1 recruitment is Snail1 and PRC2 dependent.

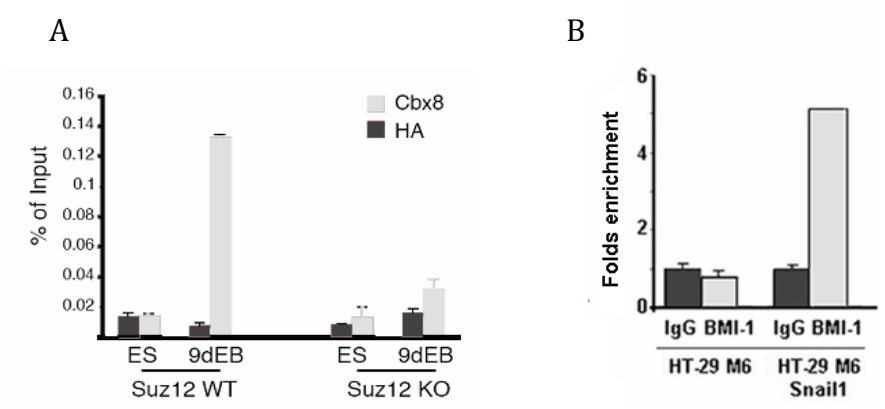


Figure R10. PRC1 binding to the CDH1 promoter is Snail1 dependent. **A.** Embryonic bodies from Suz12$^{+/-}$ or Suz12$^{-/-}$ were derived from ES cells that were grown in the absence of LIF and collected at day 9. Binding of Cbx8 in the CDH1 promoter was determined by a ChIP assay at the undifferentiated (ES) and differentiated (9d EB) states. **B**. Binding BMI-1 in the CDH1 promoter in HT-29 M6 cells stably transfected with Snai1-HA was determined by a ChIP assay.

As specified in the introduction, PRC2 has been found to functionally cooperate with DNMTs. This association is thought to strength gene silencing in cancer. Thus, we decided to investigate the relevance of DNMTs in the Snail1-mediated repression of E-cadherin. As a primary approximation, SW-620 cells and HT-29 M6 clones stably expressing Snai1 were treated with the DNMT inhibitor 5-aza-dC. As shown in Fig.R11A, inhibition of DNA methylation reactivated E-cadherin expression in SW-620 and HT-29 M6 Snail1 clones (in this last case, modestly). To further characterize DNMTs role in these tumor cells

lines we performed ChIP assays. DNMT1 binding to the CDH1 promoter was detected in both cell lines, and, importantly, it was dependent on Snail1 expression (Fig.R11B).

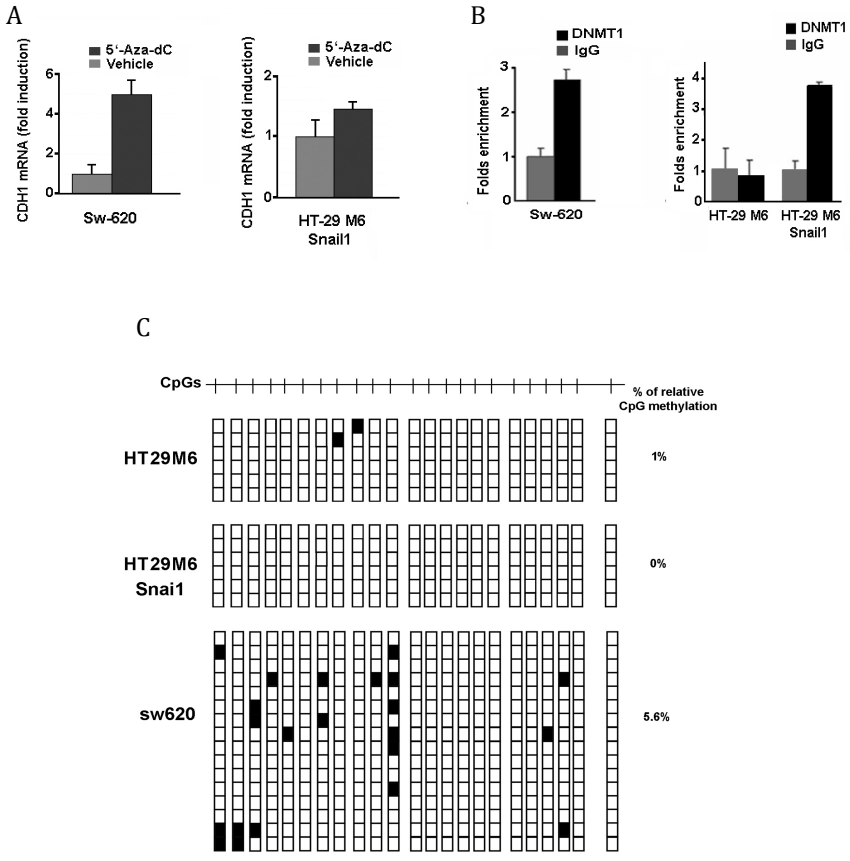


Figure R11. Characterization of E-Cadherin promoter methylation A. qRT-PCR of CDH1 in SW-620 cells and HT-29 M6 Snail1 clones after 48 h of treatment with 5-Aza-dC (5 μM) **B**. Binding of DNMT-1 in the CDH1 promoter in the referred cell lines were determined by a ChIP assay. **C.** The DNA methylation status at the CDH1 promoter was analysed by bisulfite genomic sequencing in HT 29 M6, HT 29 M6-Snail1 and sw620 cells. Each line represents a single DNA template molecule. A representative experiment is shown; black and white, respectively, represent methylated and unmethylated CpGs.

Given these results, we decided to perform bisulfite genomic sequencing of the CDH1 gene proximal promoter region in both SW-620 cells and HT-29 M6 Snail1 clones. Intriguingly, we observed promoter hypomethylation in SW-620; accordingly, Snail1 had no positive effect over promoter methylation in HT-29 M6 cells. (Fig.R11C)

1.6 PRC2 is required for Snail1 self-regulation loop

As commented in the introduction, Snail1 gene regulation has not been widely characterized. Recently, Peiró and colleagues[64] reported that Snail1 gene could be regulated by its own product, thus establishing a fine-tuning feed-back mechanism, that might be responsible for the observed sustained expression of Snail1. We studied PRC2 role in this mechanism.

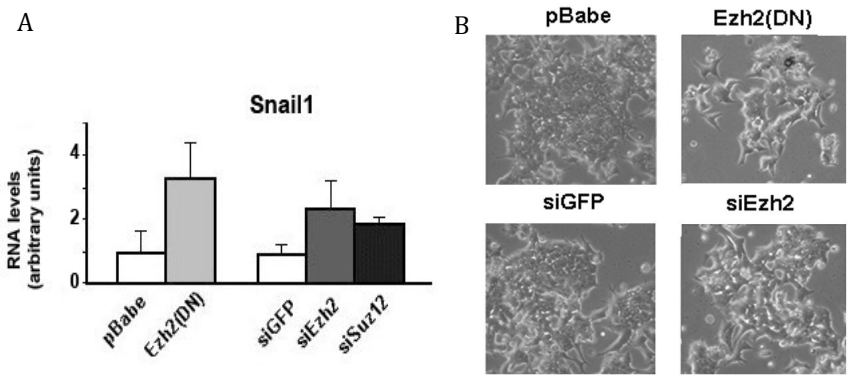


Figure R12. PRC2 is involved in Snail1 regulation A. qRT-PCR of Snail1 in RWP-1 cells infected with a mutant of Ezh2 (DN), a control plasmid (pBabe), or a retrovirus containing an shRNA specific for Suz12, Ezh2 or GFP as a control. **B.** PRC2 deficiency induced phenotypical changes in RWP-1 cells.

As shown in Fig.R12A, overexpression of a dominant-negative Ezh2 mutant as well as depletion of Ezh2 or Suz12, increased Snail1 levels in RWP-1 epithelial cells. Although E-cadherin levels remained unchanged (data not shown), indicated Snail1 mRNA upregulation induced a more mesenchymal phenotype (Fig.R12B). These results were in agreement with those we had previously observed in ES cells (see Fig.R2, right panel), in which Snail1 expression levels were also increased in the absence of Suz12. These results suggest that PRC2 is involved in Snail1 transcriptional repression.

Next, we decided to investigate if, as it is the case for other Snail1 targets such as E-cadherin (Fig.R7), PRC2 is bound to repressed Snail1 gene in a Snail1-dependent manner. As shown in Fig.R13A, whereas association of Suz12 and H3K27me3 mark enrichment were observed in the Snail1 promoter region in control cells, downregulation of Snail1 expression completely abolished Suz12 association and, consequently, the H3K27me3 levels.

Finally, taking advantage of the fact that Snail1 expression is dependent on serum in NIH-3T3 fibroblasts[226], we also investigated PRC2 role in Snail1 repression in these system. As shown in Fig.R13B, serum deprivation, which completely downregulates Snail1 protein in these cells, correlated with an important enrichment of H3K27me3 levels in the Snail1 promoter. In contrast, re-addition of serum, which is known to stimulate Snail1 protein expression, resulted in the rapid disappearance of the repressive mark in this same promoter.

Taken together, our results suggest that PRC2 is also required for Snail1 self-regulation loop. In fact, our results revealed that dynamic removal of PRC2-associated mark is necessary for Snail1 expression reactivationa

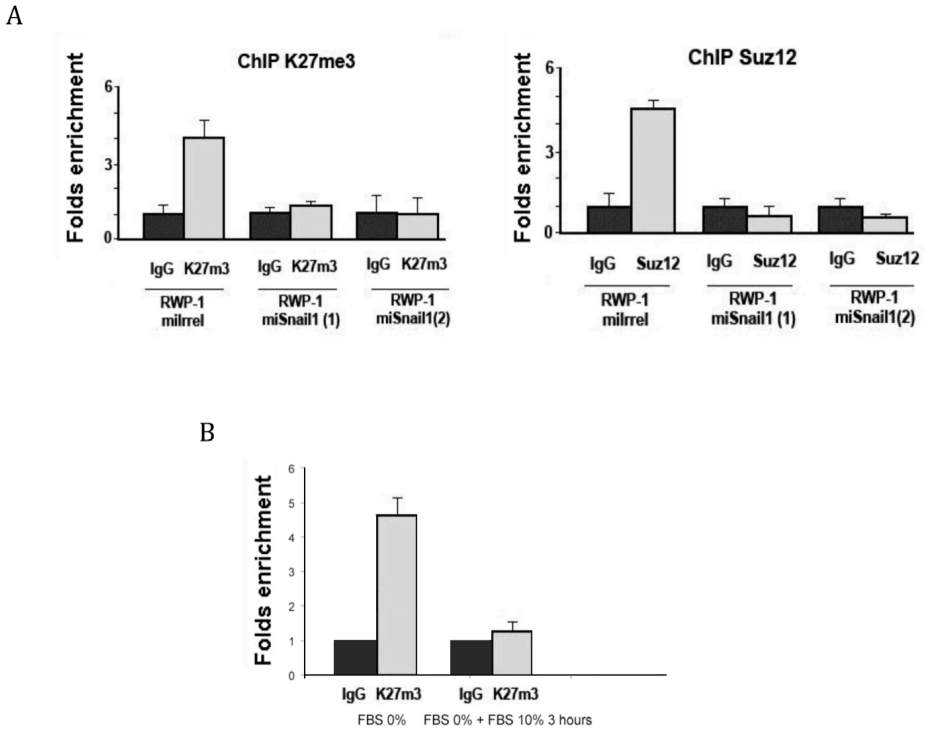


Figure R13. Suz12 binding and H3K27me3 enrichment in the Snail1 promoter are Snail1-dependent. A. ChIP assays were carried out in RWP-1 cells expressing a control pPRIME-GFP plasmid or two pools containing a miRNA specific for human SNAIL1 (miSNAIL1 c1 and c2)[64] **B.** ChIP assays were performed in NIH3T3 fibroblasts incubated in the absence of serum for 24 h and in fibroblasts treated for 3h with 10% fetal bovine serum following serum deprivation.

Results-Part II

2. LOXL2 IS A H3K4me3 DEAMINASE

2.1 LOXL2 protein is required for E-cadherin repression at a transcriptional level.

As mentioned in the introduction, LOXL2 has been associated to Snail1-dependent repression of the CDH1 gene[219]. However, its role in such mechanism remains elusive.

In order to further characterize LOXL2 function, we first depleted its expression in two different tumor cell lines (RWP-1 and MDA-MB 231) with different expression levels of LOXL2 (data not shown). LOXL2 disruption induced a more epithelial phenotype, (Fig R14A, left panel) which correlated with an increase in epithelial markers such as E-cadherin or Occludin (Fig.R14A, right panel). Importantly, in the absence of LOXL2, Snail1 no longer repressed CDH1 gene expression. (Fig. R14B) Interestingly, in our experiments, LOXL2 disruption did not affect Snail1 protein stability as other have proposed[219], thus suggesting, that LOXL2 is involved in E-cadherin repression at a transcriptional level.

In addition, LOXL2wt overexpression led to down-regulation of CDH1 mRNA in MCF-7 cells (Fig. R14C). Remarkably, a catalytically-inactive LOXL2 (LOXL2mut), in which two histidine residues of the catalytic domain involved in Cu (II) binding were mutated to glutamine (H626Q, H628Q), was unable to repress CDH1 transcription (Fig. R14C). Therefore, the transcriptional function of LOXL2 seems to depend on its catalytic activity.

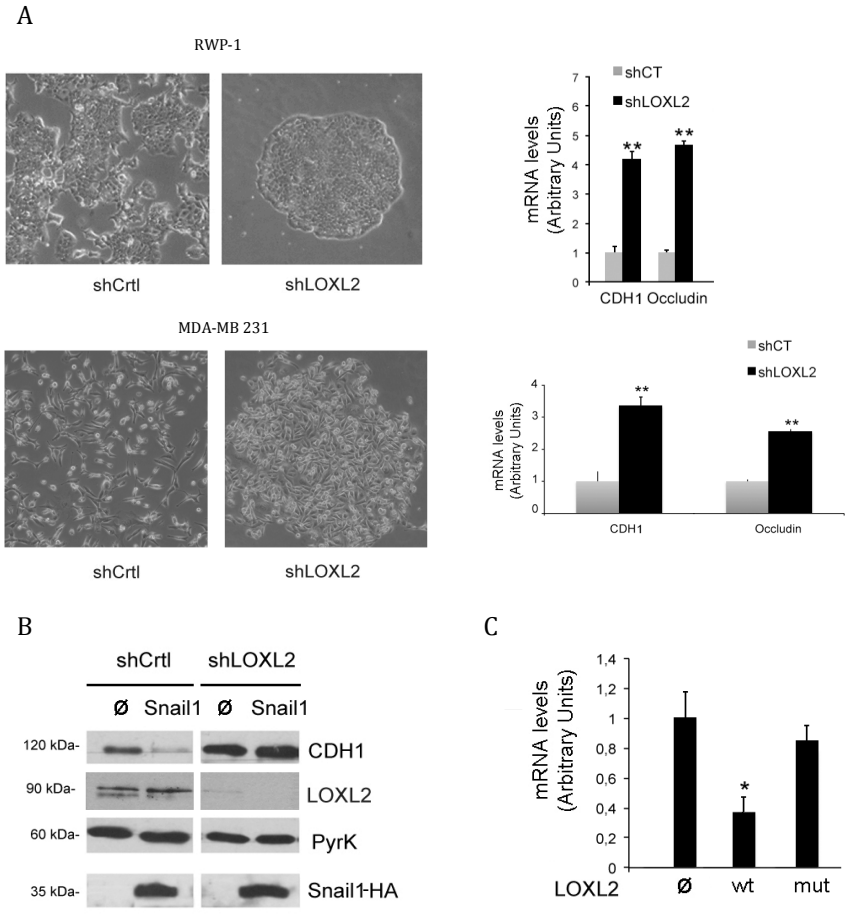


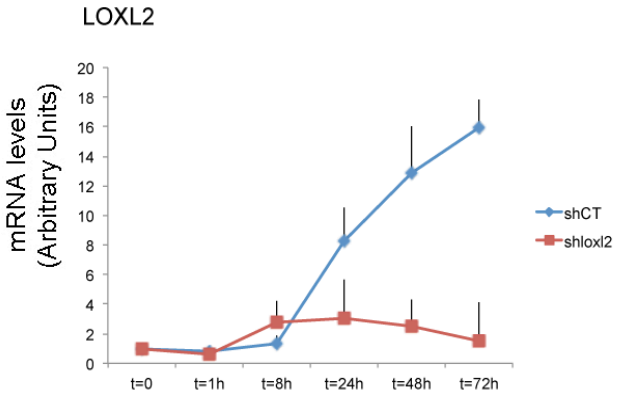
Figure R14. LOXL2 is implicated in CDH1 repression. A.LOXL2 knockdown induced phenotypical changes (left). RWP1 shLOXL2 clones and MDA-MB 231 shLOXL2 pool, were generated after puromycin selection. qRT-PCR shows the changes in expression of CDH1 and Occludin mRNAs for both cell lines upon LOXL2 depletion (right). **B.** RWP1 shLOXL2 cells were transfected with the pcDNA3 empty vector (Ø) or Snail1-HA. LOXL2, E-Cadherin, Pyruvate kinase and Snail1-HA levels were analyzed 48 h after transfection by western blot. **C.** qRT- PCR shows the changes in expression of CDH1 mRNA transfected with the pcDNA3 empty vector (Ø) or LOXL2wt or LOXL2mut.

2.2 LOXL2 depletion impairs E-cadherin repression in the context of TGF-β induced EMT.

NMuMG cells have been used as a model of TGF-β inducible EMT[229]. In order to study LOXL2 role in E-cadherin repression in a more physiological model, we knocked down LOXL2 in NmuMG cells, and prior to puromycin selection, we treated both shCt and shLOXL2 cells with TGF-β at different times (0,1,8,24,48, and 72h).

Although LOXL2 depletion did not significantly impaired TGF-β-induced phenotypic transformation (data not shown), dramatic changes in mRNA expression levels of EMT key regulators were observed (Fig.R15). In agreement with the data that has been recently reported in our lab[230], we observed that TGF-β stimulation in control cells caused a quick upregulation of Snail1 mRNA (1h). Snail1 expression remained upregulated, though diminishing afterward. On the other hand, Zeb1 mRNA expression was increased at later times; no changes were detected prior to 8 h and RNA remained increased after 72 h. As expected, Snail1 and Zeb1 induction correlated with a sustained and almost complete downregulation of E-cadherin expression.

Interestingly, LOXL2 mRNA was also strikingly upregulated upon TGF-β induction, in fact, its expression pattern was very similar to that observed for Zeb1. Conversely, LOXL2 knockdown completely prevented LOXL2 stimulation, and, as a consequence, E-cadherin expression reactivation was observed after 24h. Intriguingly, LOXL2 depletion totally prevented ZEB1 induction and Snail1 sustained upregulation.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14
12
10
8
6
4
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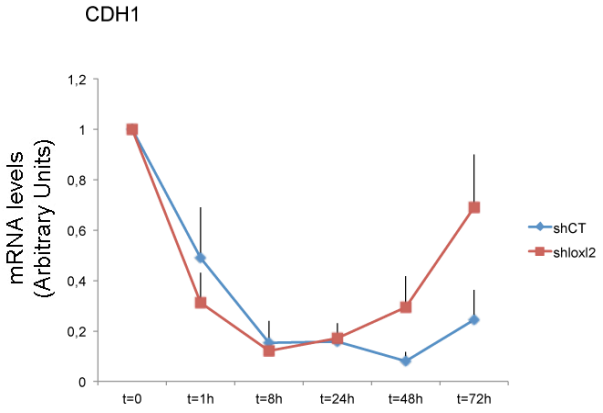
t=72h
shCT
shloxl2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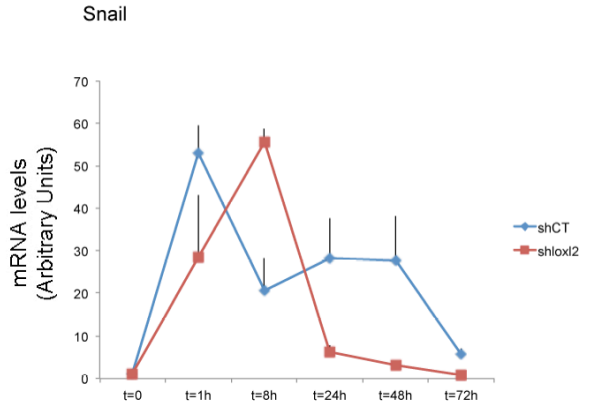
shloxl2


Snail
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shloxl2

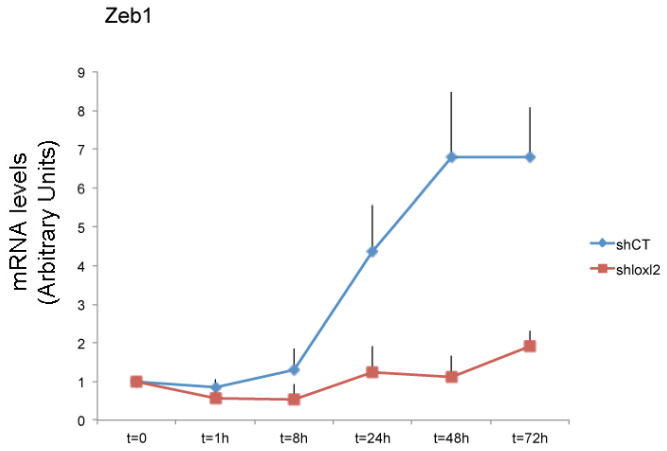


Figure R15. LOXL2 depletion impairs CDH1, Snail1 and Zeb1 mRNA expression during TGF-β induced EMT in NMuMG cells. Following two rounds of infection (with shCT or shLOXL2 lentivirus), NMuMG cells were subjected to puromycin selection (3 μg/ml) for 48h. Next, they were replated and incubated with TGF-β (5 ng/ml) for the indicated times; RNA was isolated and analysed by qRT-PCR.

These results suggest that LOXL2 is an important player in TGF-β induced EMT. However, it is likely that, at least in this cellular system, LOXL2 is not only contributing to E-cadherin repression in a direct manner, but also through the transcriptional regulation of EMT-TFs, such as Snail1 or Zeb1.

2.3 LOXL2 protein is highly enriched in the chromatin fraction and is bound to CDH1 gene promoter.

To broaden study LOXL2 putative transcriptional role, we determined LOXL2 subcellular localization by immunofluorescence and subcellular fractionation in several tumor cell lines. We observed that LOXL2 was predominantly nuclear (Fig.R16A); indeed, its expression was almost restricted to the chromatin fraction (Fig.R16B, upper panel). To ensure that LOXL2 association with chromatin was not due to protein insolubility, we treated chromatin pellets with nucleases. As shown in Fig.16B (lower panel), LOXL2 was released after nuclease treatment, thus indicating that LOXL2 presence in the chromatin fraction is DNA- dependent.

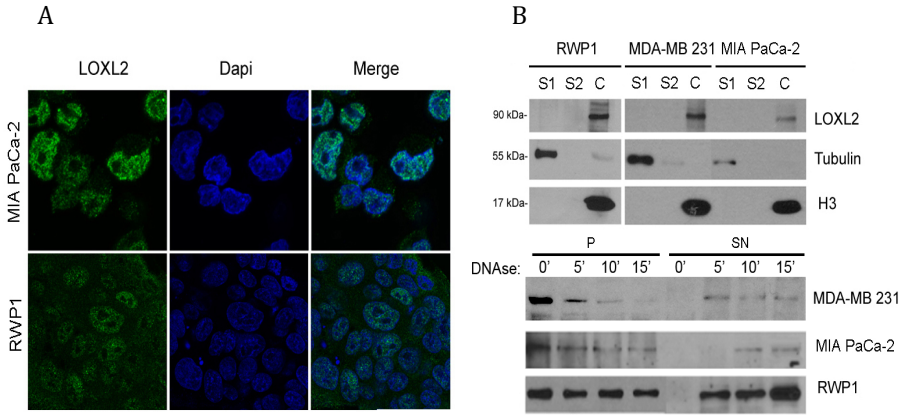


Figure R16. LOXL2 is located in chromatin. A. Confocal microscopy of LOXL2 (green) in cancer cell lines. Nuclei were stained with DAPI. **B.** Subcellular fractionation (S1, soluble; S2, nuclear soluble; C, chromatin-enriched) of the indicated cell lines (upper panel). Chromatin fraction was digested with nuclease at the indicated times and the presence of LOXL2 in the supernatant and in the pellet was detected by western blot (lower panel).

Taken together, our results pointed to a scenario in which LOXL2 has a direct effect over the CDH1 gene. Accordingly, ChIP assays demonstrated that LOXL2 was bound to the proximal promoter region of CDH1 locus in RWP1 and MDA-MB231 cells (Fig.R17).

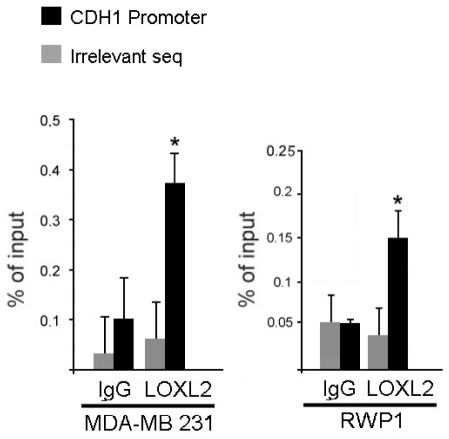


Figure R.17 LOXL2 is bound to the CDH1 promoter. LOXL2 ChIP in the CDH1 promoter in MDA-MB-231 and RWP1 cells. Error bars provide the s.d., n=3. One asterisk indicates $p<0.05$; two asterisks, $p<0.01$

2.4 LOXL2 is a co-repressor and interacts with repressive complexes.

Considering our previous observations, we considered that LOXL2 could function as a co-repressor. In order to determine it, we cloned the full-length open reading frame fused to the GAL4 DNA binding domain (G4LOXL2wt) and we performed reporter assays. The GAL4-LOXL2 fusion protein was able to repress the G4-TK-Luc reporter gene in a dose-dependent manner (Fig.R18), whereas a mutant version (G4LOXL2mut) was not, although some residual activity was observed (Fig.R18). As expected, G4LOXL2wt had no effect on TK-Luc

reporter gene lacking GAL4 binding sites (Fig.R18, left panel). These results suggest that LOXL2 behaves as a general repressor.

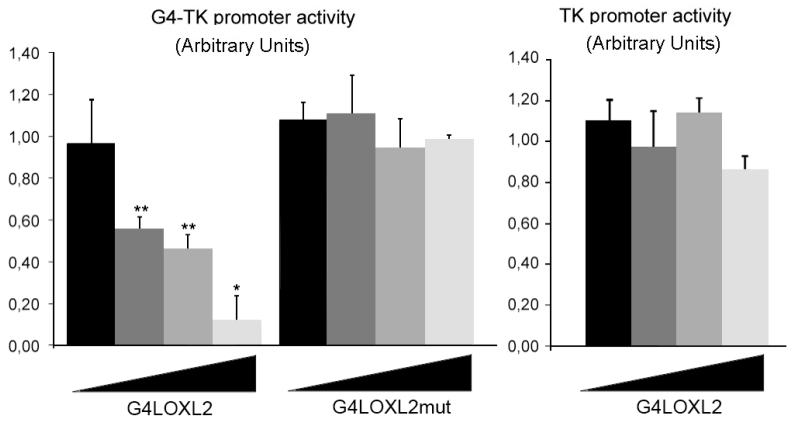
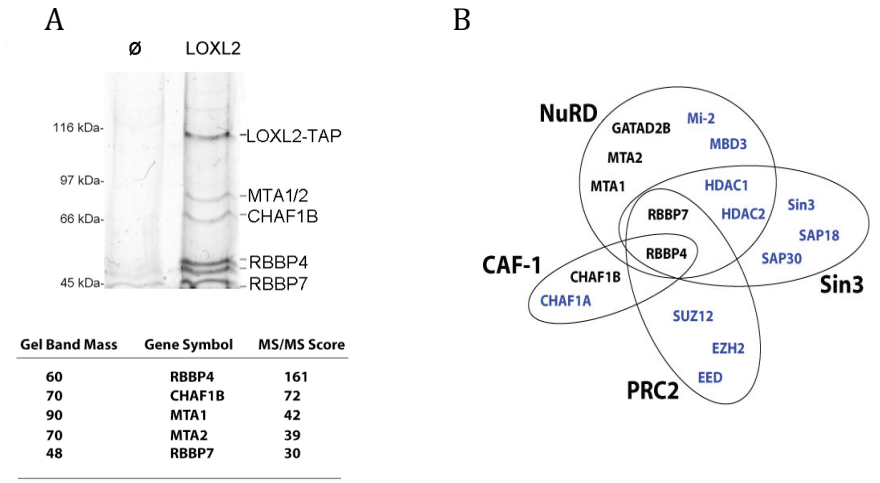


Figure R.18 LOXL2 is a corepressor. G4LOXL2 or G4LOXL2mut fused proteins were transfected into HEK 293 cells, together with the G4TK-Luc reporter gene or TK-Luc reporter gene. Reporter activity in the presence of GAL4DBD was designated as 1. Error bars provide the s.d., n=5. One asterisk indicates $p<0.05$

To strength our theory, we attempted to isolate LOXL2 interactors from HEK 293 cells, in which we overexpressed LOXL2wt protein. By using tandem affinity purification approach and Mass spectrometry analyses, we were able to identify several putative LOXL2 partners; including RBBP4(RbAp48), RBBP7 (RbAp46), CHAF1B (CAF-1p60) and MTA1/2 (Fig.R19A). Most of these proteins are members of repressive complexes, such as PRC2[231], CAF-1[232] or the HDAC-containing complexes, NuRD and Sin3[70,233-235] (Fig.R19B). These complexes have pivotal roles in epigenetic regulation involving gene silencing, histone exchange or heterochromatin formation.



Gel Band Mass	Gene Symbol	MS/MS Score
60	RBBP4	161
70	CHAF1B	72
90	MTA1	42
70	MTA2	39
48	RBBP7	30

Figure R19. LOXL2 interacts with members of repressive complexes. A. Total extracts of HEK 293 cells transfected with LOXL2 were subjected to affinitychromatography using anti-Flag resin of LOXL2-Flag. Silver staining of a NuPAGE 4-12% Bis-Tris gel depicting LOXL2 interactors is shown. Proteins identified by MS/MS are indicated on the right. MS score is shown below. **B.** Schematic representation of the multiprotein complexes in which LOXL2 partners (in black) have been identified.

In order to confirm LOXL2 interaction with these complexes, we performed co-immunoprecipitation (Co-IP) assays in HEK 293 cells in which Flag-LOXL2wt was overexpressed. Following Flag-purification and Western Blot we validated LOXL2 association with HDAC1, CHAF1B, RBBP4, EZH2 and Sin3A proteins. β-Catenin was used as negative control (Fig.R20). Although the demethylase LSD1 was not detected in the Mass Spectometry analysis, LSD1 and LOXL2 share a great number of partners, such as NuRD components[95] or Snail1 protein[236]. Therefore, we decided to check if LSD1 was also present in purified Flag-LOXL2wt immunocomplexes. Indeed, it was (Fig.R20, left panel). To further validate LOXL2 interactions, we performed some reciprocal CoIPs in which we successfully immunoprecipitated endogenous Ezh2, Sin3A or HDAC1 proteins together with Flag-LOXL2wt protein (Fig.R20, right panel).

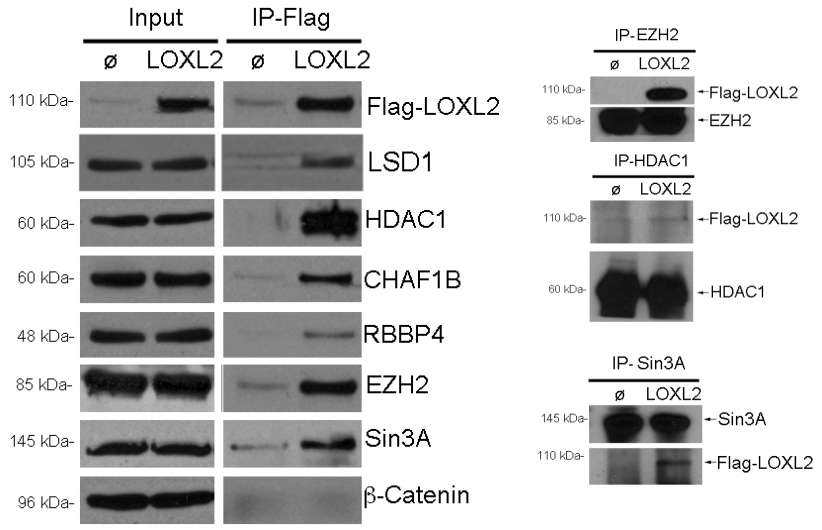


Figure R20. LOXL2 co-immunoprecipitates with proteins involved in chromatin remodelling. Flag-LOXL2 co-immunoprecipitated with CHAF1B, HDAC1/2, RBBP4, EZH2, Sin3A and LSD1 in HEK 293 cells expressing high levels of ectopic Flag-LOXL2wt. β-catenin was tested as a negative control. (Left Panel). Extracts from HEK293T cells, transiently transfected with LOXL2wt or an empty vector, were IP with anti-HDAC1, anti-Ezh2 or anti-Sin3A antibodies. The immunocomplexes were analyzed by Western blotting, using the indicated antibodies. (Right panel)

The collective association of all these proteins with LOXL2 strongly pointed to a LOXL2 role in transcriptional repression through direct or indirect regulation of epigenetic events. Consequently, we decided to analyse the functional effects of manipulating LOXL2 expression on histone marks.

2.5 LOXL2 modulates activation histone marks (H3K4me2/3) *in vivo.*

As expected, knockdown RWP-1 cells displayed a reduced LOXL2 occupancy at the CDH1 promoter (Fig.R21A, left panel). LOXL2 binding interference was associated with a concomitant increase of H3K4me3/me2 activation marks (Fig.R21A, right panel); these results correlated with the observed increase in the CDH1 mRNA

levels (Fig.R14A, right panel). As previously indicated, LOXL2wt, but not LOXL2mut overexpression, led to CDH1mRNA downregulation in MCF-7cells (Fig. R14C). Thus, we also checked the status of histone marks in this context. Although both ectopic LOXL2wt and mutant bound to the CDH1 promoter (Fig.R21B, left panel), only the active LOXL2 led to a decrease of H3K4me3/me2 marks in the same promoter (Fig.R21B, right panel). Neither depletion nor overexpression of LOXL2wt significantly altered other methylation marks (Fig.R21A and B, right panel).

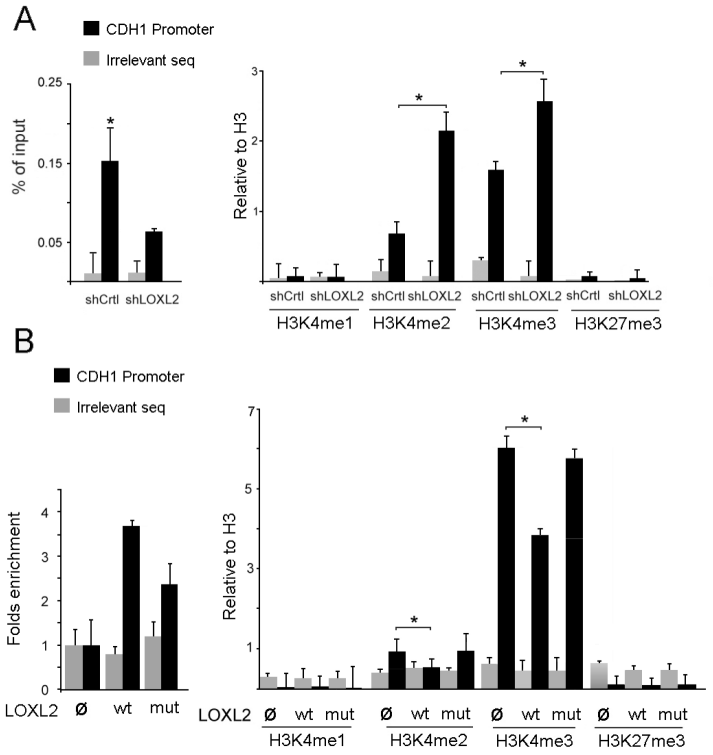


Figure R21. LOXL2 modulates H3K4 methylation in the CDH1 promoter. A. Loss of binding of LOXL2 and histone methyl marks in the CDH1 promoter in control and in the LOXL2 knockdown conditions were analyzed by ChIP. **B.** Flag-LOXL2 or inactive Flag-LOXL2 (mut) was expressed in MCF7 cells, and LOXL2 binding and histone methyl mark levels were analyzed by ChIP in the CDH1 promoter**.** Error bars provide the s.d., n=3. One asterisk indicates $p<0.05$.

Once we had demonstrated that LOXL2 could specifically regulate H3-associated lysine- methyl marks within the CDH1 promoter, we found interesting to investigate if LOXL2 could also have a global effect over these methyl marks. Surprisingly, overexpression of LOXL2wt in HEK 293 cells led to a huge global decrease of H3K4me3 (Fig.R22, left panel). Although to a less extent, di- and mono-methylated H3K4 (H3K4me2/me1) were also downregulated. We did not detect any significant decrease in other repression-associated marks, such as H3K9me3/2 and H3K27me3. LOXL2mut expression had no significant effect in any of the analysed marks. Accordingly, LOXL2 depletion in RWP-1 cells resulted in an increase in the global levels of H3K4me3/me2 (Fig.R22, right panel), again, without interfering in the levels of other methyl marks

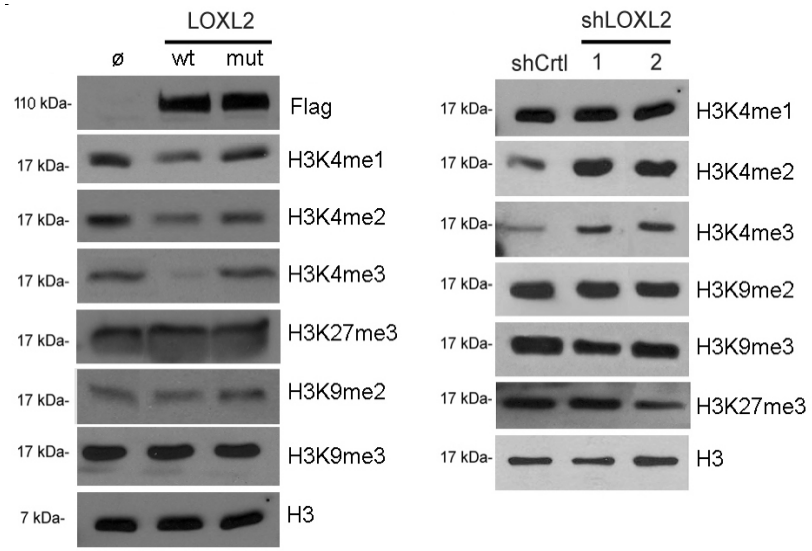


Figure R22. LOXL2 affects global H3K4 methyaltion *in vivo*. Histone methylmark levels were analyzed by western blot in HEK 293 cells transfected with Flag-LOXL2 or inactive Flag-LOXL2 (mut) and in two independent shLOXL2 RWP1 clones.

Taken together, these results indicated that LOXL2 specifically regulates the methylation status of H3K4 and that this regulation depends on its catalytic activity. Since we observed dramatic changes at a global level, it is very likely that LOXL2 effect within the cell goes beyond CDH1 gene regulation.

2.6 LOXL2 alters chromatin structure

The notorious global effect that LOXL2 overexpression caused over H3K4 methyl marks (Fig.R22), prompted us to investigate if LOXL2 could, in fact, affect nucleosomal compaction.

In order to determine it, we used micrococcal nuclease (MNase) digestion at different times in HEK 293 cells expressing high levels of ectopic LOXL2 (wt or mut). Analysis of MNase-digestion patterns of nuclei isolated from these cells, showed that chromatin from cells expressing LOXL2wt was much less sensitive to MNase digestion when compared to the chromatin from control cells or cells expressing a mutant LOXL2 (Fig.R23).

Thus, this result strongly suggests that LOXL2 may alter global chromatin structure; indeed, taken together, our observations associate LOXL2 catalytic activity with a less “open” chromatin state.

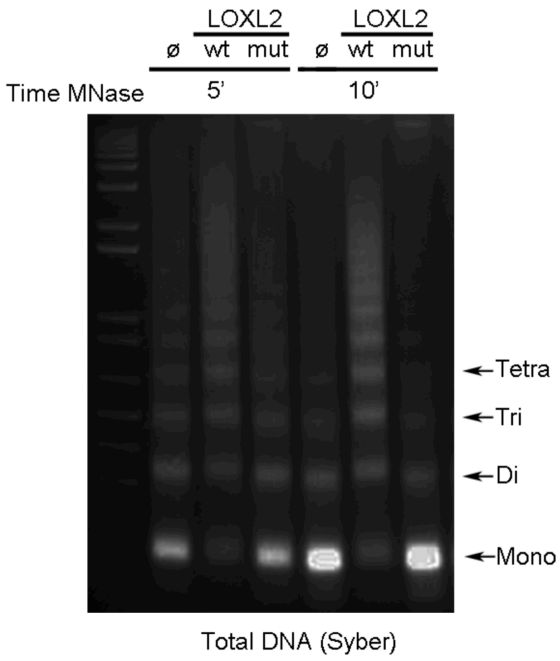


Figure R23. LOXL2 favors nucleosomal compaction. Nuclei isolated from HEK 293 cells transfected with LOXL2wt, mut, or an empty vector were digested with micrococcal nuclease (MNase) for 5 or 10min. Total genomic DNA was analyzed using agarose gel electrophoresis. Positions of the oligonucleosomes are indicated (Mono-, di-, tri-, tetra-).

2.7 LOXL2 is a H3K4me^3 deaminase *in vitro.*

Since LOXL2 is a lysine oxidase that, as our results had demonstrated, affects H3K4 methylation in vivo, we hypothesized that it may catalyse the conversion of lysine 4 to allysine in histone H3.

To analyze LOXL2 putative role as histone deaminase, LOXL2 recombinant proteins (wt and mut) were purified from baculovirus-infected Sf9 cell extracts using anti-Flag beads (Fig.R24). Next, LOXL2wt/mut activities towards different H3 peptides (corresponding to amino acids 1-16) were analysed by Attenuated Total Reflection-Fourier Transform Infrared spectroscopy (ATR-FTIR). Since the infrared light is sensible to side chains bond vibrations, we took advantage of it to detect specific modifications in the lysines of the different H3 peptides. Therefore, if a lysine suffers a chemical modification in its side chain, we will be able to observe a change in the peak wavenumber corresponding to the vibration of the new lysine bond.

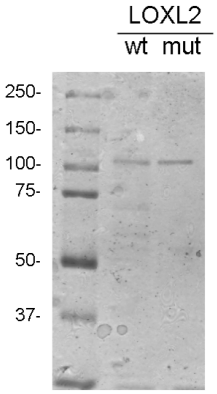


Figure R24. Affinity-purified Flag-tagged LOXL2wt and LOXL2mut recombinant proteins were analyzed by Coomassie blue staining. Recombinant LOXL2wt and LOXL2 mutant were expressed in Sf9 insect cells and purified with Flag M2 beads. A 105kDa protein was identified by Comassie blue staining in both cases. In LOXL2 mutant, two histidines (amino acid positions 626 and 628, respectively) residues were mutated to glutamine. These residues are predicted as Cu (II) binding sites and conserved in all LOX family members.

All ATR-FTIR difference spectra are shown in Fig.R25 (A-I). To initially characterize LOXL2 action, we incubated the wild type recombinant protein with an unmodified H3 peptide (unmethylated K4). This assay revealed a positive band at 1740 cm-1 (Fig.R25A), which corresponds to the formation of an aldehyde group[237], and two negative bands at 1577 and 1540 cm-1 (Fig.R25B) corresponding to the disappearance of a lysine-amine group. LOXL2 mutant failed to deaminate this peptide since no aldehyde group was formed (Fig.R25C). To further determine the substrate specificity of LOXL2, we analysed aldehyde formation using different H3 mutant peptides as a substrate. Whereas mutation of K9 or K14 to arginine (R) did not prevent aldehyde formation (Fig.R25D), aldehyde was not form when K4 was mutated (Fig.R25E). We neither detect aldehyde formation when we used an irrelevant peptide (Flag) as a substrate (not shown). These results showed, for the first time, that a LOX family protein could specifically deaminate lysines in histones; indeed, our results demonstrated that LOXL2 action was specific for K4.

Although LOX proteins activity towards methylated lysines had never been reported, our in vivo data (Fig.R21 and 22), prompted us to check if LOXL2 could also deaminate methylated K4. Surprisingly, a band at 1740 cm-1 also appeared when LOXL2wt was incubated with a H3K4me3 peptide (Fig.R25F). In contrast, difference spectra remained unaltered when LOXL2wt was incubated with H3K4me2 or H3K4me1 peptides (Fig.R25G and I). Interestingly, kinetic analysis of the aldehyde band formation demonstrated that LOXL2 presented much higher activity for the H3K4me3 peptide than for the unmethylated one (H3K4)(Fig.R25H).

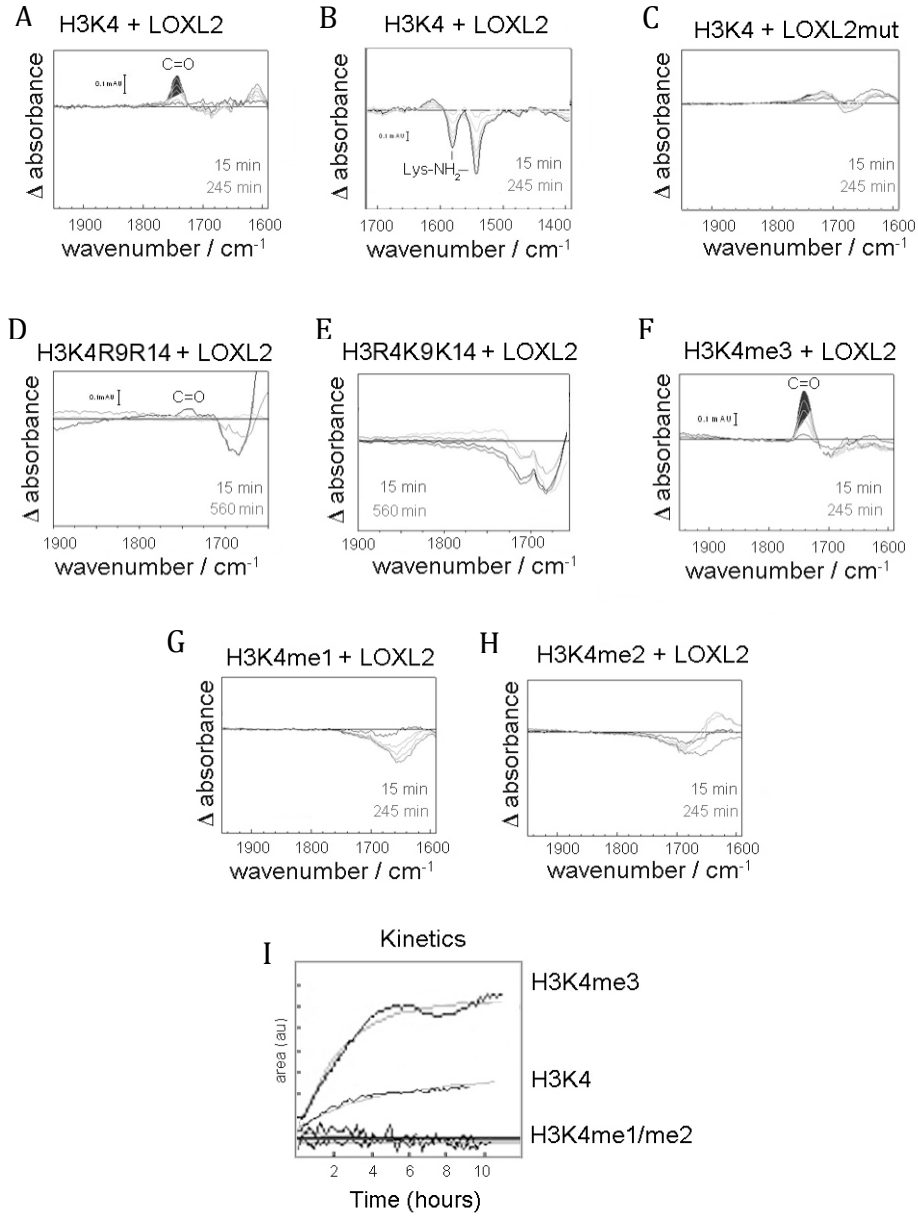


Figure R25. LOXL2 is a H3K4me3 deaminase in vitro (I). A-H. ATR-FTIR difference spectra after LOXL2 addition at 15 min and 245 min with the indicated peptides. Vibration bands corresponding to aldehyde or amino groups are shown. **I.** Kinetics of the formation of the aldehyde group is shown.

To strength Infrared spectroscopy data, we also performed Mass spectrometry analyses. As shown in Fig.R26, LOXL2 incubation with the H3K4me3 peptide revealed a net loss of 44 Da. This molecular weight decrease perfectly matches with the release of $N(CH3)^3$ and the formation of an aldehyde. In agreement with ATR-FTIR results, mass spectrometry analyses also showed that H3K4me2 and H3K4me1 peptides were not affected by LOXL2 activity (Fig.R26).

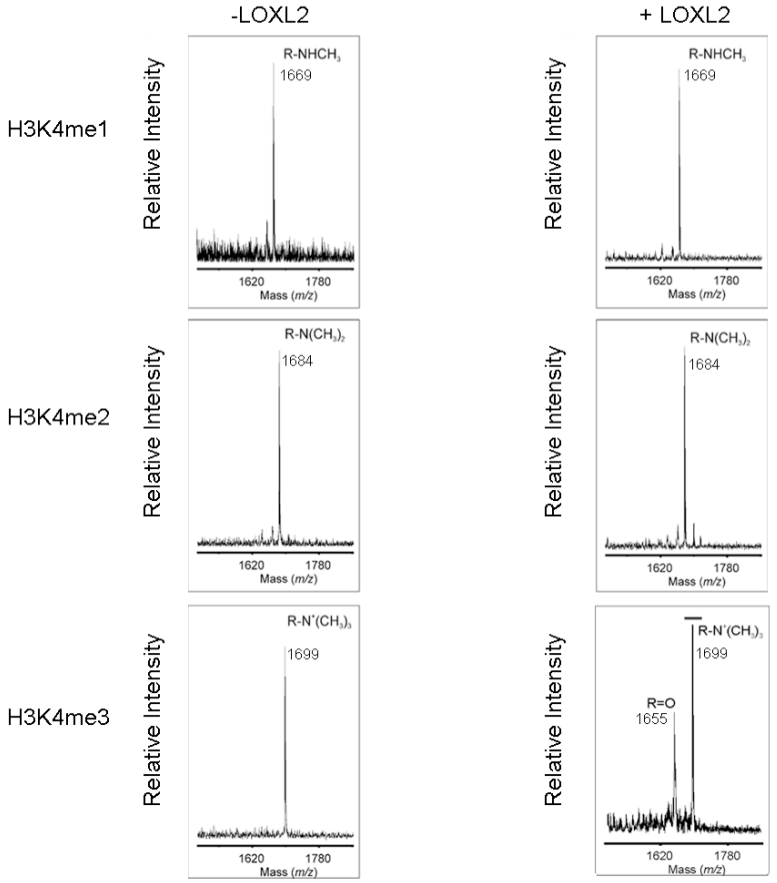


Figure R26. LOXL2 is a H3K4me3 deaminase in vitro (II). H3K4me3/me2/me1 peptides were incubated with or without recombinant LOXL2wt (ratio 1:100) and analysed by mass spectrometry.

To further validate our observations, we performed additional LOXL2 activity in vitro assays using more physiological substrates, such as purified bovine bulk histones or oligonucleosomes. In this case we determined LOXL2 effect by analysing the histone lysine methylation pattern by western blot. As shown in Fig.R27 (left panel), LOXL2wt effectively and specifically reduced H3K4me3 levels on bulk histones, again suggesting that H3K4me3 is the preferred substrate. Similar results were obtained when oligonucleosomes were use as substrates (Fig.R27, right panel). It is worth noting that higher amounts of LOXL2 (approx. 100x) were required to observe methylation changes in oligonucleosomes when compared to purified histones; this matter will be discussed later.

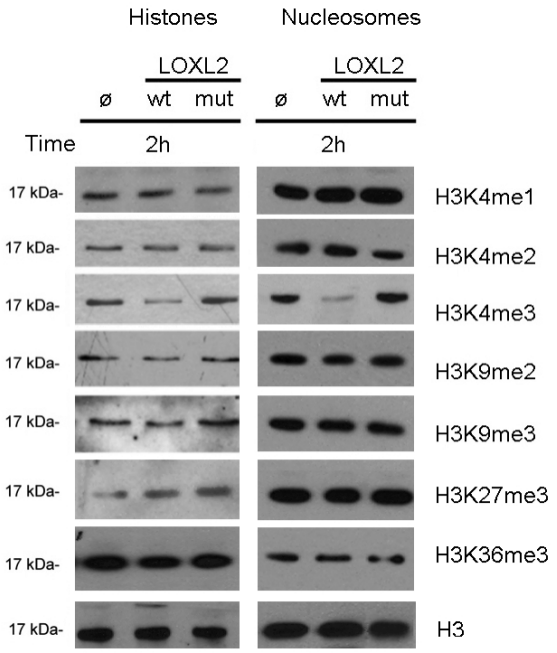


Figure R27. LOXL2 is a H3K4me3 deaminase in vitro (III). Purified histones and nucleosomes were incubated for 2h at 37ºC with LOXL2wt or mutant (200ng) and analysed by western blot with the indicated antibodies.

Taken together, our results clearly demonstrate that LOXL2 specifically deaminates trimethylated lysine 4 on histone H3 (H3K4me3) in vitro.

In order to further confirm this LOXL2-mediated enzymatic mechanism we performed additional experiments. First, by ATR-FTIR assays, we could detect O^{18} in the generated aldehyde group when LOXL2wt and H3K4me3 were incubated in the presence of H_2O^{18}, meaning that the source of oxygen comes from water. (The isotopic shift caused by O^{18} incorporation is shown in Fig.R28B). Second, as previously described for LOX mechanism of catalysis, hydrogen peroxide release was also detected. H_2O_2 was produced only when LOXL2wt, but not mutant, was incubated with the H3K4me3 peptide (Fig.R28B).

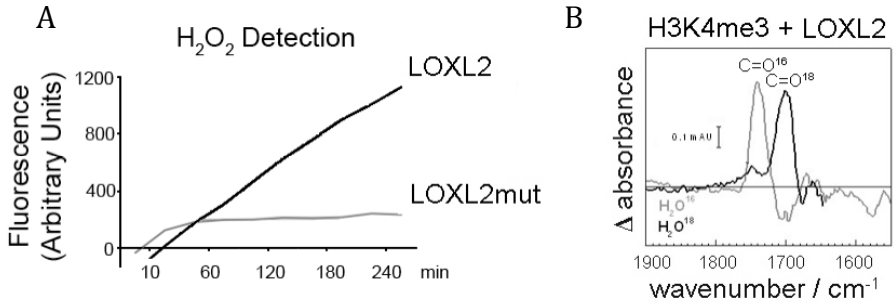


Figure R28. LOXL2 is a H3K4me3 deaminase in vitro (IV). A. Hydrogen peroxide release in the presence of LOXL2 with the H3K4me3 peptide was measured by fluorescence using the Amplex Red assay. **B.** Isotopic shift of the vibration of the aldehyde group formed after LOXL2 addition with H3K4me3 peptide in presence of $H2O^{18}$

2.8 LOXL2 modulates H3 oxidation *in vivo*.

In order to check whether this reaction occurs *in vivo*, and since it was impossible to generate an antibody against this new modification due to aldehyde group high reactivity, we used an indirect approach based on an activated biotin (biotin-hydrazide) that reacts with aldehyde groups.

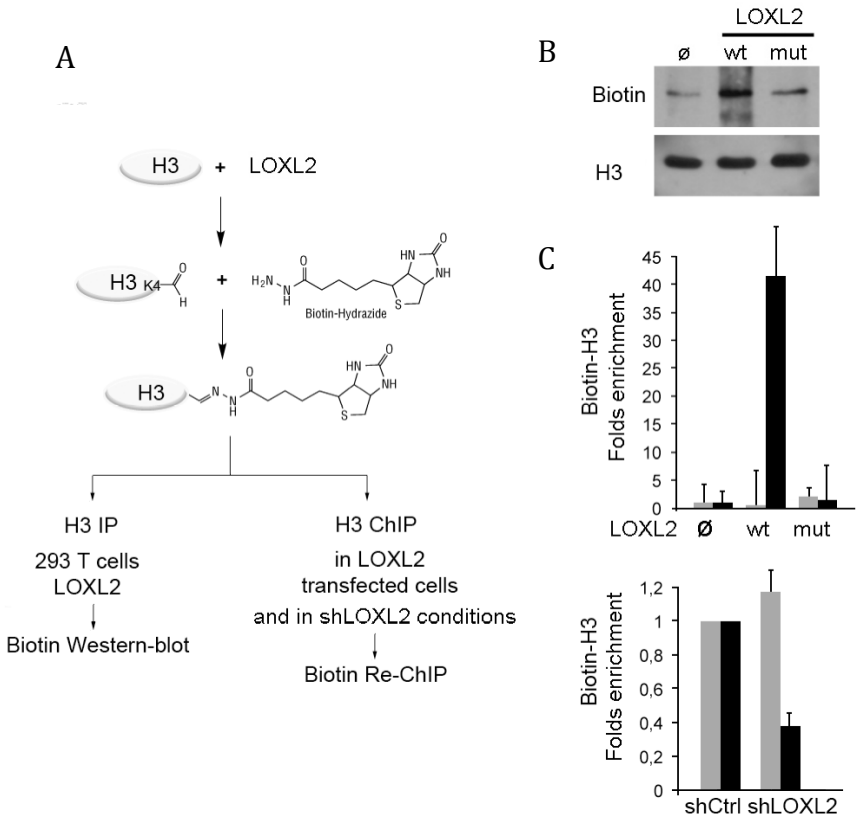


Figure R29. LOXL2 affects H3 oxidation *in vivo*. A. Schematic representation of the *in vivo* detection of deaminated H3 **B.** Total extracts of HEK 293 cells transfected with LOXL2 (wt and mut) were incubated with biotin-hydrazide. H3 was immunoprecipitated and biotin incorporation (oxidized H3) was checked by western blot using streptavidin-HRP **C.** Re-ChIP for Biotin H3 in MCF7 cells transfected with LOXL2wt and mutant (top) and in shCtrl and shLOXL2 in MDA-MB-231 cells (bottom) in the CDH1 promoter. The lysate was incubated with biotin-hydrazide before Re-ChIP. Extracts were sequentially IP with anti-H3 and streptavidin-beads

Following experimental procedures represented in Fig.R29A, we could observe a convincing increase of biotinylated H3 in total extracts of HEK 293 cells expressing ectopic LOXL2wt (Fig.R29B), thus indicating that LOXL2 promotes H3 oxidation in vivo. Moreover, by using similar methodology combined with Chip assays (Fig.R29A), we could also detect this modification at the chromatin level; indeed, oxidized H3 was present at the CDH1 promoter of MCF-7 cells only upon LOXL2 expression (Fig.R29C, upper panel). Conversely, LOXL2 depletion in MDA-MB 231 cells decreased the extent of this modification in the same promoter (Fig.R29C, lower panel), reinforcing again LOXL2 role in oxidizing histone H3 in vivo.

2.9 LOXL2 counteracts histone methyltranferase activity *in vitro*

In this work we have characterized a new H3K4 modification, deamination. This discovery naturally presents an obvious next question; which are the functional consequences underlying this new modification? One of the scenarios we first considered is that LOXL2 might counteract the action of H3K4 methyltransferases. As a first approximation to investigate such hypothesis, we analysed the effect of LOXL2 in the methylation of H3K4 by the MLL protein, a classical methyltransferase. As shown in Fig.R30, MLL-mediated methylation was strongly impaired in the presence of LOXL2wt protein. As expected LOXL2 mutant had no effect. This result demonstrates that LOXL2 counteract H3K4 methylation in vitro.

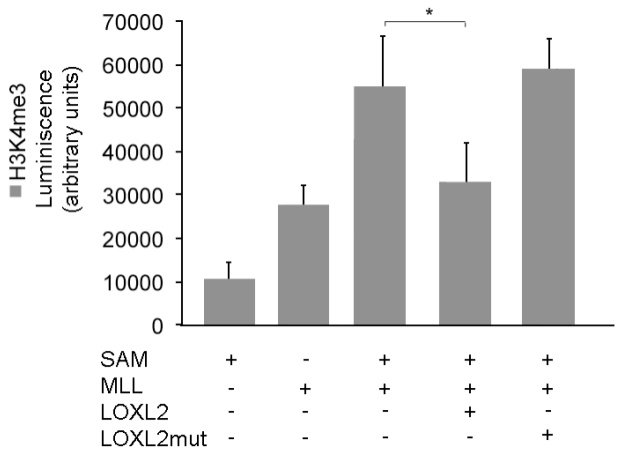


Figure R30. LOXL2 prevents MLL-mediated trimethylation of histone H3 lysine 4 in vitro. Unmethylated H3 peptide was incubated with MLL (200ng) in the absence or presence of 20μM S-adenosyl-L-methyonine (SAM) and LOXL2 proteins (300 ng) as indicated. In order to detect trimethylated lysine 4 levels, samples were incubated with an antibody against H3K4me3 and an HRP-secondary antibody. Chemiluminescence measurement was performed following HRP substrate addition.

2.10 Microarray analysis provides new insights into LOXL2 role in tumor progression.

As previously indicated, LOXL2 depletion induced a strong epithelial phenotype in both RWP-1shLOXL2 clones and MDA-MB 231shLOXL2 cell pools. Remarkably, when we tried to generate stable clones of mesenchymal cells (MDA-MB 231 or Mia Paca-2) upon LOXL2 depletion, proliferation was inhibited and cells underwent apoptosis. To understand the mechanistic basis behind these LOXL2-mediated dramatic effects we used Affymetrix microarrays and, consequently, established genome-wide gene expression profiles of MDA-MB 231 cells transiently infected with control or LOXL2 shRNAs,. When compared with control cells, 412 and 312 genes were respectively

derepressed and induced significantly ($P < 0.05$) in LOXL2-deficient cells. Heat map showing the most deregulated genes as well as the main pathways and functions affected by LOXL2 depletion are represented in Fig.R31 and R.32, respectively.

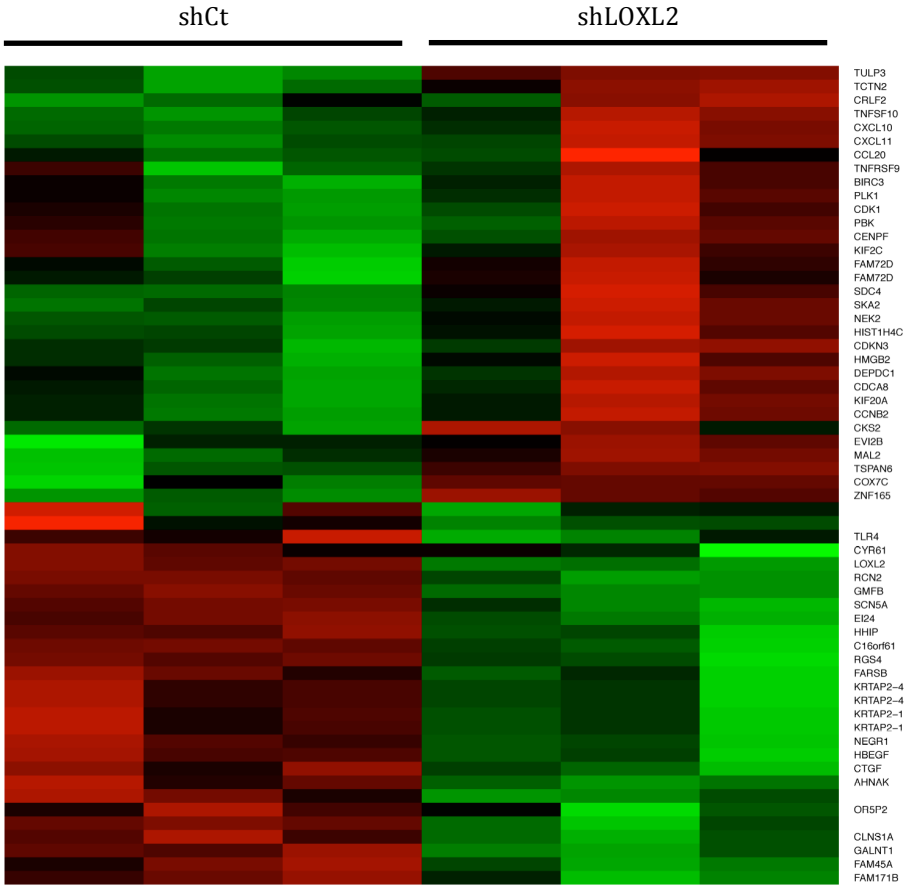


Figure R31. Analysis of LOXL2 target genes in mesenchymal cells (I).) Heat map of microarray data for three biological replicates of MDA-MB 231 cells infected with a control or a LOXL2-specific shRNA. RNA samples were processed. Cells were subjected to two rounds of infection and RNA was isolated 24h after the last infection. The most highly up-regulated and down-regulated transcripts are presented. The levels of mRNAs expressed from are depicted as green (low) or red (high), where black indicates no change.

Although obtained gene expression profiles require further study and validation, they disclosed a large amount of information regarding LOXL2 putative roles in mesenchymal cells. Indeed, we observed that LOXL2 depletion derepressed genes involved in epithelial differentiation, cell cycle regulation, activation of cell death programs, centromere organization or histone transcriptional regulation. Conversely, LOXL2 knock down induced the activation of genes involved in ETM and metastasis such as TGF-β, Notch2 or MMP-1, among others.

Overall, these data is in agreement with our previous observations and, in addition, extend our understanding for the mechanisms by which LOXL2 could be involved in EMT and tumor progression.

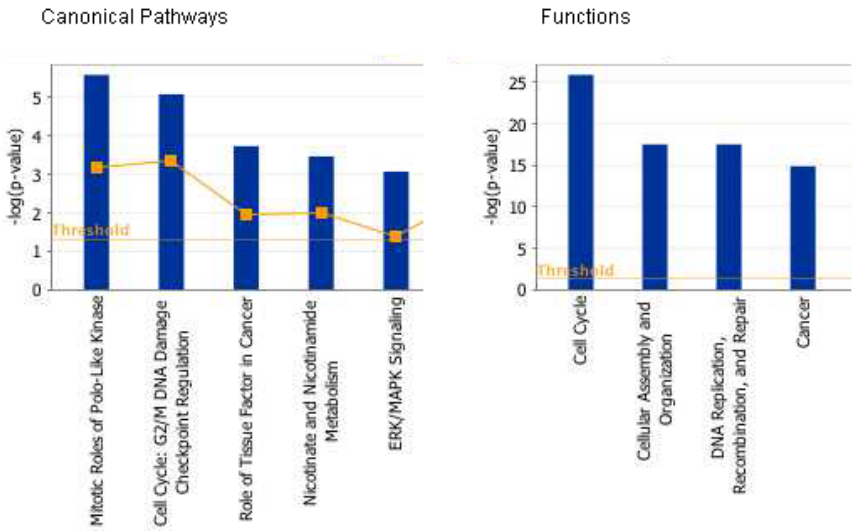


Figure R32. Analysis of LOXL2 target genes in mesenchymal cells (II). Ingenuity analysis revealed biological pathways and functions differentially expressed in LOXL2 deficient MDA-MB 231 cells.

Discussion

1. PRC2 is required for Snail1-mediated E-cadherin repression.

PRC2 role in Snail1-mediated repression

As previously indicated, this thesis has been focused on the characterization of the epigenetic mechanisms underlying Snail1-mediated CDH1 repression. In our first project we investigated the importance of Polycomb proteins in this process.

Snail1 recruits the PRC2 complex to the CDH1 promoter; indeed, binding of Suz12 is dependent on Snail1 and on the integrity of Snail1-binding sites in this promoter. As a consequence, transcriptional repression by Snail1 is associated with H3K27me3. Accordingly, CDH1 repression by Snail1 is impaired in cells in which Suz12 has been depleted. As this alteration was observed in both embryonic stem cells and tumoral cells, it could be assumed that Snail1-PRC2 interplay has a physiological relevance. Moreover, our results indicate that Snail1 can associate, directly or indirectly, with PRC2 components. Thus, providing a physical explanation for the recruitment of these factors to Snail1-targeted promoters.

The altered effect of Snail1 in Suz12 knockdown cells is not limited to CDH1; repression of other Snail1 target genes, such as PTEN, Mucin-1 or Snail1, is also prevented in the absence of PRC2 activity. These results suggest that PRC2 recruitment could be a general mechanism underlying Snail1-mediated repression.

Regarding PRC2 role in Snail1 self-regulation, we observed that, while modest, endogenous Snail1 increase upon PRC2 depletion was sufficient to induce a more mesenchymal phenotype without, as

expected, significantly altering CDH1 expression levels due to the absence of PRC2. This observation suggests that Snail1 is able to directly or indirectly activate mesenchymal genes independently of PRC2. Since it has been recently reported in our lab that Snail1 could also function as an activator when associated with specific co-activators (unpublished data) and since our results predict PRC2 recruitment as a Snail1-mediated repression general mechanism, it is plausible that the observed phenotypical changes respond to Snail1 direct activation of mesenchymal genes.

Snail1-mediated epigenetic regulation in the context of EMT

Importantly, in our work we also show that Snail1-SNAG domain is required for Snail1-mediated repression, PRC2 binding, and PRC2 recruitment to the CDH1 promoter. As mentioned in the introduction, prior to our work some have already highlighted the importance of the SNAG domain in the repression of CDH1 gene expression by Snail1[47,67]. In fact, in 2004, Peinado and colleagues reported that Snail1-mediated CDH1 repression requires the association of the SNAG domain with HDAC1/2, an interaction that is mediated by the corepressor Sin3A[69]. Therefore, our findings emphasized the idea that the SNAG domain is crucial for Snail1 transcriptional repression due to its ability in recruiting co-repressors and epigenetic modifiers. Accordingly, following the publication of our work, other groups have identified new co-repressors and epigenetic regulators involved in Snail1-mediated CDH1 repression; in all cases, the SNAG domain is crucial for a proper physical and/or functional cooperation.

First, it was reported that the AJUBA family of LIM proteins function as co-repressors of Snail1[238], shortly after; this functional connection was linked with AJUBA ability to recruit the arginine methyltransferase PRMT5. Snail1 requires the AJUBA-mediated recruitment of PRMT5 to repress E-cadherin, in fact depletion of PRMT5 stimulates E-cadherin expression, and the SNAIL1, AJUBA, and PRMT5 ternary complex can be found at the proximal promoter region of the E-cadherin gene, concomitant with increased arginine methylation (H4R3) at the locus[239].

More recently, two independent studies have shown that Snail1 directly interacts with the histone demethylase **LSD1**[236,240]. Lin T. and colleagues[240] demonstrated that Snail1 recruits LSD1 to the E-cadherin and other epithelial gene promoters, resulting in down regulation of the active H3K4me2 mark and promoter activity; hence, depletion of LSD1 substantially impairs Snail1-mediated repression.

Lin Y. and colleagues approximation to this topic is much more attractive[236]. Apart from showing very similar data to that exposed above, they added a few remarkable observations. For example, they insinuated that Snail1 might represent the first unique example of a transcription factor that uses a histone-mimicking motif, SNAG domain in this case, as a 'hook' for recruiting chromatin-modifying enzymes. The sequence of the SNAG domain is highly similar to that of the N-terminus of histone H3 and adopts a conformation similar to that of histone H3 at the catalytic cavity of LSD1. Mutation of the critical contact residues of the SNAG domain of Snail1 completely disrupts the interaction of Snail1 with LSD1 and blocks its suppressive activity on the E-cadherin promoter. Importantly, LSD1 enzymatic inhibitors and histone H3 peptides disrupt Snail1–LSD1

interaction. Interestingly, LSD1 has been found as a component of the HDACs-containing repressor complex CoREST. Accordingly, the authors showed that CoREST, form a ternary complex with LSD1-Snail1 and enhance the stability of the individual components of the complex.

Taken together, all these findings indicate that Snail1 uses different co-repressor complexes to achieve its repressor function during the EMT process. Indeed, a picture in which binding of Snail1 to the CDH1 promoter leads to epigenetic gene silencing in a coordinated multistage process seems plausible. At this respect, some have suggested that epigenetic modifications may be interdependent and successive. If we consider this scenario, it would be logical to assume that proteins recruited to Snail1 target genes are somehow connected. At this concern, PRC2 has been found to physically[131] and functionally[241,242] associate with HDACs. In addition, Suz12 has been found to interact with MEP50[243], a PMRT5-associated protein[244]. In the case of LSD1, although no direct interaction have been described, the possibility of PRC2 and LSD1 association in the context of HDACs-containing complexes, such as CoREST or Sin3, does not seem unlikely.

But, regardless of the nature of their interactions, how these proteins coordinate their enzymatic activities to achieve E-cadherin silencing? It is known that, at least in Drosophila, transcriptional silencing is initiated through the removal of H3K4 methylation by the LSD1 homologue SU(VAR)3-3[245]. In addition, it has also been reported that acetylation prevents the establishment of the H3K27me3 mark[108]. Given these observations we could conceive a scenario in which HDAC1/2 within CoREST or Sin3A complexes deacetylate histone H3

and H4 and LSD1 demethylates H3K4me2/1. Subsequently, PRC2 and PRMT5 are drafted to direct trimethylation of H3K27 and methylation of H4R3, respectively.

Description of the functional role of the indicated epigenetic modifiers in E-cadherin repression has significantly improved the understanding of how Snail1 exerts its repressive function during tumor progression. However, there are some epigenetics events known to be important for gene silencing that has not been clarified in these context. For example, it is widely accepted that robust silencing may involve H3K27me3 along with demethylation of H3K4me3[246]. And as far as is known Snail1 has not been associated with any H3K4me3 demethylase. At this regard, PRC2 has been functionally related with H3K4me2/3 demethylase RBP2[247], hence, it would be interesting to determine the role of this protein as well as of others H3K4me3 demethylases in Snail1-mediated repression.

On the other hand, H3K27me3 is not the only mark associated with a repressive state. Although it has been mostly associated with heterochromatin formation, di- and trimethylation of H3K9 are also found in repressed promoters. In fact, it was reported that transcriptional repression by Snail1 is associated with an increase of H3K9me2[69]. Thus, it would be interesting to study the significance of this histone modification during EMT-induced repression of the E-cadherin gene.

Another point of interest for the understanding of how E-cadherin is silenced relies on another epigenetic modification, DNA methylation. Indeed, E-cadherin promoter aberrant methylation has been found in many cancer cell lines and tumour samples[248]. Interestingly, it has been reported that whereas cell lines with CDH1 somatic mutations

show epithelial features, cells with CDH1 promoter hypermethylation normally present a mesenchymal morphology and a gene expression profile very similar to those cells that have undergone EMT[249,250]. These observations suggest that, unlike mutational inactivation, CDH1 promoter hypermethylation is involved in tumor progression and that, in fact, aberrant methylation might be triggered by EMT-TFs and/or the epigenetic regulators associated with them. However, how the initial silencing of the E-cadherin gene promoter converts into a long-term repression by DNA hypermethylation remains to be resolved.

At this regard, we demonstrate that DNMTs inhibition increase E-cadherin expression; in fact DNMT1 is bound to the E-cadherin promoter only in cells expressing high levels of ectopic or endogenous Snail1, hence, DNMT1 recruitment seems to be Snail1-dependent. Intriguingly, Snail1 expression and DNMT1 binding did not correlate with promoter hypermethylation when analysed by bisulfite genomic sequencing. At this regard, it was observed in our lab that E-cadherin promoter aberrant methylation within transformed cells is more frequent in tumor samples that in tumoral cells lines, thus suggesting that DNA methylation might be lost in cultured cells. In fact, it has previously been reported that not always tumoral cells presenting mesenchymal features are associated to DNA aberrant methylation, nor the opposite[251,252]

Nevertheless, our results show that Snail1 has a positive effect over DNMTs recruitment, and this becomes even more relevant if we take into account that PRC2 function in cancer it has been strongly associated with its ability to recruit DNMTs to target promoters[132]. A further investigation of Snail1 and PRC2 role in a broad panel of

tumor samples, tumoral cell lines, as well as in EMT-induced models, would be required to elucidate the role of these proteins in E-cadherin promoter methylation.

Snail1 and Zeb1 use different epigenetic mechanisms to inhibit CDH1.

Another point of discussion within our results came from the observation that PRC2 depletion only partially reactivates E-cadherin expression, hence, indicating that its repression is only partially dependent on PRC2. Accordingly, we observed that Zeb1-mediated CDH1 repression was not compromised in the absence of Suz12, thereby suggesting that Snail1 and Zeb1 might use different mechanism to exert their repression functions. Importantly, a recent report have reinforce our observations by showing that PRC2- and Zeb1-mediated CDH1 repression are independently and comparably critical for CSC growth[253].

Whereas, as discussed above, Snail1 associated epigenetic regulators have been intensively studied in recent years, Zeb1 ones remain poorly understood. Indeed, Zeb family proteins have only been functionally associated with CtBP co-repressor[22,24]. However, when CtBP co-repressor core complex was identified[254], ZEB1 and ZEB2 were detected together with CtBP1/2 HDAC1/2, G9a (a H3K9 HMT), chromodomain-containing proteins, CoREST, CoREST associated proteins and LSD1. Although none of Zeb putative interactions has been functionally validated, these results permit to visualize a very interesting picture. In this presumed situation Snail1 and Zeb1 would share those interactors involved in initiating the repression process

(as indicated before, HDACs, CoREST and LSD1). However, they would contribute in a different way to the establishment of a repressive mark, Snail1 via H3K27me3 (PRC2) and Zeb1 via H3K9me3 (G9a). Interestingly, as well as PRC2[132], G9A can direct de novo DNA methylation to certain loci[255].

Our results are not the first example of Snail1 and Zeb1 differences within CDH1 repression. In fact, as mentioned in the introduction, it has been suggested that whereas Snail1 function might be more for relevant for the initial CDH1 repression, Zeb1 role could be more relevant for the maintenance of this repression. Thus, the fact that Snail1 and Zeb1 are not equally depending on PRC2 may provide a mechanistic explanation at this regard.

PRC2 function and recruitment

At the time our results were published, the most accepted working model to explain how PcG complexes modulate chromatin was that transcription factors and their associated machinery dictate loci destined for silencing, and PRC2 tags these target loci by methylating lysine 27 of histone H3. This mark was then recognized and bound by the chromodomain of CBX proteins within PRC1, which mediates transcriptional silencing by binding methylated histones in the vicinity and by monoubiquitylating Lys 119 of histone H2A (H2AK119ub) via RING1A/B[124,125]. Accordingly, in our work we showed that both in ES cells (CBX8) and in tumoral cell lines (BMI1), PRC1 proteins binding completely correlated with that of PRC2. Importantly, in both cases PRC1 recruitment also seemed Snail1-dependent. Even though we did not check H2AK119ub in the CDH1

promoter, our results strongly fitted into the proposed model. However, in the last three years, genome-wide analyses have provided a large amount of information and this new data has challenged the above-mentioned theories for PRC2 function and recruitment. For example, increasing evidences indicate that the hierarchical recruitment model in which PRC2 and PRC1 bind sequentially is not accurate. Although PRC2 and PRC1 are often both required to maintain gene repression[110], it has been reported that there are genes targeted by PRC2 that lack H2AK119ub[141], and just the opposite, genes targeted by PRC1 in the absence of PRC2[256-258].

An even more studied topic has been the PRC2 recruitment to target genes in Mammals. As mentioned in the introduction, first large-scale analysis revealed that PRC2 is broadly distributed across many kilobases of mammalian developmental genes[141-143], thus suggesting that many different mammalian TFs might contribute to PcG recruitment. At this regard, some authors have proposed[126] that 'cell fate' TFs (CFTFs), those that regulate cell fate decisions during embryogenesis or adult cell differentiation, are strong candidates for PcG proteins recruitment to and dissociation from their target genes. Interestingly, most, if not all, CFTFs are themselves PcG target genes[142,143,161]. Accordingly, functional cooperation of PcG proteins and mammalian CFTFs has been described in several reports. (reviewed in Bracken et al, 2009[126]). Definitely, Snail1 could be considered one of these CFTFs. Indeed, as mentioned in the introduction, Snail1 is a well-known regulator of cell fate decisions during gastrulation and other developmental processes, and, as we have demonstrated, it is able to recruit PRC2 to target genes. Importantly, we have also shown that Snail1 itself is also a PcG target gene.

The main about concern about considering CFTFs as Polycomb recruiters is that genome-wide analysis does not show a clear global overlap between any of the analyzed CFTCs and the Polycomb proteins[122]. This circumstance, as well as the lack of specific DNA sequence consensus, is promoting an increasing interest on the described function of non-coding RNAs (ncRNAs) as recruiters of PcG proteins to target genes[259]. Long ncRNAs are known to promote PRC2 binding *in* cis (X inactivation and imprinting[260,261] and *in trans.* PRC2 binding *in trans* is associated with the ncRNA HOTAIR[262,263], which is expressed from the HOXC locus but is associated with repression of 40kb of the HOXD locus. Interestingly, it has been reported that HOTAIR interacts with PRC2 and LSD1 complexes simultaneously, with the two protein complexes associating with different sites of the RNA[264].

Regarding PRC2-ncRNAs interplay, in our lab it has been recently demonstrated that other ncRNAs, such as Lef-1 natural antisense transcript (NAT), can recruit PRC2 to repress Lef-1 transcription in the context of EMT regulation (unpublished data). Interestingly, a CDH1 NAT has also been identified. Preliminary data indicate that its expression could be upregulated upon Snail1 expression. Therefore, the possibility of an RNA-dependent mechanism for CDH1 repression should not be discarded. Interestingly, this additional mechanism might be also dependent on Snail1 and PRC2.

New insights into PRC2 role in tumor progression

As mentioned in the introduction, the role of PcG proteins in stem cell fate and pluripotency is firmly established[106,160,265]. Several studies

have revealed that PcG complexes bind to and repress numerous genes encoding key developmental regulators and signalling proteins in embryonic stem cells (ESCs). Importantly, these repressed PcG target genes preferentially activated during ESC differentiation[130,142,143,161]. This correlates with the loss of PRC2 binding, loss of the H3K27me3 and increase in the active H3K4me3 modifications. Consequently, knockout of the PRC2 proteins leads to a loss of H3K27me3, a spontaneous increase in developmental gene expression, and consequently, differentiation defects of embryonic stem cells[258,266,267]. Accordingly, we observed that in ESCs null for Suz12, H3K27me3 mark was lost in the E-cadherin gene and, consequently, CDH1 expression was upregulated. Thus, our results suggest that CDH1 gene could be an important Suz12 target in ES cells. Indeed, since CDH1 expression is associated to cellular differentiation, it is reasonable to predict that its PRC2-mediated repression could be important to maintain ESCs pluripotency and regulate cell fate. Importantly, as discussed above, we have also found that PRC2-dependent CDH1 repression is relevant in the context of EMT, hence, suggesting that PRC2 have analogous functions in cancer and development. Regarding this hypothesis, it has been recently reported that Suz12-mediated CDH1 repression affects the process of CSC formation[253]. Iliopoulos and colleagues demonstrate that in comparison to non-stem cancer cells (NSCCs), CSCs in the same population show strongly increased Suz12 levels and dramatically reduced CDH1 levels. Accordingly, differentiation of CSCs into NSCCs results in inhibition of Suz12 and upregulation of CDH1 at both the mRNA and protein level.

Taken together, all the results discussed here strongly suggest that PRC2-dependent CDH1 repression is a key mechanism in EMT and

CSCs-driven tumor progression. Besides, since PRC2 proteins overexpression has been widely reports in tumors, we speculate that PRC2 could drive to malignancy my aberrantly mimicking its role in repressing cell-differentiation markers in ESCs

2. LOXL2 is a H3K4me3 deaminase

LOXL2 is a co-repressor and modulates H3K4 methylation and chromatin structure in a global manner.

In the second part of this thesis we kept on investigating the mechanisms underlying Snail1-mediated repression. However, since in the first part we investigated if a well-known epigenetic modifier as PRC2 was involved in CDH1 repression, in this second part we studied LOXL2, a protein of unidentified function, but known to be relevant in the EMT process.

As explained in the introduction, although LOXL2 mechanisms of action remained poorly characterized, LOXL2 effects have been related to both extra- and intracellular processes within both physiological and pathological situations. Interestingly, Peinado and colleagues[219] reported that LOXL2 is required for Snail1-mediated CDH1 repression. Accordingly, LOXL2 interacts with Snail1 through its SNAG domain. Regarding LOXL2 function in this process, the authors suggest that LOXL2 might be regulating Snail1 protein stability in the cytoplasm.

Conversely, our results indicated that LOXL2 protein is required for E-cadherin repression at a transcriptional level. Indeed, LOXL2 is bound to the E-cadherin promoter and, more importantly, LOXL2 depletion strongly impairs Snail1-mediated CDH1 repression without affecting Snail1 protein levels. Thus, although LOXL2 role as regulator of Snail1 protein stability should not be discarded and requires further comprehension, our results clearly point to a LOXL2 direct effect on the CDH1 gene. In fact, endogenous LOXL2 is mainly located

in the chromatin fraction in all analysed tumoral cell lines, independently of their relative LOXL2 expression levels.

Since LOXL2 is required for Snail1-mediated repression, is bound to the E-cadherin promoter and, as we observed, acts as a repressor when directed to an ectopic target promoter, we anticipated that it could function as a co-repressor. Indeed, like the entire machinery associated with Snail1 transcriptional repression, LOXL2 interacts with the SNAG domain of Snail1.

In an effort to better understand the mechanistic roles of LOXL2, we employed affinity purification and mass spectrometry to identify LOXL2 partners. As shown in the results section, our experiments revealed that most of the proteins that copurified with LOXL2 are members of repressive complexes, such as PRC2[231], CAF-1[232] or the HDAC-containing complexes, NuRD and Sin3[70,233-235]. Indeed, subsequent coimmunoprecipitation assays revealed that LOXL2 interacts with HDAC1, CHAF1B, RBBP4, EZH2 and Sin3A proteins.

Obviously, these results prompted much speculation. First, they reinforce LOXL2-Snail1 functional cooperation. Indeed, they discovered that PRC2, HDACs and Sin3A complexes associate with both Snail1 and LOXL2 proteins. Since Snail1 has also been associated with LSD1 activity, we decided to check if LOXL2 and LSD1 also copurified. In fact, though it was not detected in our Mass spectrometry analysis, LSD1 was found to interact with LOXL2. This observation further validated Snail1-LOXL2 functional interplay and, additionally, provided a new link between well-known epigenetic modifiers such as PRC2 and LSD1. Second, LOXL2 interacts with the histone chaperone chromatin assembly factor 1 (CAF-1). CAF-1 is essential in human cells for the de novo deposition of histones H3 and

H4 at the DNA replication fork[232,234]. This function is known to be crucial for chromatin assembly following replication and DNA repair as well as for coordinated inheritance of states of gene expression. Regarding its role in gene expression regulation, CAF-1 has been found to bind to HP1 proteins and functionally cooperate with them in the replication and transcriptional regulation of pericentric heterochromatin[268,269]. In fact, HP1 proteins and the epigenetic mark they recognised (H3K9me3) are crucial for condensing pericentric regions to maintain transcriptional repression and to prevent these regions from being aberrantly replicated[269].

Although the physiological relevance of the described interactions requires further comprehension, their collective presence in LOXL2-associated complex(es) indicated that LOXL2 might be involved in transcriptional repression both in euchromatin and heterochromatin contexts. Indeed, the nature of its interactors predicted that LOXL2 could function as a general co-repressor, more likely, through direct or indirect regulation of epigenetic events. This possibility was further reinforced when we analysed the effects of manipulating LOXL2 expression on histone marks, either at specific loci (E-cadherin promoter) or at a global level.

As shown in the results section, whereas LOXL2wt overexpression specifically reduced the levels of H3K4me3/2 in the E-cadherin promoter, LOXL2 depletion induced the increase of these same marks. Interestingly, both LOXL2wt and mutant were bound to E-cadherin gene although the mutant version did not affect H3K4 methylation, or E-cadherin expression. Thus, suggesting that LOXL2 catalytic domain is required for protein activity, but not for its binding. As indicated, LOXL2 could affect H3K4me also in a global

manner. Indeed, LOXL2wt overexpression induced a remarkable global decrease in the levels of H3K4me3 and, to a less extent H3K4me2/1. Importantly, other histone lysine marks (K9me, K27me) or H3 total protein levels were not significantly altered. Accordingly, depletion of LOXL2 induced a significant global increase of H3K4me3/2.

In summary, our findings demonstrated that LOXL2 regulates H3K4 methylation and revealed that this effect is beyond E-cadherin repression. Regarding LOXL2 global effect, MNase-digestion patterns showed that LOXL2wt overexpression strikingly increased global nucleosomal compaction. While this effect has already been reported in the literature after alteration of other epigenetic regulators[270,271] (HDAC3, CAF-1), nucleosome compaction was increased to a higher extent in the case of LOXL2. Although LOXL2 effect could be overestimated due to the high levels of ectopic LOXL2 expression, our results indicate that LOXL2 may regulate chromatin structure.

Characterization of a new LOXL2 function: H3K4me3 deamination

Since LOXL2 is a lysyl oxidase, we speculated that it could directly act over H3K4 mark. By using ATR-FTIR technique, we observed that LOXL2 specifically deaminates unmethylated H3K4 and H3K4me3 peptides but not H3K4me2/1. Since LOX proteins associated deamination has only been addressed in unmodified lysines, it was surprising to detect that LOXL2 enzymatic reaction was much more efficient for the H3K4me3 peptide than for the unmethylated one as observed on the kinetics data. Importantly, Mass spectrometry (MS) analysis further confirmed H3K4me3 as a specific substrate for

LOXL2. It is worth to remark that ATR-FTIR technique has never previously been used to identify epigenetic modifications. Indeed, this technique resolves some of the limitations of the MS system. For example, it determines the generation and release of the modified chemical groups, it allows to monitor the kinetics reaction at real time and, importantly, it can predict the substrate secondary structure changes produced. Last but not least, very little amount of enzyme is required to perform these experiments.

LOXL2-mediated specific H3K4me3 deamination was further validated in more physiological substrates, such as purified bovine bulk histones or oligonucleosomes. Importantly, it was required to add a much higher amount of LOXL2wt protein (around 100x more) to detect significant changes when oligonucleosomes were used as a substrate. At this regard, some have nicely addressed that demethylases requires the functional cooperation of their associated partners to modify nucleosomes but not histones. For example it has been described that CoREST complex is crucial to enable LSD1 demethylation of nucleosomal substrates[272,273]. However, it has also been reported that recombinant demethylases alone (RBP2, JHDM1) can successfully modify nucleosomes[174,274]. Importantly, in most of the cases, it has not been further investigated if the observed effects really respond to the enzyme autonomous function or they could be motivated by the high amounts of enzyme used in the reaction, as our results might suggest.

Taken together, our in vitro experiments led to the characterization of LOXL2 as a H3K4 deaminase. Importantly, LOXL2 deaminates H3K4 preferentially when it is tri-methylated. Thus, indicating for the first time, that LOX proteins can also specifically deaminate modified

lysines in vitro. However, this new finding raised a concern regarding its chemical-associated reaction; indeed, the well-characterized LOXL mechanism of catalysis for unmodified lysines is not compatible with the deamination of a trimethylated lysine. LOX-catalysed reaction model described by Lucero and Kagan (explain in detail in Fig.D1), involves the initial formation of a Schiff base between the primary amine and the LTQ group[192]. The formation of this Schiff base is not possible in the presence of a methylated lysine.

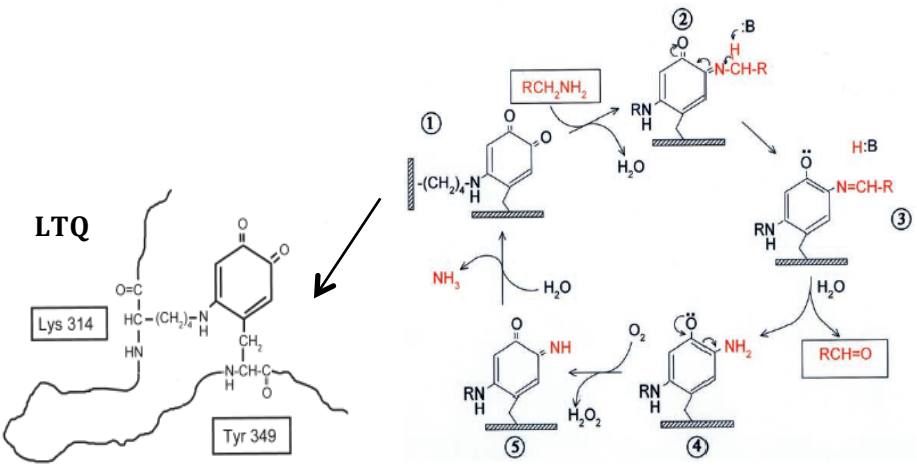


Figure D1. LOX-catalyzed reaction model. The LOX-catalyzed reaction falls into the oxidation- reduction category of enzymes. In detail, the Ɛ-amino group of the substrate lysine residue condenses with one of the two carbonyls of LTQ ①. The resulting Schiff base linkage permits the flow of two electrons into LTQ ② generating the reduced peptidyl lysine tyrosyl aminoquinol ③. Hydrolysis of the resulting imine linkage releases the peptidyl aldehyde (AAS) product from the reduced cofactor ③ – ④. Subsequent reoxidation of the peptidyl aminoquinol ④ occurs by transfer of two electrons to molecular oxygen, forming and releasing hydrogen peroxide ④ – ⑤. The quinoneimine so formed is then hydrolyzed to regenerate LTQ ① and to release the free ammonia product of the reaction.

As a consequence, we proposed a putative mechanism for LOXL2-catalyzed deamination of H3K4me3 (Fig.D2). This model involves the nucleophilic attack by a OH- to Cε and the release of $N(CH3)^3$. The generated alcohol is rapidly oxidized by the LTQ generating the aldehyde group. Similar to what has been described for LOX[192], LTQ would be reoxidized with the simultaneous release of H_2O_2. According to this model, deamination occurs with quaternary ammonium and not with primary, secondary or tertiary amines that are bad leaving groups, thus, indicating, that this reaction model would not be suitable for deamination of di-, mono- or unmethylated lysines.

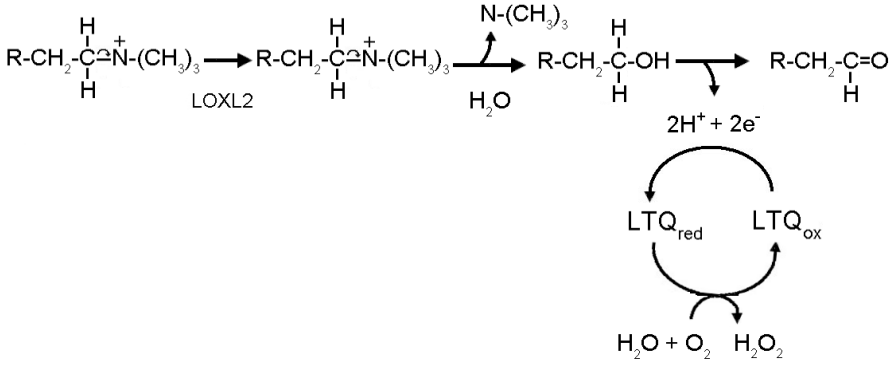


Figure D2. Chemical reaction model for LOXL2-mediated H3K4me3 deamination.

However, since we have observed that LOXL2 is also able to deaminate unmethylated H3K4, it is still possible that the enzyme follows Lucero and Kagan's mechanism, when challenged with high amounts of unmethylated K4 although at a much lower rate. Importantly, our results showing that LOXL2-mediated H3K4me3

deamination causes the release of hydrogen peroxide and that oxygen bound to Lys4 comes from the H_2O source, perfectly fit in our model. It is worth to remark that we also tried to detect $N(CH3)^3$ release. Unfortunately, since trimethyl ammonium (TMA) is a very volatile gas, production of this compound could not be determined since it was below the detection limit.

Characterization of the above-mentioned LOXL2 function uncovered a new H3K4 modification: deamination. However, this epigenetic modification has never been described within the cell. In order to check whether this reaction occurs *in vivo*, and since it was impossible to generate an antibody against this new modification due to aldehyde group high reactivity, we used an indirect approach based on an activated biotin (biotin-hydrazide) that reacts with aldehyde groups. This method is commonly used to identify carbonylated proteins and their oxidation sites within oxidative stress cellular contexts[275]. Remarkably, we observed that LOXL2wt overexpression caused a very significant increase of global H3 oxidation levels. In agreement, LOXL2 binding to the repressed E-cadherin promoter perfectly correlates with an increase of H3 oxidation in this promoter. To further validate H3K4me3 deamination *in vivo*, it should be demonstrated that, in fact H3K4 is the residue that is being oxidized. However, although we have not yet clarified this issue, all our in results in cell lines, both at a local and global level, reveal a strong and specific inverse correlation between LOXL2-mediated H3K4 trimethylation and H3 oxidation.

In summary, our findings strongly suggest that LOXL2 could deaminate H3K4me3 in vivo. As a consequence, our work constitutes the first example of a LOX protein that deaminates lysines in a

substrate different from fibrillar forms of collagen and elastin in the ECM[199]. Indeed, putative LOX proteins effect over histones had been already contemplated a long time ago when in vitro assays demonstrated that the LOX purified enzyme readily oxidized a number of basic globular proteins with pI values ≥ 8.0, among which H1 histone was included[276]. More recently, Giampuzzi and colleagues[277] demonstrated that LOX could specifically interact not only with histone H1, but also with histone H2. Furthermore, our observations suggest that lysine deamination might have different outcomes depending on the cellular context. As mentioned in the introduction, oxidized lysines in the ECM tend to instantly crosslink due to aldehyde groups high reactivity. We speculate that if the same would happen in oxidizied lysines of histones we would have not been able to detect LOXL2-mediated H3 oxidation in vivo, since biotin-hidrazyde cannot bind to aldehyde groups once they have crosslinked.

Functional and physiological relevance of H3K4 deamination

As well as LSD1 and some JmjC domain-containing H3K4 demethylases, LOXL2 can decrease H3K4 methylation at a global level and it has been found in large multiprotein complexes together with HDACs, PcG proteins, NuRD, and other epigenetic regulators[95] However, LOXL2 is not a direct demethylase, but a deaminase. Thus, our findings raise a certain question, which are the functional differences between H3K4 demethylation and deamination?

At this regard, a very similar mechanism to that mediated by LOXL2, was recently described; two different reports characterized a new

modification in arginines, arginine deimination[181,182]. They indicated that this modification antagonizes arginine methylation by converting methyl-arginine into citrulline. Considering these observations, we contemplated that, in a similar fashion, LOXL2 might counteract the action of H3K4 methyltransferases. Indeed, our in vitro experiments revealed that in the presence of LOXL2wt, methylation of H3K4 by MLL, the classical H3K4m3/2 methyltransferase, was strongly impaired. Although this mechanism requires further comprehension these observations may suggest that, unlike demethylation, deamination cannot be directly reversed by subsequent methylation. (Fig.D3). Importantly, this hypothesis might clarify why LOXL2 modulates H3K4me3/2/1 levels in vivo, although our in vitro data demonstrate the LOXL2 is specific only for H3K4me3.

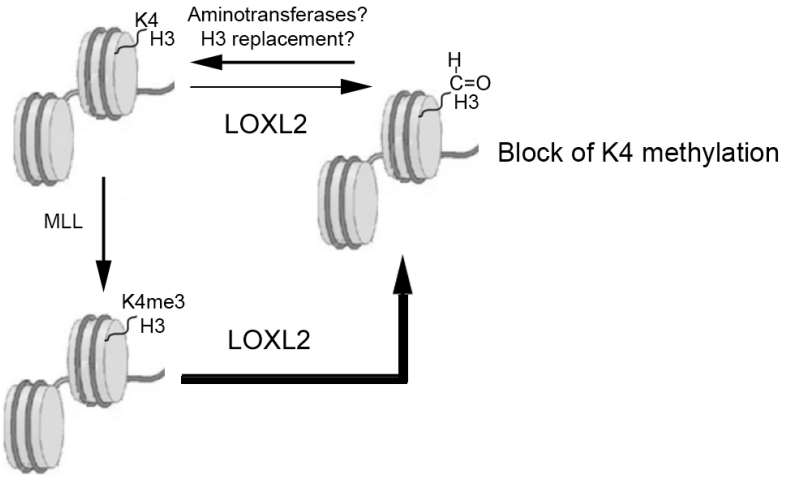


Figure D3. Model for H3K4me3 deamination mediated by LOXL2.

If not by methylation, how deamination can be reversed *in vivo*? We speculate that the aldehyde group generated upon LOXL2 activity could react with aminotransferase enzymes, which could catalyse the incorporation of a new amino group. Alternatively, it is also possible that a histone variant replacement might exchange the deaminated histone by a new one that contains an unmodified H3K4 available for further methylation. (Fig.D3)

Under this hypothesis we could suggest that reversal of H3K4 deamination is a more energetically demanding process than H3K4 demethylation. Consequently, deamination could also be a more stable and powerful repressive mark. Although this is just speculation, this idea could partly explain why LOXL2 mediates such a strong effect over H3K4 methylation and chromatin structure. At this regard, LOXL2, H3K4me3/2/1, and H3K4 deamination coupled genome wide-analysis would offer a huge amount of essential information. Apart from providing a better understanding of the observed LOXL2 and H3K4 deamination functional relevance within different cellular contexts, the indicated large-scale analyses might clarify if H3K4 deamination is indeed involved in counteracting all H3K4 methyl marks or if H3K4 deamination could also be triggered independently of LOXL2.

Regarding LOXL2 physiological relevance, we have observed that this protein is highly expressed in embryonic stem cells (ESCs) (data not shown). As a consequence, it could be speculated that as well as other epigenetic modifiers, such as PcG and TrxG proteins, LOXL2 might regulate cell fate genes expression and/or stem cell pluripotency.

LOXL2 may function beyond H3K4 deamination

Considering our results, we could predict a scenario in which LOXL2-mediated H3K4me3 deamination synergizes with other repressive epigenetic modifications in order to switch off transcription in active sites. However, not all the proteins found to be associated with LOXL2 fit in this classical repression context, hence, suggesting that LOXL2 effect might not be limited to H3K4 deamination.

For example, as previously indicated, LOXL2 interacts with the histone chaperone CAF-1. Among other functions, this histone exchange regulator cooperates with HP1 proteins in the replication and transcriptional regulation of pericentric heterochromatin. At this regard, we found that, as well as CAF-1, LOXL2 contains HP1 binding motifs and indeed interacts with this protein. In addition, genome-wide analysis performed in our lab, revealed that Snail1 is enriched in multiple regions of pericentric heterochromatin (unpublished data). Significantly, Snail1 was also found to bind to HP1. However, since Snail1 protein does not contain HP1 binding motifs, we speculate that Snail1 can bind to HP1 through LOXL2. Taken together, these observations might indicate that LOXL2 is somehow involved in the regulation of heterochromatin domains.

In addition, although not indicated in the results section, we also found that LOXL2-associated complex integrated five different proteins belonging to the SRCAP/TIP60 complexes. Since, both mammalian SRCAP and TIP60 are chromatin-remodelling complexes known to modulate the exchange of histone H2A for the H2A.Z variant[278-281], this observation provided another example of LOXL2 putative function within histone exchange dynamics. However, is difficult to speculate about LOXL2 role in this process, especially if we

consider that H2A.Z deposition effect at a transcriptional level is not fully characterized. H2A.Z was initially associated with activation. However, it was found that at individual loci H2A.Z was preferentially enriched over the promoters of inactive genes[282]. Subsequent genome-wide mapping studies confirmed this principle of H2A.Z occupancy, leading to the hypothesis that H2A.Z nucleosomes are deposited at repressed/basal promoters but could facilitate activation through their rapid turnover rates[283].

Finally, LOXL2 was also found to interact with the Menin protein. Menin binds to histone methyltransferase MLL and promotes MLL-mediated H3K4me3/2 methylation[284]. Although Menin is a well-know tumor suppressor in the endocrine lineage[285], its functional cooperation with MLL has been reported to be essential for transforming activity of at least several oncogenic MLL fusion proteins in the hematopoietic lineage[286-288].

Considering the literature, LOXL2-mediated H3K4 deamination would not be suitable in any of the above-mentioned cellular contexts. Then, how LOXL2 is cooperating with the indicated partners? On one hand, we could speculate that LOXL2 might promote the deamination of other histone residues. Indeed, a very similar scenario was described when human LSD1 was found to function as an H3K9 demethylase and a transcriptional activator in the presence of the androgen receptor (AR)[172]. On the other hand, as it has previously been suggested, LOXL2 might also modify non-histone proteins within the cell. Thus, we foresee that LOXL2 might positively or negatively regulate the activity of some of its partners by directly oxidizing specific lysines on them. Since LOXL2 and Menin present completely opposite effects over H3K4 methylation, it would

be appealing to investigate if LOXL2 could also modulate H3K4 methylation by diminishing Menin activity.

In summary, we speculate that if LOXL2 truly acts over different substrate, its specificity might depend on protein-protein interaction.

New evidences on LOXL2 role in tumor progression

As referred in the introduction, LOXL2 is highly expressed in many human cancers. Indeed, its aberrant expression has been mainly associated with the metastasis process. At this regard, the literature suggests that LOXL2 may contribute to cancer through different mechanisms, more likely, depending on its localization. With our work we have reinforce this scenario and, more importantly, we have characterized a new and relevant function for nuclear LOXL2, H3K4me3 deamination. Thus, we predict that LOXL2 deregulation might contribute to tumor progression due to H3K4me3 deamination transcriptional effects over key genes in tumor progression; such as we have demonstrated for E-cadherin.

Remarkably, our results reveal that LOXL2 effects in the context of EMT and tumor progression do not seem restricted to direct repression of CDH1. In the discussion that follows, we provide important novel insights into LOXL2 significance in the EMT process.

Prior to our work, it has been reported that LOXL2 could induce a complete EMT process. Although we did not observed a complete EMT, our results further confirmed that LOXL2 overexpression promotes CDH1 repression in cells with low or null levels of endogenous Snail1 expression[219,220]. This fact suggests that LOXL2

might be able to regulate Snail1 expression and/or that it could also participate in Snail1-independent pathways. In agreement with this hypothesis, we observed that LOXL2 depletion in the more physiological context of TGF-β-induced EMT not only impaired E-cadherin repression, but also, and even more strongly, Snail1 and Zeb1 upregulation. Concerning putative LOXL2-mediated Zeb1 regulation, our preliminary data have indicated that LOXL2 might be inhibiting miRNA-200 expression in both mesenchymal and TGF-β-induced cells. MiR-200 family induce epithelial differentiation and is associated with inhibition of stemness features and tumor progression[253,289,290]. Accordingly, several reports have addressed that miR-200 family regulates Zeb1 in an interesting fashion; they are reciprocally linked in a feedback loop, each strictly controlling the expression of the other[291-295]. Regarding miR-200 regulation, it is worth to remark that Snail1 has also been associated with its repression[230,291]; however, if Snail1 action is direct or dependent on Snail1-mediated Zeb1 activation remains unclear. In addition, Suz12 has also been found to be a target of miR-200[253]. Interestingly, loss of miR-200 during CSC formation increases Suz12 expression, Suz12 binding, H3-K27 trimethylation, and Polycomb-mediated repression of the E-cadherin gene.

Taken together, these observations prompted us to speculate that Snail1 and LOXL2 could also enhance E-cadherin repression in an indirect manner. In fact, we could imagine a scenario in which Snail1 and LOXL2 repress miR-200, thus permitting ZEB1 and Suz12 expression and consequent E-cadherin silencing. As described, Zeb1 expression would also further control miR200 expression. Interestingly, this mechanism itself could provide an explanation for the described Snail1-mediated Zeb1 activation. If confirmed, our

results would also further support the idea that whereas Snail1 role is crucial for E-cadherin repression initiation, Zeb1 activity is required for its maintenance.

Others and we have demonstrated Snail1-LOXL2 functional cooperation. However, as it could be the case for other epigenetic modifiers (HDACs, LSD1), it should not be discarded that LOXL2 also cooperates with other EMT effectors such as Zeb1. In fact, we have observed that LOXL2 and Zeb1 expression levels strongly correlate, not only in TGF-β induced cells, but also in the entire panel of analysed tumoral cell lines (data not shown). Interestingly, our results have revealed that LOXL2 and ZEB1 parallel expression is especially high in the most undifferentiated cells, although, in some cases, Snail1 mRNA levels in these cells are low.

Apart from shedding some light in the molecular network controlling CDH1 repression, our findings have indicated that LOXL2 have a key role in the acquisition and maintenance of the mesenchymal features. Indeed, LOXL2 depletion induced a very significant change to a more epithelial phenotype, both in cells with normal (RWP-1) or high (MDA-MB 231) expression levels of the protein. Moreover, when we tried to generate stable clones of mesenchymal cells (MDA-MB 231 or Mia Paca-2) upon LOXL2 depletion, cell division was inhibited and cells underwent apoptosis. In agreement with these observations, microarrays analysis in MDA-MB 231 cells revealed that expression of genes involved in epithelial differentiation or cell death program activation was increased in the absence of LOXL2. Microarray data also pointed to a LOXL2 role in the regulation of cell cycle. Though, the heterogeneous nature of the cell cycle regulators identified does not clarify which could be the putative role LOXL2 in cell

proliferation. At this regard, the literature does not either provide a clear conclusion. Indeed, opposite results regarding LOXL2 role in cell proliferation and primary tumor growth have been reported[217-219].

Strikingly, gene expression analysis also revealed that a large number of different histone clusters representing almost all histone variants were upregulated upon LOXL2 depletion. The majority of vertebrate histone genes are replication dependent and are primarily expressed during S phase; in fact, histone gene expression is one of the major events that mark entry into S phase[296,297]. Accordingly, it has been reported that downregulation of proteins which promote histone transcription results in S-Phase block[298]. Given these observations, we could speculate that LOXL2-mediated repression of histones might block cell cycle progression. Bearing in mind that cell proliferation is much more inhibited in metastatic cells than in primary tumor cells, our hypothesis might provide new insights into the widely demonstrated LOXL2 significance in metastasis.

In summary, our results reinforce the idea that LOXL2 have a relevant function during tumor progression. Indeed, it is likely that LOXL2 regulates the initiation and maintenance of the EMT process through different mechanisms.

Concluding Remarks

Following the initial objectives of this thesis, our work has revealed two different epigenetic mechanisms underlying Snail1-mediated E-cadherin repression: PRC2-mediated H3K27 methylation and the previously uncharacterized LOXL2-mediated H3K4 deamination. As mentioned in the discussion, other repressive epigenetic modifiers (HDACs, LSD1, etc.) have also been found to cooperate in this mechanism in recent years. Interestingly, we and others have observed that LOXL2 and PRC2 bind to most of these epigenetic modifiers as well as to each other. Thus, our findings reinforce the idea that cells have developed the strategy of assembling protein complexes containing complementary enzymatic activities to co-ordinately regulate histone modifications and, subsequently, chromatin structure and gene transcription. However, what it has not been yet fully elucidated is how these epigenetic modifiers are directed to target genes. Indeed, this topic is currently a matter of controversy. At this regard, at least in the context of CDH1 repression in EMT, most of the studies, including ours, have demonstrated that Snail1 TF is required for the function and recruitment of its associated epigenetic regulators.

As previously indicated, both LOXL2 and PRC2 proteins aberrant expressions have been reported in a wide range of tumors. Importantly, our results offer novel mechanistic explanations for the connection of these factors with tumor progression, which, in fact, may impact the already recognized usefulness of these proteins as therapeutic targets in cancer.

Last but not least, LOXL2 involvement in CDH1 repression has enabled us to characterize a new epigenetic modification mediated by this enzyme: H3K4me3 deamination. LOXL2 has remarkable global

effects over H3K4 methylation levels and chromatin structure. Thus, considering the well-defined occupancy and functional relevance of H3K4me3 mark at active transcription start sites, we speculate that LOXL2 may exert a very significant repression function within different cellular contexts. Future experiments to further understand the specific consequences of LOXL2-mediated deamination include the determination of specific LOXL2 occupancy within the genome and, at a more molecular level, the understanding of the precise mechanism by which LOXL2-mediated deamination is reversed (a working model is depicted in FigD3). It is lysine deamination a highly dynamic modification? It is associated with different transcriptional outcomes if compared with lysine demethylation? Better understanding of these topics as well as of LOXL2 functional cooperation with its interactors will likely provide significant new insight into fundamental mechanisms that regulate chromatin structure and transcription.

Materials and Methods

Cell lines

HEK 293 and HEK 293T, HT-29 M6, RWP1, MCF7, SW-620 and MDA-MB 231 were maintained in Dulbecco's modified Eagle's medium (Invitrogen) with 10% fetal bovine serum (Invitrogen) at 37ºC. In the case of NMuMG murine cells, media was additionally supplemented with 10 μg/ml of insulin (Sigma), 100 units/ml of penicillin, and 50 μg/ml of streptomycin. Sf9 insect cells (Gibco) were grown in Grace's medium supplemented with 10% FBS at 27ºC. The generation and properties of RWP1 cells stably transfected with Snai1-hemagglutinin (HA) or with a SNAIL1 micro-interference RNA (miRNA) has previously been described[64] RWP-1shLOXL2 clones were generated as described[39] using the Mission shLOXL2 from Sigma.

RWP-1 and SW-620 cells as well as RWP1 shLOXL2 clones were transiently transfected with the indicated vectors by using the Lipofectamine Plus reagent (Invitrogen). MCF-7 cells were transfected with pFLAG-CMV-*hLoxl2wt/mut* or an empty pFLAG-CMV (Sigma) by using Polyethylenimine (PEI) reagent.

Retroviral and lentiviral infection

Phoenix Gag-polymerase producer cells were used to generate retroviral stocks, using the calcium phosphate transfection method. Phoenix cells were transfected with the indicated RNA interference plasmids expression as well as an adyuvant vector (pVSV-G). Subsequently, SW-620 and HT29-Snail1 were infected twice with viral supernatant in the presence of polybrene (4 μg/ml; Sigma).

To knockdown LOXL2 in MDA-MB 231 and NMuMG cells, shLOXL2 vectors (human or murine), as well as the three adjuvants vectors:

pMDLg/pRRE, pRSV-REV, and pVSV-G, were transiently transfected into HEK293T cells. Viral supernatants were collected 48 h and 72h later, clarified by filtration and used to infect cells with 4mg/ml polybrene. MDA-MB 231 cells were processed 48h after the initial infection. NMuMG cells were selected with puromycin (2,5 μg/ml) and further treated with TGF-β at the indicated times.

Cloning procedures

pcDNA3-*Snail1*-HA vector was generated as previously described[47,219]. Cloning and characterization of the wild-type and mutated CDH1 and SNAI1 promoters and the Snai1-P2A mutant have previously been described[64]. Mammalian expression vectors encoding the Ezh2 mutant H694L[299] and RNA interference expression plasmids specific for Suz12[224], Ezh2, or green fluorescent protein (GFP)[225] have previously been described. RNA interference plasmids specific for SNAIL1 were designed (5'-GATCCCCCTCAACTGCAAATACTGCAATTCAAGAGATTG CAGTATTTGCAGTTGATTTTTGGAAA-3') together with their complementary counterparts, annealed, and subcloned into a pSUPER vector digested with BglII and HindIII. pFLAG-CMV-*hLoxl2* and pBXG1-GAL4-*hLoxl2* plasmids were generated by cloning the BamHI/SmaI and BamHI/XbaI fragments of full-length humanLOXL2 cDNA (Origene) respectively. pFastBac- *hLoxl2*-Flag was generated by PCR with primers containing SalI and XbaI restriction sites and cloned into the SalI/XbaI sites of the pFastBac-HTB plasmid. Double mutants (H626Q, H628Q) of pFLAG-CMV-*hLoxl2*, pBXG1-GAL4-LOXL2 and pFastBac-*hLoxl2*-Flag vectors were obtained by using the QuickChange site-directed mutagenesis kit (Stratagene). The sense

oligonucleotide sequence was 5'-T GGATCTGGCACGACTG TCAAAGG CAATACCACAGCATGGA-3'. Mutation was verified by sequencing.

Antibodies and other reagents.

The following antibodies were used in this study: H3K4me1 and H3K9me2 from Upstate, H3K4me2, H3K4me3, H3K9me3, BMI-1, and CBX8 from Millipore, H3K27me3 from Diagenode or Abcam, H3, LOXL2, RbAp48 and Pyruvate kinase 1 from Abcam, Flag and Tubulin from Sigma, Sin3A, HDAC1, DNMT-1 and Suz12 (for WB) from Santa Cruz, CDH1 and β-catenin from BD Biosciences, Streptavidn-HRP from Zymed, anti-HA tag from Roche and Goat anti-rabbit antibody–Alexa Fluo-568 and goat anti-mouse antibody–Alexa Fluo-488 were from Invitrogen.The preparation and use of monoclonal antibodies against Ezh2 (AC22 or BD43), Suz12 (2AO9), and Snail1 has previously been reported[154,155,224,226] .The hybridoma E910 was used to analyse the myc tag.

FLAG Peptide (F3290), Histone from calf thymus (H9250) and anti-FLAG M2 Affinity Gel (A2220) were purchased from Sigma. Biotin Hydrazyde was purchased from Thermo Scientific. H3 (1-16), H3K4R9R14, H3R4K9K14, H3K4me1, H3K4me2, H3K4me3, H3K9me3 and Flag peptides used for IR experiments were synthesized by UPF/CRG Proteomics Facility at Parc de Recerca Biomèdica de Barcelona.

Real-time RT-PCR.

Total RNA samples were isolated by using a Gene Elute mammalian total RNA kit (Sigma) or Trizol reagent (Sigma). Quantitative determination of RNA levels was performed in triplicate, using the

indicated primers at the end of the section. RT-PCR and data collection were performed with an ABI PRISM 7900HT sequence detection system. Alternatively, RNA was reverse transcribed by using Transcriptor First Strand cDNA Synthesis Kit (Roche) and oligo-dT primers. In this case determination of RNA expression levels by real-time quantitative PCR (Roche LightCycler) was performed using the LightCycler 480 SYBR Green Kit from Roche. In all cases we normalized values to the expression of housekeeping genes (HPRT, Pumilio). The corresponding oligonucleotides are also indicated at the end of this section.

Western Blot and Immunofluorescence.

Cells were lysed in SDS buffer (1% SDS, 65mM Tris-HCl (pH 8.8); Western blots were performed according to standard procedures. For immunofluorescence and confocal microscopy, cells were grown in cover slips, fixed with 4% p-formaldehyde, treated with 0.3% PBS-Triton X-100 and incubated with an anti-LOXL2 antibody. DAPI was used for nuclei staining.

ChIP assays

Chromatin immunoprecipitation (ChIP) assays were performed as previously follows: Cells were cross-linked with 1% formaldehyde for 10 min. and lysed in a buffer IP1 (50 mM Tris, pH 8, 2 mM EDTA, 0.1% NP-40, and 10% glycerol) for 10 min at room temperature. The pellet obtained after centrifugation (3.000rpm for 15') was then lysed in IP2 buffer (50 mM Tris, pH 8, 10 mM EDTA, and 1% sodium dodecyl sulfate [SDS]) and sonication was performed five times at 40% for 10 seconds (Branson) to generate 200- to 1,500-bp DNA fragments. Immunoprecipitation was carried out in IP buffer (16.7

mM Tris, pH 8, 167 mM NaCl, 1.2 mM EDTA, 1.1% Triton X-100, 0.01% SDS). Samples were treated with elution buffer (100 mM Na2CO3, 1% SDS, and proteinase K) and incubated at 65°C overnight to reverse formaldehyde cross-linking. DNA was purified using a GFX PCR DNA and gel band purification kit (Amersham). Promoter regions were detected by quantitative PCR Sybr green staining (Qiagen or by using the LightCycler 480 SYBR Green Kit from Roche. PCR and data collection were performed with the ABI PRISM 7900HT system or with Roche LightCycler The ChIP results were quantified relative to the input amount, as previously described[300] or, when indicated, to the amount of immunoprecipitated H3. In the case of the exogenous CDH1 promoter, 1 μg of a CDH1 promoter [pGL3-E-cad (-178/ +92)], either the wild type or a form with the three E boxes mutated, were transfected in RWP-1 stably transfected with Snail1 with either the wild type or the P2A mutant. The transfection efficiencies of the two promoters (wild type and mutated) were analysed by quantitative PCR in the input samples, using specific primers for the luciferase gene. The oligonucleotides that were used in all the experiments are found at the end of the section.

Coimmunoprecipitation assays

Snail1-HA stably transfected RWP-1, HT 29 M6-Snai1 cells, and SW-620 were lysed in 50 mM Tris, 2 mM EDTA, 0.1% NP-40, 10% glycerol supplemented with protease and phosphatase inhibitors for 5 min on ice. The samples were centrifuged at 3,000 rpm for 15 min, and the supernatant was discarded. The nuclear pellet was lysed in radioimmunoprecipitation buffer for 30 min at 4°C, and the lysate was subjected to immunoprecipitation with anti-Suz12 (Abcam), mono- clonal anti Snail1, or irrelevant immunoglobulin G overnight at

4°C. Protein G-Sepharose beads (Roche) were added for 1 h at 4°C. Precipitations were washed with radioimmunoprecipitation buffer and then resuspended in Laemmli buffer. Proteins were resolved in SDS-polyacrylamide gel electrophoresis gel and analysed with anti-Suz12 (Santa Cruz) and anti-HA (Roche).

Alternatively, Coimmunoprecipitation assays were carried out in HEK 293 cells. Cells were transfected with pFLAG-CMV-*hLoxl2*wt/mut or an empty pFLAG-CMV by using Polyethylenimine (PEI) reagent. 48h after transfection, cell lysis and purification of the immunocomplexes was carried out as described for the Sf9 cells (see recombinant LOXL2 purification) by using whether Flag M2 beads or EZH2, Sin3A or HDAC1 antibodies. Protein interactions were analysed by Western Blot.

Luciferase reporter assay

For the luciferase reporter assay, 50 ng of a CDH1 promoter [pGL3-E-cad (-178/+92)], either the wild type or a form with the three E boxes mutated, was transfected in RWP-1 cells. Cells were cotransfected with pcDNA3- Snai1-HA in the presence of small interfering RNA (siRNA) against Suz12 (200 ng) or GFP (200 ng) as a control. A simian virus 40-Renilla luciferase plasmid (1 ng) was cotransfected to control the efficiency of transfection. Transfection was performed by using Lipofectamine The activities of the firefly and Renilla luciferases were analysed at 48 h posttransfection according to the manufacturer's instructions.

Reporter assays were also carried out in HEK293T cells by using 50 or 100 ng of the 4xGal4-*tk*- luciferase reporter. Cells were contransfected with 0,10,20 and 30 ng of both wt and mutant LOXL2.

Derivation, culture of ES cells, and embryoid body formation

Derivation, culture of embryonic stem (ES) cells, and embryoid body formation (ES differentiation) were performed as described previously[258,301].

Immunohistochemistry.

Paraffin sections were obtained from embryonic day 7.5 (E7.5) wild type or Suz12-deficient[224] murine embryos or E9.5, E15, and E18 wild-type embryos. Sections were deparaffined in xylene and rehydrated. Snail1 antigenic recovery was carried out with a pressure cooker for 15 min in Tris-EDTA buffer, pH 9. For the Suz12 immunohistochemistry, antigenic recovery was carried out with 2 N HCl for 30 min at 37°C. The slides were washed in borate buffer and digested in 0.01% trypsin for 2 min. After the endogenous peroxidase was blocked with 4% H2O2 for 15 min, sections were incubated for 2 h in phosphate-buffered saline supplemented with 3% bovine seroalbumin and with monoclonal antibodies anti-Snail1[226] and anti-Suz12[301]overnight. E7.5 embryos were stained with rabbit polyclonal antibody anti-Suz12 (Upstate). Bound antibodies were detected using the Envission system (Dako, Glostrup, Denmark), following the manufacturer's instructions. Sections were counterstained with hematoxylin.

Bisulfite Genomic Sequencing

Bisulphite genomic sequencing was performed on bisulphate-treated genomic DNA as described[302]. The bisulphite reaction was carried out for 16 hours at 55ºC on 1 mg of *Hind* III digested DNA. A semi-nested PCR was performed using the following primers: outer 1: 5'-ATTTAGTGGAATTAGAATAGTGTAGGTTTT-3'; outer 2: 5'-CTACAACT

CCAAAAACCCATAACTAAC-3'; inner 1: 5'-GTTTAGTTT TGGGGA GGGGTT-3'; inner 2: 5'-CTACAACTCCAAAAACCCATAACTAAC-3'.[303]

Subcellular fractionation.

RWP1, MIA PaCa-2 and MDA-MB 231 cells were lysed in 10mM Hepes (pH 7.9), 10mM KCl, 1.5mM $MgCl_2$, 340mM Sucrose, 10% Glycerol, 0.1% Triton X-100 and 1mM DTT supplemented with protease and phosphatase inhibitors for 8 min at 4ºC. Samples were then centrifuged at 13.000rpm at 4ºC for 5 min. Supernatant was collected (cytoplasmic fraction) and the pellet was lysed in 3mM EDTA, 0.2mM EGTA and 1mM DTT supplemented with protease and phosphatase inhibitors for 30 min and centrifuged at 13.000rpm at 4ºC for 5 min. Supernatant (soluble nucleus) was separated from pellet (chromatin fraction). Histone H3 and β-tubulin were used as chromatin and cytoplasmatic markers, respectively. To release chromatin-bound proteins by nuclease treatment, cell nuclei were resuspended in 10mM Tris (pH 7.4), 10mM NaCl, 3mM $MgCl_2$, 300mM Sucrose, and 0.2mM PMSF plus 10mM $CaCl_2$ and 1 unit of micrococcal nuclease. After incubation for different time periods at 37 °C, the nuclease reaction was stopped by the addition of 1 mM EGTA. Nuclei were collected by low speed centrifugation and lysed according to the chromatin isolation protocol described above.

Affinity Purification of TAP-Tagged LOXL2.

The stable EcR-HEK 293 cell line carrying inducible tandem affinity purification (TAP)-tagged LOXL2, the purification of the TAP-tagged complex, and the identification of specific interacting proteins by tandem mass spectrometry (MS) were performed as previously described{Jeronimo, 2007 #2020}. Briefly, approximately 75mg of

whole cell extract prepared from mock EcR-HEK 293 cells and from stable EcR-HEK 293 LOXL2-TAP cells induced with 3μM ponasterone A (Invitrogen) for 48 hours were subjected to purification by the TAP method. The TAP eluates were separated on NuPAGE 4-12% Bis-Tris gel (Invitrogen) and subjected to MS analysis after silver staining. The MS-based protein identification proteomic analysis was carried out in the UPF/CRG Proteomics Facility (Parc de Recerca Biomèdica de Barcelona)

Recombinant LOXL2 purification.

LOXL2-Flag recombinant proteins (wt and mutant) were purified from Sf9 insect cells. Briefly, LOXL2-encoding baculovirus were amplified and the proteins were produced in Sf9 cells according to standard procedures. Cell lysis was performed as previously described[304]. Cell extracts were incubated with Flag M2 beads for 4 hours at 4ºC and washed 4 times with 20mM Hepes (pH 7.4), 1mM $MgCl_2$, 300mM NaCl, 10mM KCl, 10% Glycerol and 0.2% Triton X-100. Elution was carried out with Flag peptide (1μg/μl) for 1 hour at 4ºC.

Infrared measurements.

A sample of 200 μl of H3 peptide solution (1.3 μg/μl) was homogenously spread on one side of a germanium crystal (50 x 10 x 2 mm, Harrick, Ossining, NY, yielding 12 internal reflections at the sample side). The sample was left at 35ºC for 30 min and a spectrum of 4000 scan was acquired. LOXL2 enzymatic kinetics started by the addition of 50 μl of LOXL2 (25ng/μl) and then a spectrum was recorded every 10 minutes during 560 minutes. A total of 50 spectra of 2000 scans at a resolution of $4cm^{-1}$ were taken and averaged in order to increase to signal-to-noise ratio. All the spectra were

acquired at 35ºC with a thermostatic circulatory bath connected to a stainless plate, using a FT6000 Bio-Rad spectrometer. For the H_2O_{18} experiments, KPI 100mM buffer was lyophilized and resuspended in water O_{18}. The contribution of water O_{16} arising from LOXL2 addition (50 µl) was subtracted with the kinetics spectra of trimethylated H3 peptide in the same conditions without LOXL2. Several corrections were applied to the experimental data prior to the analysis of the LOXL2 enzymatic kinetics of the H3 peptides. All the spectra were first corrected for water atmospheric contribution[305]. The increase in the protein concentration during the kinetics was subtracted using kinetics of each H3 peptide without adding LOXL2. The kinetics spectra were obtained subtracting each spectrum with the spectrum at time = 0 (without LOXL2). The absorbance spectra of each H3 peptide in solution were subtracted with each buffer absorbance spectrum. A correction for TFA contribution was also performed for H3 peptides.

MALDI Mass Spectrometry

Sample incubations were processed prior to mass spectrometric analysis, using manually confectioned POROS R2 containing Geloader tips (Applied Biosystems). 20-50 µl Sample solutions were applied to preconditioned tips, wash with 50 µl 0.1 % TFA and peptides eluted with 0.5 µl 80 % acetonitrile in 0.1 % TFA, containing 20 g/l α-cyano-4-hydroxycinnamic acid (Sigma) directly onto the MALDI target.

Mass spectrometric analyses carried out on a Voyager-DE™ STR Biospectrometry workstation (Applied Biosystems), equipped with a N2 laser (337 nm). Typically, spectra were acquired in reflectron mode with positive polarity. The array detector was set to high

resolution and mass scans were accumulated in the mass range between 800-5000 Da. External calibration of the spectrometer was performed using the SequazymeTM Peptide Mass Standards Kit of the desired range (PerSeptive Biosystems). Recorded data were processed with Data ExplorerTM Software (Applied Biosystems).

Nucleosome purification

After harvesting by centrifugation, 293T cells were resuspended in Buffer A (10mM Tris (pH 7.4), 10mM NaCl, 3mM $MgCl_2$, 300mM Sucrose, 0.2% NP-40 and 0.2mM PMSF) and homogenized by 10 strokes in a Dounce homogenizer with a B-type pestle. After centrifugation, nuclei were resuspended Buffer A plus 10 mM $CaCl_2$ and 4 units of micrococcal nuclease. After incubation at 37 °C, the nuclease reaction was stopped by the addition of 1mM EGTA.

Deamination assays

Bulk histones and nucleosomes (1µg) were incubated with the indicated amounts of recombinant LOXL2-Flag proteins in 20mM Hepes (pH 7.4), 100mM NaCl, 1mM $MgCl_2$, 0.1µg/µl BSA and 5µM $CuCl_2$ (deamination buffer). Reaction was carried out for 2 hours and O/N at 37ºC in a final volume of 65µl and then analysed by SDS-PAGE and Western blotting.

Hydrogen peroxide detection

Hydrogen peroxide detection was evaluated using Amplex Red fluorescence assay (Molecular Probes). This assay detects the presence of hydrogen peroxide produced from the activity of any amine oxidase. Briefly, H3 and H3K4me3 peptides (40 pmols) were incubated in presence of recombinant LOXL2 proteins (500mg) in

deamination buffer. The samples were incubated at 37ºC and fluorescence was measured by emission at 590nm after excitation at 560nm using a Bio-Tek fluorescent microplate reader following manufacturer's instructions.

In vivo H3 Oxidation Experiments

In order to detect H3 global oxidation levels, HEK293T cells were cotransfected(10:1) with pcDNA3-*hLoxl2wt/mut*-Flag or an empty pcDNA3 and pEGP-C1. 36h after transfection, cells expressing high GFP levels were sorted by a fluorescence-activated cell sorter. Cell pellets ($4 \cdot 10^6$ cells for each experiment) were resuspended in Soft lysis buffer (50 mM Tris pH 8, 10 mM EDTA, 0.1% NP-40, and 10% glycerol) for nuclear enrichment. After 15min of centrifugation at 3000rpm at 4ºC, the resulting pellet was lysed with 2%SDS, 10mM EDTA, 50 mM Tris pH 8 and 5mM Biotin Hydrazyde. Obtained lysates were then incubated at 25ºC for 2h and further diluted with Dilution buffer (0,01% SDS, 1% Tritó X-100, 1,2 mM EDTA, 16,7 mM Tris pH 8, 167 mM NaCl), to make a final 0,1%SDS concentration. Samples were then incubated with 5ug of anti-H3 O/N. Precipitations were washed with high salt buffer(0,1% SDS, 1% Tritó X-100, 2 mM EDTA, 20 mM Tris and 500 mM NaCl). Proteins were resolved in SDS-polyacrylamide gel electrophoresis gel and analyzed with Streptavidin-HRP. To detect H3 oxidation in an specific promoter, ChIP assays were performed as described[39], but extracts were lysed in presence of Biotin Hydrazide reagent (5mM). Samples were then incubated at 25ºC for 2h. Immunoprecipitation was carried out with anti-H3 and reimmunoprecipitation with Streptavidin beads.

MLL activity

MLL activity was essentially performed following manufacturer's instructions (Bioscience) in the absence or presence of recombinant LOXL2 proteins (500ng). After anti H3K4me3 antibody incubation, samples were treated with HRP-labeled secondary antibody followed by the addition of the HRP substrate to produce chemiluminiscense. Luminescence was measured after 4 hours of incubation.

Gene Expression Analysis

We measured gene-expression levels of three biological replicates of shCt and shLOXL2 MDA-MB 231. For microarray analysis, amplification, labeling, and hybridizations were performed according to protocols from Ambion and Affymetrix. Briefly, 250 ng of total RNA were amplified using the Ambion® WT Expression Kit (Ambion/Applied Biosystems, Foster City, CA, USA), labeled using the WT Terminal Labeling Kit (Affymetrix Inc., Santa Clara, CA, USA), and then hybridized to Human Gene 1.0 ST Array (Affymetrix) in a GeneChip® Hybridization Oven 640. Washing and scanning were performed using the Hybridization Wash and Stain Kit and the GeneChip® System of Affymetrix (GeneChip® Fluidics Station 450 and GeneChip® Scanner 3000 7G).

Mnase digestion

HEK293T cells were cotransfected (10:1) with pcDNA3-*hLoxl2wt/mut*-Flag or an empty pcDNA3 and pEGP-C1. 36h after transfection, cells expressing high GFP levels were sorted by a fluorescence-activated cell sorter. Cell pellets ($1.5 \cdot 10^6$ cells for each experiment) were lysed in 500ul of Buffer A (10mM Tris[pH 7.4], 10mM NaCl, 3mM $MgCl_2$ and 0.3M Sacarose supplemented with

protease and phosphatase inhibitors) for 10min at 4ºC. After NP-40 addition to a final 0.2% (v/v) and 10 min of incubation at 4ºC, the lysate was centrifuged 10' at 1200rpm at 4ºC. The resulting pellet was resuspended in 100ul of BufferA ($CaCl_2$ was added to a final concentration of 10mM) and digestion with MNase(0.08 units) was carried out (5 and 10 min). Enzyme was inactivated with 50mM EDTA. After treatment with Rnase A (2min at RT) and Proteinase K (10min at 56ºC), DNA was purified (GFX™ PCR DNA Purification Kit, GE Healthcare). Digestion products were analyzed by agarose gel electrophoresis. Total DNA was visualized by ethidium bromide staining.

Primers (Real Time RT-PCR)

Human LOXL2 mRNA

5'-ACTGACTGCAAGCACACGGA-3'

5'-TCAGGTTCTCTATCTGGTTGATCAA-3'

Mouse LOXL2 mRNA

5'-GCGAAGGCCCCATCTGGTTG-3'

5'- GTGCTTGCAGTCAGTGACACCCC-3'

Human CDH1 mRNA

5'-GAACGCATTGCCACATACAC-3'

5'-ATTCGGGCTTGTTGTCATTC-3'

Mouse CDH1 mRNA

5'-TTCAACCCAAGCACGTATCA-3'

5’-ACGGTGTACACAGCTTTCCA-3’

Mouse Snail1 mRNA

5’-GCGCCCGTCGTCCTTCTCGTC-3’

5’- CTTCCGCGACTGGGGGTCCT-3’

Mouse Zeb1 mRNA

5’-TCAGCTGCTCCCTGTGCAGT-3’

5’-AAGGCCTTCCCGCATTCAGT-3’

Human Occludin mRNA

5'-ACAATCAGCCATGTCATCCAGG-3'

5'-CTGGAGGAGAGGTCCATTTGTAG-3'

HPRT mRNA

5’- GGCCAGACTTTGTTGGATTTG-3’

5’-TGCGCTCATCTTAGGCTTTGT-3’

Pumilio mRNA

5’-CCAGTACAGCATACAGGGCAGATG-3’

5’-CCCAGGAATATCTTGGTCGGC-3’.

Human PTEN:

5’-AATCCTCAGTTTGTGGTCT-3’

5’-GGTAACGGCTGAGGGAACT-3’

CDH1 human promoter:

5’-ACTCCAGGCTAGAGGGTCAC-3’

5’-GCCCGACCCGACCGCACCCG-3’

CDH1gene (Intron 5)

5’-GGCTGGAAGTCCCTGACCTAA-3’

RNA polymerase II 3’UTR

5’-GCTTTTTCCTCCCAACTCG-3’

5’-TAGGTGCTCAGACCTCGTCA-3’

PTEN human promoter:

5’-CCGTGCATTTCCCTCTACAC-3’

5’-GAGGCGAGGATAACGAGCTA-3’

Snail1 human promoter:

5’-GGCGCACCTGCTCGGGGAGTG-3’

5’-GCCGATTCGCGCAGCA-3’

Snail1 mouse promoter:

5’-CGCACCTGCTCCGGTCTCAG-3’

5’-CTACGATCCCCTAGCAGCAG-3’

Genomic sequence:

5’-ACTCCAGGCTAGAGGGTCAC-3’

5’-CCGCAAGCTCACAGGTGCTTTGCAGTTCC-3’

PGL3 antisense primer:

5'-GGTGGGTTTACCAACAGTACCG- 3'

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Annex

Part of the work presented in this thesis was published in:

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Independently of the work published on this thesis, the author worked in other projects, reflected in the articles:

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