ROLE OF RING1B IN EPITHELIAL TO MESENCHYMAL TRANSITION, MIGRATION AND INVASION OF MAMMARY EPITHELIAL CELLS.

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"It is our choices that show what we truly are,

far more than our abilities"

A. Dumbledore

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ABSTRACT

The Polycomb group (PcG) family of proteins form chromatin-modifying complexes essential for embryonic development, and stem cell renewal and are commonly deregulated in cancer. There are several reports that address the possible implication of PcG proteins in tumor progression and metastasis, but very little is known about the specific role of these proteins in tumor progression and invasion. On the other hand, the molecular processes of the worst cancer prognosis, metastasis, which leads to an incurable disease, are yet incompletely elucidated. Here we show a role for Ring1B, a PcG protein, in three processes related to metastasis: in the *Epithelial–mesenchymal transition (EMT),* a critical morphogenic event that occurs during embryonic development and during the progression of various epithelial tumors, an in the *migration* and the *invasion* of mammary epithelial cells..

Las proteínas del grupo Polycomb (PcG) forman complejos modificadores de la cromatina esenciales en el desarrollo embrionario y en la renovación de las células madre, y su desregulación ha sido asociada al cáncer. Varios estudios muestran la posible implicación de las proteínas de PcG en la progresión tumoral y en la metástasis, pero a pesar de ello se sabe muy poco de los procesos moleculares en los que estas proteínas están

participando. Por otro lado, los procesos moleculares responsables del peor pronóstico en cáncer, la metástasis, que continua siendo una enfermedad incurable, siguen sin estar completamente elucidados. En esta disertación mostramos el papel de Ring1B, una proteína del PcG, en tres procesos implicados en la metástasis: en la *transición epitelio-mesénquima (EMT),* un proceso morfogénico crítico en el desarrollo embrionario y durante la progresión de varios cánceres epiteliales, y en la *migración* y la *invasión* de las células epiteliales mamarias.

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1. List of abbreviations

3

3met-K27H3: trimethylation of lysine 27 at the histone H3 tail

A

ATM: ataxia telangiectasia mutated

ATR: ataxia telangiectasia and Rad3 related.

B

BMI1: BMI polycomb ring finger oncogene

BRCA1: breast cancer

BSA: bovine serum albumina

C

CDH1: Cadherin1, E-cadherin.

CDK: cyclin dependent kinase

CDKN2A: cyclin-dependent kinase inhibitor 2A

cDNA: complementary DNA

ChIP: chromatin Immunoprecipitation

CKI: cyclin kinase inhibitor

CpG: cytosine phosphate

D

DAB+: diaminobenzidine tetrahidrochloride plus

DCIS: ductal carcinoma in situ

DMEM: Dulbecco s modified eagle medium

DNA: deoxyribonucleic acid

DNMT1: DNA methyltransferase 1

DNMT3A: DNA methyltransferase 3A

DNMT3B: DNA methyltransferase 3B

E

ECL: enhanced chemiluminescence

ECM: extracellular matrix

EDTA: ethylenediamine tetraacetic acid

EED: embryonic ectoderm develpment

EGFR: epidermal growth factor receptor

EMT: epithelial to mesenchymal transition

ERK: extracellular signal-regulated kinase

Ezh1: enhancer of zeste 1

Ezh2: enhancer of zested 2

E2F: electro-acoustic 2 factor

F

Fak: focal adhesion kinase

FBS: foetal bovine serum

FGF: fibroblast growth factor

G

GDP: guanine diphosphate

GEF: GDP/GTP exchange factor

GFP: green fluorescent protein

GH: growth hormone

GTP: Guanine triphosphate

H

HAT: histone acetyltransferases

HEK: human embryonic kidney

Hgf: hepatocyte growth factor

HLH: helix-loop-helix

HPC: human polycomb

HPH: human polyhomeotic

I

IDC: infiltrating ductal carcinoma

IGF: Insuline-like growth factor

ILC: infiltrating lobular carcinoma

J

JNK: c-Jun N-terminal kinase

L

LCIS: loubular carcinoma in situ

M

MAPK: mitogen activated protein kinase

MDM2: Murine double minute 2

miRNA: microRNA

MMP: matrix metalloproteinase

N

NMuMG: normal murine mammary gland

O

O.N: over night

P

PBS: phosphate buffered saline

PcG: polycomb group proteins

PI3K: phosphoinositide 3 kinase

PRC1: polycomb repressive complex 1

PRC2: polycomb repressive complex 2

R

Ring1B: ring finger protein 2

RIPA: radioimmunoprecipitation assay

RNA: ribonucleic acid

S

SDS: sodium dodecy sulfate

SIM2: single minded homolog

SIS3: specific inhibitor of smad3

SUZ12: supressor of zeste 12

T

TDLU: terminal duct lobular unit

TEB: terminal end bud

Tgf-α: transforming growth factor alpha

Tgf-β: transforming growth factor beta

U

Ub-H2A: ubiquitinated histone 2A

W

Wnt: Wingless/type MMTV integration site family

Introduction

Research goals

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2 INTRODUCTION

2.1 The mammary gland.

The mammary gland is an anatomic structure present in both male and female, although in male is usually atrophied. The main function of mammary gland is milk production to lend the breeding of the new born. The mammary gland is enclosed by the pectoral muscle and is composed by a conical disk of glandular tissue, which is encased in variable quantities of fat that give it its characteristic shape. The glandular tissue itself is made up of 15–20 lobes composed of solid cords of ductal cells; each lobe is subdivided into many smaller lobules [1]. Lobules are composed of terminal ducts and acini and their specialized supporting stroma.

Each lobe is drained by a separate excretory duct. These converge beneath the nipple, where they widen into milk reservoirs, before branching another time to form openings at the tip of the nipple.

Except for a small portion of the collecting ducts at the nipple where squamous epithelium lines the duct, the entire duct system is lined by two cell layers: luminal epithelial cells and basally located myoepithelial cells. Myoepithelial cells are located in close contact with cytoplasm of the epithelial cells and are surrounded by basal lamina. Myoepithelial cells often show ovoid to elongated bipolar dense nuclei and small cytoplasm. [2]

In figure I.1 are shown a diagram of mammary gland and a section of a terminal duct-lobular unit with haematoxylin-eosin staining.

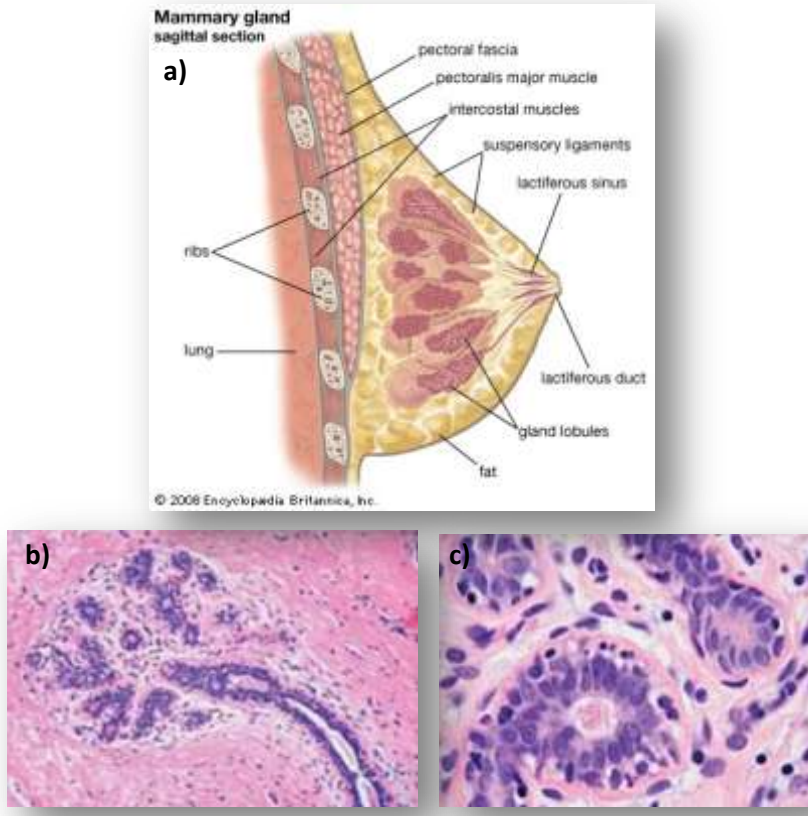


Figure I.1 The mammary gland a) Diagram of the mammary gland anatomy [1] **b)** A terminal duct-lobular unit (TDLU) with regular acinar structures and a small segment of extralobular (terminal) duct [2]. **c)** The acini are lined by luminal epithelial and basally located myoepithelial cells. The myoepithelial cells often show elongated or bipolar nuclei. The acini are surrounded by a continuous layer of basal lamina [2].

2.2 Mammary gland development

The process by which mammary gland and other ramiform tissues are formed is called branching morphogenesis. The mammary gland branching happens mainly during puberty in the female. This hormone-dependent development initiates at the end of the ducts, known as terminal end buds (TEB). The TEB is composed by two cell types, the cap cells, a single layer of cells in contact with the basal

lamina, and the body cells, composed by multiple layers, that are the majority of the TEB.

The TEB undergoes ductal arborization via sprouting through the mammary fat pad. With pregnancy, differentiation of the ducts occurs, resulting in the formation of luminal structures required for lactation. Lumen formation is intrinsic to milk production and transport and is controlled by cell death of the body cells bordering the TEB [3, 4].

At this stage, body cells of ducts and lobules of the human breast are made up of two morphologically distinct cell populations: the inner, milk producing luminal epithelial cells, which express low molecular-weight cytokeratins and the estrogen receptor (ER), and the outer, supporting basal myoepithelial cells, which express high molecular-weight cytokeratins and smooth muscle markers. A number of paracrine, juxtacrine, and autocrine factors are known to affect mammary gland branching morphogenesis [5].

Finally mammary ducts and alveoli, in the adult, are bilayered epithelial structures enclosing a central lumen. Luminal cells adhere to one another via E-cadherin. Myoepithelial cells surround the luminal layer and adhere to one another via P-cadherin. Desmosomal Cadherins, Desmogleins and Desmocollins interconnect the luminal and myoepithelial layers and are critical for establishing this bilayered arrangement [6].

2.3 Factors implicated in mammary gland development.

Growth and development of the mammary gland at puberty is regulated at the systemic level by ovarian and pituitary hormones and, at the local level, by paracrine interactions between ductal epithelial cells and their surrounding stroma. The systemic hormones that regulate this process were identified by hormone depletion and replacement studies. It is known that estrogens can rescue mammary development in ovariectomized but no hypophysectomiced animals. Moreover estrogens can restore TEB and duct development in hypophysectomized and ovariectomized rats if growth hormone (GH) (but not prolactin) or insulin-like growth factor-1(FGF-1) is also provided [7]

These systemic hormones are very powerful regulators of development; however, with the advent of knockout mice and tissue transplantation experiments, it has become clear that the wide-ranging effects of these agents are not due to direct hormone action but rather to the actions of multiple secondary paracrine effectors. At the local level, the branching pattern of epithelial cells seems to be controlled by signals from themselves and also from the stroma [8] [9].

2.3.1 Tgf-β

The precise pattern of the mature ductal structure in the mammary gland would suggest a need for localized growth inhibitors. One such molecule, which is known to play a significant role in

mammary morphogenesis, is the transforming growth factor beta (Tgf-β), secreted in a latent form from ductal epithelium, activated extracellularly and acting on its receptor to inhibit lateral branching and ductal growth [10].

Since it is known that Tgf-β acts primarily on stromal receptors [11], its growth inhibitory function on mammary epithelial cells must be mediated by stimulation or inhibition of diffusible paracrine factors. One promising candidate is hepatocyte growth factor (Hgf), an amitogenic cytokine secreted by fibroblasts which promotes cell proliferation, survival and motility by binding to its tyrosine kinase receptor, c-Met. Hgf induces tubule formation in many epithelial cell lines [12], and it is known to be negatively regulated by Tgf-β [13]. Overexpression of Hgf in mouse mammary epithelial cells resulted not only in increased lateral branching but also in a marked increase in the number and size of end buds [14] .

Tgf-β released from epithelial cells is also crucial for the control of ductal elongation: in Tgf-β heterozygous knockout mice, which have less than 10% of the wild-type Tgf-β levels, both ductal and alveolar development were increased 2 to 4 fold, consistent with its role as a growth inhibitor [10]

2.3.2 EGFR

The epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases, which consists of EGFR, ErbB2, ErbB3, and ErbB4, is important in many normal developmental processes and is often over-expressed or mutated in human cancers. EGFR is an integral

membrane tyrosine kinase that is activated upon binding of a family of polypeptides that includes Epidermal Growth Factor (Egf), Transforming Growth Factor α (Tgf-α), Amphiregulin (Ar), Heparin-binding Egf (Hb-Egf), Betacellulin (Btc), and Epiregulin (Epr). These ligands share a conserved, three disulfide loop structure (the Egf-like motif), that is released by proteolytic cleavage of transmembrane precursor proteins [15].

In respect to the ErbB members, an important defining feature is that two members of the family, ErbB2 (also known as Her2/neu) and ErbB3, are non-autonomous: ErbB2 lacks the capacity to interact with a growth-factor ligand, whereas the kinase activity of ErbB3 is defective. Despite this lack of autonomy, both receptors form heterodimeric complexes with other ErbB receptors that are capable of generating potent cellular signals [16].

Several of the EGFR pathway members are important in mammary gland development. EGFR ligands rescue ductal development in ovariectomized and Estrogen Receptor alpha (ER-α) deficient mice, and exogenous estrogen elicits EGFR activation in ovariectomized mice, suggesting that EGFR promotes mammary branching downstream of ER-α [7].

About the EGFR pathway ligands, Amphiregulin is the only one that is upregulated at puberty and the only that is required, because ductal outgrowth is impaired in Amphiregulin-deficient mice but not in mice lacking Egf, Tgf-α, heparin-binding Egf-like growth factor, or Betacellulin [7]

Despite Tgf-α null mouse has no defective effect in mammary branching during puberty, in others epithelial tissues both Amphiregulin and Tgf-α null phenotypes suggest abnormal epithelial cell migration or adhesion, while the mechanisms by which these ligands mediate these processes are not understood. Candidate signaling pathways include Integrins and their downstream targets as Focal adhesion kinase (Fak) and other non-receptor tyrosine kinases [17]

2.3.3 Matrix metalloproteinases

A family of extracellular or membrane-bound, Zn^{2+}-dependent proteases known as matrix metalloproteinases (MMPs), capable of digesting various proteinaceous components of the extracellular matrix (ECM), has also been shown to regulate localized growth during mammary development. Functions of the MMPs include cell signaling as a result of alteration of the microenvironment, release of bioactive components from the ECM such as Fibronectin and Laminin, activation of growth factors and cleavage of cell-cell and cell-matrix adhesion proteins [18]. MMPs are also proposed to "clear a path" for the invading ducts in the mammary gland during puberty due to their matrix remodeling activities. This remodeling process is an essential part of the ductal development, as was shown for instance for MMP2, which is highly expressed in the stroma in front of advancing TEBs. In addition, MMP2 null mice exhibited retarded ductal invasion and increased lateral branching, suggesting a differential effect depending on its location. Thus, MMP2 promotes ductal elongation while repressing lateral budding [19].

2.4 Breast Cancer

Breast cancer is the most frequently diagnosed form of cancer among women in the Western World. Detection of breast cancer has increased dramatically since the introduction of screening mammography and is expected to increase exponentially as women's life expectancy increases and more developing countries begin breast cancer screening programs.

The most common types of breast cancer begin at the breast milk ducts (ductal carcinoma) or at the lobules and terminal ducts of the breast (lobular carcinoma). Both of these types could be *in situ* (non invasive), when they have not spread to breast tissue around the duct (ductal carcinoma *in situ*, DCIS) or lobule (lobular carcinoma *in situ*, LCIS). Among these two, the most common is DCIS, that has an excellent prognosis upon appropriate treatment.

Invasive (infiltrating) breast cancers spread outside the membrane that lines a duct (infiltrating ductal carcinoma, IDC) or lobule (infiltrating lobular carcinoma, ILC), invading the surrounding tissues.

2.4.1 Lobular carcinoma

LCIS was described in detail in 1941 by Foote and Stewar, emphasizing the morphologic similarity of the cells comprising LCIS and ILC.

The LCIS is usually found incidentally during the workup of suspicious breast lesions. Initially it was thought to be a

premalignant lesion that would progress to invasive lobular carcinoma, and ipsilateral mastectomy was recommended for all patients. Later, it was demonstrated that most of the women with LCIS did not develop invasive cancer [20, 21] and now LCIS is considered only to be a marker of increased risk of breast cancer.

Histopathologically, the LCIS is characterized by a proliferation of small cells, which lack cohesion and appear dispersed through a fibrous connective tissue or arranged in single file linear cords that invade the stroma. These infiltrating cords frequently present a concentric pattern around normal ducts. About 70-95% of lobular carcinomas is Estrogen Receptor (ER) positive, a rate higher that in DCIS.

50% of LCIS cases have been found to have chromosomal abnormalities. The most common genetic alteration is the loss of the long arm of chromosome 16. The epithelial-cadherin gene (cdh1, E-cadherin gene) maps in this region, and a correlation has been found between deletion of 16q and the loss of cdh1 expression. In fact, immunohistochemical analysis has shown complete loss of cdh1 expression in 80-100% of LCIS in contrast with the 30-60% of DCIS.

2.4.2 Ductal carcinoma

The term ductal intraepithelial neoplasia (DIN) refers to the spectrum of diverse intraductal proliferations that are associated with an increased risk of developing invasive ductal cancer. In the conventional classification, this spectrum has been divided into benign and malignant and designated as intraductal hyperplasia

(IDH), atypical intraductal hyperplasia (AIDH) and the above mentioned DCIS.

DCIS is considered to be a precursor lesion for development of invasive breast cancer. However, conservative treatment (complete local eradication) is usually curative. 1,9% of patients diagnosed with DCIS between 1984 and 1989 died of breast cancer in 10 years in United States. But, for these women, it seems that DCIS *per se* was not responsible for their deaths, but were due to an undetected invasive carcinoma present at the time of initial diagnosis of DCIS, to the progression of residual incompletely excised DCIS to invasive carcinoma, or to the development of a *de novo* invasive carcinoma elsewhere in the breast [22].

DCIS is further subdivided into low, intermediate and high grade [22].

2.4.2.1 Low grade DCIS

Low grade DCIS is composed of small monomorphic cells growing in arcades, micropapillae, cribiform or solid patterns. The nuclei are of uniform size and have a regular chromatin pattern and mitotic figures are rare. There may be occasional desquamated cells within the ductal lumen, but the presence of necrosis is unacceptable within low grade DCIS. It can present microcalcifications.

2.4.2.2 Intermediate grade DCIS.

Intermediate grade DCIS lesions are often composed of cells cytologically similar to those of low grade DCIS, forming solid, cribiform or micropapillary patterns, but with some ducts

containing intraluminal necrosis. The distribution of microcalcifications is generally similar to low grade DCIS or it may display characteristics of both low grade and high grade patterns of microcalcifications.

2.4.2.3 High grade DCIS.

High grade DCIS is composed of highly atypical cells proliferating as one layer, forming micropapillae, cribiform or solid patterns. Nuclei are high grade, markedly pleomorphic, poorly polarized, with irregular contour and distribution, coarse, clumped chromatin and prominent nucleoli. Mitotic figures are usually common but their presence is not required. Characteristic is the comedo necrosis with abundant necrotic debris in duct lumens surrounded by generally solid proliferation of large pleomorphic tumor cells. However, intraluminal necrosis is not obligatory. Even a single layer of highly anaplastic cells lining the duct in a flat fashion is common. Amorphous microcalcifications are common.

2.5 Genetic alterations in breast tumors.

The developing mammary gland displays many of the properties associated with tumor progression, such as invasion, reinitiation of cell proliferation, resistance to apoptosis and angiogenesis. Thus, it is not surprising that many of the factors essential for mammary gland development are also associated with cancer [23].

2.5.1 P53 Family

p53 was discovered 30 years ago. The 53 KDA protein was immunoprecipitated from SV40-transformed mouse cell lysates using anti-large T serum isolated from rabbits, hamsters, mice and monkeys [24]. The molecular function of p53 was defined to be that of a transcription factor by showing that it binds specifically to its target DNA fragments [25]. p53 is a tumor suppressor and is known as “the guardian of the genome” [26], because of its central role coordinating the cellular responses to a broad range of cellular stress factors. p53 regulates processes such as apoptosis, cell cycle arrest, senescence, DNA repair, cell metabolism or autophagy, initiating several programs that ultimately arrest proliferation (by both blocking cell cycle progression and promoting apoptotic cell death) and preventing the generation of genetically-altered cells.

p53 remains the most commonly mutated gene in many common human cancers, with mutations estimated to occur in 50% of all cancers. Mutant proteins are almost always defective for sequence-specific DNA binding, and thus for transactivation of genes upregulated by the wild type protein [27]. Interestingly, the

proportion of missense mutations in p53 is higher than that seen in other tumor suppressor genes, suggesting that expression of p53 mutants may confer selective advantage over and above loss of wild-type function [28].

Numerous studies have identified coding mutations in p53 in breast cancer and this is now recognized as a common, but by no means ubiquitous, somatic genetic change in breast cancer. Indeed, a comprehensive meta-analysis revealed that only approximately 20% of all cases express mutant p53 [29], that means that p53 mutation in breast cancer is significantly lower than that in many other common cancers, and suggesting that there are other inactivation mechanism of wild-type p53. Importantly, the presence of p53 mutation frequently correlates with a poor grade of differentiation in human breast cancers [30].

An early insight into mechanisms of p53 inactivation in breast cancer was afforded by a study of breast cancer with different p53 mutation status [31]. It was shown that significant number of wild-type p53 tumors display only cytoplasmic protein staining. Exclusion of the wild-type protein from the nucleus thus represents a potential mechanism for p53 inactivation independent of mutation. Subsequently, alterations both in upstream regulatory proteins and in downstream p53-induced proteins have been identified, that may disable or compromise the pathway in breast cancer lacking p53 mutations [31]. For instance, patients with ataxia-telangectasia, illness due to mutations in the p53 upstream regulator ATM, display high incidence of breast cancer [32]

It has been also shown that mutant p53 can empowers Tgf-β proinvasive and migration abilities, whereas loss of mutant-p53 expression in aggressive tumors impairs their metastatic potential [33]. The results from this work show that p53-mutant is able to form a ternary complex with Smad and p63, in which p63 transcriptional functions are antagonized [33]

The p53 family also comprises two other subfamilies: p63 and p73, which were identified to be homologues of p53 [34, 35]. Using knockout studies in mice, p63 and p73 were found to play very different roles to p53 during development and in adult tissue. p63 is essential for the proper development of skin and epithelial structures during embryogenesis [36, 37]. In contrast, p73 is involved in the development of neural structures such as the hippocampus, as well as in the proper pheromonal signaling and in the regulation of the dynamics of the cerebrospinal fluid [38].

Both the p63 and p73 genes contain two transcriptional start sites and make use of alternative splicing to produce at least six p63 different isoforms and a larger number p73 isoforms. Importantly, all p63 and p73 proteins contain a DNA binding domain having 60% sequence identity to the DNA binding domain of p53 [38]. At least three of each of the p63 and p73 proteins also contain a transactivation (TA) domain with 25% sequence homology to that in p53, and are referred to as TAp63 and TAp73. Other p63 and p73 isoforms lack the transactivation domain and are designated as ΔNp63 and ΔNp73 [38]. It is thought that ΔNp63 and ΔNp73 could act as dominant negative proteins to inhibit the function of TAp63 and TAp73 forms, but also p53 [38], although in the case of

ΔNp63α, which is the most abundantly expressed p63 protein in many different squamous epithelial cells and glandular tissues [39], the protein exhibits transcriptional repressor activity and represses transcription of various p53 target gene promoters *in vitro* [40].

Very few human cancers exhibit mutations in p63 or p73 [41]. Examination of mice heterozygous null for p53, p63 and p73 or combinations of the three family members revealed the spontaneous generation of tumors in mice of all genotypes. The p53, p63 and p73 heterozygous null mice displayed tumor spectra unique for each genotype. Mice heterozygous null for both p53 and p63 or p53 and p73 exhibited higher tumor size and tumors with greater metastatic potential than mice heterozygous null for p53, p63 or p73 alone [42].

In spite of these phenotypes, there is a current controversy on whether p63 and p73 act as tumor suppressors, like p53, and their precise roles in the development of cancer in humans. Based in several reports that study the implication of the p63 isoforms in cancer [43-45], it seems that ΔNp63 could act as an oncogene working as a dominant negative of p53 and TAp63, while p63 isoforms containing the transactivation domain (TAp63) might perform tumor suppression activities, although there is an open debate over this topic because TAp63α, which is known to induce apoptosis in many assays, can promoter tumor progression and metastasis in mice [46].

Supporting the possibility of ΔNp63 working as an oncogene, it has been found to be overexpressed in a number of epithelial cancers, often as a result of genomic amplification of the p63 locus [47-49].

Several functions of ΔNp63α have been reported that could mediate ΔNp63-mediated oncogenesis, such us activation of the β-catenin signaling [50] or suppression of p73-dependent apoptosis [51].

In contrast to a role in promoting tumorigenesis, loss of p63 expression has been reported to be associated with aggressive tumor progression and poor prognosis [49, 52, 53]. p63 expression is associated with favorable prognosis in patients with lung [54] and also loss of p63 expression in bladder cancer is associated with progression to more invasive and metastatic tumors [55]. Moreover, loss of p63 expression in squamous cells results in an up-regulation of genes associated with tumor invasion and metastasis, likely mediated by ΔNp63α [56].

Then, taking together these findings, it is possible that ΔNp63a acts to promote early steps in tumorigenesis by protecting the cells from growth arrest and apoptosis, while in the late stages of the tumorigenesis it could act as a metastasis suppressor by maintaining the epithelial character of cancer cells [56].

2.5.2 BRCA1 and breast cancer

It is very well known that germ-line mutations in the BRCA1 transcription factor predispose carriers to breast and ovarian cancer. BRCA1 is phosphorylated after DNA damage by ATM, ATR and Chk2. BRCA1 associates with the C-terminus of wild-type p53 and stimulates transcription from p53-responsive promoters, whereas tumor-associated mutants of BRCA1 are deficient in coactivating activity [57]. Although somatic mutations in BRCA1

have not been described in sporadic breast cancer, expression of BRCA1 is decreased in the majority of cases, implying a further mechanism whereby p53 function can be inhibited in breast cancer carcinomas. Down regulation of BRCA1 expression is attributable to methylation-dependent silencing in a small proportion of cases [58], but the mechanism underlying the low level of expression seen in many sporadic breast cancers awaits clarification.

Tumors associated with germ-line mutations of BRCA1 are characterized by the loss of the second BRCA1 allele, concomitant loss of p53 function and undifferentiated, basal-like phenotype [59, 60]. Consistent with their basal-like characteristics, BRCA1-deficient breast tumors exhibit aggressive behavior and are associated with poor survival. At the cellular level, an important consequence of loss of BRCA1 function is impaired DNA double-strand break repair [61]. As unresolved double-strand breaks will activate p53, resulting in either cell cycle arrest or apoptosis, there is a strong selection pressure on loss of p53 function in BRCA1 associated breast tumorigenesis. In addition, recent evidence indicates that loss of BRCA1 inhibits differentiation into ER-positive luminal cells, which might contribute to the observed undifferentiated phenotype [62].

2.5.3 Cyclin D1

Cyclin D1 gene is amplified in 15% of breast cancers. However, cyclin D1 is overexpressed at the mRNA and protein level in over 50% of the breast cancers in the presence or absence of gene

amplification and qualifies as one of the most commonly overexpressed proteins in breast cancer.

The stimulation of growth-arrested cells in response to various oncogenes such as Ras, Myc and mitogenic growth factors such as members of the Egf or the Igf families results in the induction of the D-type cyclins. These cyclins link the extracellular signals to the cell-cycle machinery, being the Cyclin D1 the most predominantly associated with human tumorigenesis.

Cyclin D1 overexpression has been reported in more that 40% of cases of invasive breast cancer, while gene amplification is seen in about 5-20% of tumors. This indicates that though Cyclin D1 amplification correlates well with the overexpression of the protein, high expression of Cyclin D1 is not always secondary to gene amplification, implying that other mechanisms contribute to maintain Cyclin D1 overexpression. Various factors that could contribute to protein overexpression in breast cancer include ER and p53 through p21. [63]

2.5.4 Myc

Myc is a basic helix–loop–helix leucine zipper (bHLH-Zip) transcription factor which induces the expression of around 15% of all known genes through consensus sequences like E-boxes and by recruiting histone acetyltransferases (HATs). Different signaling pathways like Wnt, Shh or the MAPK/ERK induce Myc expression, which has been reported to promote cell proliferation but at the same time it also has a role in regulating cell growth, cell differentiation, apoptosis and stem cell self-renewal [64]

Myc deregulation has been associated with oncogenesis in a variety of human tissues including breast [65]. However, the information about Myc expression in normal human breast remains quite limited.

Cells of the TDLU are either negative or weakly positive (a minor percentage), whereas the staining increased in frequency and intensity in the epithelial cells lining extralobular ducts, and was found to be particularly strong in nipple ducts. In contrast to the normal TDLU, cases of DCIS and LCIS were characterized by the presence of some strongly-labeled cells. Also strong was the staining of Myc-positive invasive cancers that usually exhibited a variable mosaic pattern [66].

2.5.5 Notch

The Notch signaling pathway is a highly conserved developmental pathway first identified early in the 20th century through genetic mutation screens in Drosophila [67].

There is accumulating evidence implicating Notch activity in breast carcinogenesis. Aberrant activation of the Notch signaling pathway in both mouse and human mammary cells causes the development of malignant phenotype. These cells change shape and form disorganized, multi-layer cell masses in 3D-cultures and exhibit invasion of the underlying collagen matrix, an indicator of loss of cell adhesion molecules [68-70]. In human mammary epithelial cells, this was specifically due to the loss of E-cadherin [71]. Human lesions have also been examined for aberrant Notch signaling and it was demonstrated that Notch1 overexpression was related to

increased tumor grade, with the highest expression of Notch1 in high grade tumors. High Notch1 expression was also associated with significantly poorer prognosis [72].

2.6 Breast cancer metastasis

The most common complications associated with breast cancer are due to metastasis developing in regional lymph nodes and in distant organs, including lung, liver and brain. Recently the rates of metastasis and mortality in breast cancer patients have decreased as a result of early diagnosis by mammographic screening and the implementation of systemic adjuvant therapy. However, chemotherapy has a wide range of acute and long-term side effects that substantially affect the patient quality of life [73].

By this reason, it is necessary to identify new prognostic markers, but despite the importance of metastasis, the mechanisms underlying this process remain unclear.

Approximately 10-15% of patients with breast cancer have an aggressive disease and develop distant metastases within 3 years after the initial detection of the primary tumor. However, the manifestation of metastases at distant sites 10 years or more after the initial diagnosis is also not unusual [74]. Patients with breast cancer are therefore at risk of experiencing metastasis for their entire lifetime. The heterogeneous nature of breast cancer metastasis makes it difficult not only to define cure for this disease, but also to assess risk factors for metastasis.

The risk of metastasis development increases with the presence of lymph-node metastasis, a larger sized primary tumor and the loss of histopathological differentiation (grade), which are established breast cancer prognostic markers [73]. In patients with tumor-

negative axillaries lymph nodes, vessel invasion is an additional predictor for distant recurrence [75, 76].

Marker	Use in clinic	Metastatic determinants	Details
Tumour size	Established	Tumours under 2 cm in diameter have a low risk of metastasis; tumours of 2–5 cm have a high risk of metastasis; tumours over 5 cm have a very high risk of metastasis	Independent prognosis marker
Axillary lymph-node status	Established	If there are no lymph-node metastases, the risk of metastasis is low; if lymph-node metastases are present, the risk of metastasis is high; the presence of over 4 lymph-node metastases is associated with very high metastasis risk	Related to tumour size
Histological grade	Established	Grade 1 tumours have a low risk of metastasis; grade 2 tumours have an intermediate risk of metastasis; grade 3 tumours have a high risk of metastasis	Related to tumour size
Angioinvasion	Established in patients with lymph-node-negative tumours	The presence of tumour emboli in over 3 blood vessels is associated with metastasis	In patients with lymph-node-negative tumours
uPA/PAI1 protein level	Newly established marker	High protein levels of uPA and PAI1 are associated with high metastasis risk	Independent prognosis marker
Steroid-receptor expression	Established for adjuvant therapy decision	Low steroid-receptor levels are associated with metastasis	Short-term predictor of metastasis risk (5 years); related to histological grade
ERBB2 gene amplification and protein expression	Established for adjuvant therapy decision	*ERBB2* amplification/overexpression is associated with metastasis	In patients with lymph-node-positive tumours
Gene-expression profiling	Currently being tested	A 'good signature' of 70 genes is associated with low metastasis risk; a 'poor signature' of 70 genes is associated with high metastasis risk	Tested in patients with lymph-node-negative tumours

Figure I.4 Breast cancer metastasis prognosis markers. Adapted from [71]. bcm: m and models

Today, these traditional prognostic markers are able to confidently identify the group of approximately 30% of patients who are most likely to have either a very favorable or a very poor outcome. For the remaining 70% of patients of whom approximately 30% will still develop metastasis [77], new prognostic markers are needed to help identify low-risk and high-risk groups.

Considering the heterogeneity of the disease, prediction of the metastatic potential of a tumor might require the analysis of many different markers at once. This is made possible by the introduction of DNA-microarray technology, which can analyze gene expression in a genome-wide manner.

One of the last approaches to determine gene-expression patterns that can predict the clinical behavior of tumor is the supervised classification method. Such classification method was used to identify an expression profile of 70 genes that predicted the likelihood of distant metastases in young patients with lymph-node-negative tumors [78]. The primary breast tumors were classified as having either a poor-prognosis signature, which means they were likely to metastasize, or a good-prognosis signature, meaning that the development of metastases was unlikely. The poor–prognosis signature included genes involved in the cell cycle, invasion and metastasis, angiogenesis and signal transduction [73].

This method was validated in a cohort of 295 patients and was shown to be the strongest predictor for metastasis-free survival and overall survival, and was independent of the other clinical and pathological prognosis markers. Among the 60% of patients with lymph-node negative disease, 60% were classified as having high metastatic risk and 40% as having a low metastatic risk. After a follow-up period of 20 years, 65% of the poor-prognosis patients developed a metastasis whereas only 13% of the good-prognosis patients did. Classifying the patients who are at risk of metastasis on the basis of traditional clinical parameters, only 15% (versus 40%) were classified in good-prognosis group [79].

The new prognosis markers are a good tool to avoid the overtreatment in newly diagnosed breast cancer patients, but to allow microarray testing in all hospitals, the technology and access to it needs to be improved. Moreover the pathways that are responsible to convert primary tumors into metastatic cell populations remain unclear and better understanding of these mechanisms is necessary to determine new therapeutic targets.

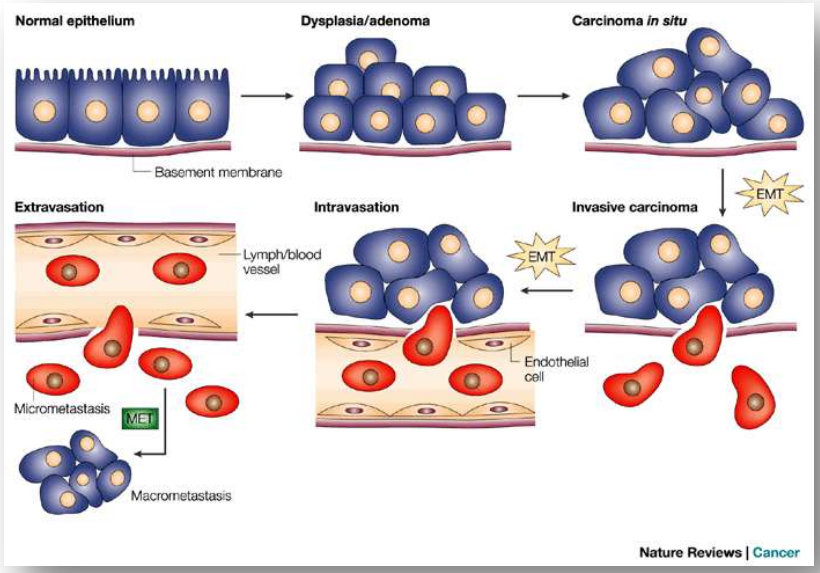


Figure I.2 Sites of EMT and MET in the emergence and progression of carcinoma. The metastasis happens in two different phases. In the first phase, epithelial cells loss cell-cell contact, degrade extracellular matrix, acquire invasive abilities, etc. In the second phase cells have to be able to survive non-attached and to invade a new tissue. *Figure obtained from [80]*

2.7 Epithelial to mesenchymal transition

The metastasis process can be divided in two phases with interrelated rate-limiting steps. The first phase begins with the cell's acquisition of invasive ability via changes in adhesiveness, initiation of motility and extracellular matrix proteolysis, which culminates in shedding of the cells into the circulation directly or via the lymphatic system [80]. The second phase of the metastasis process is colonization, which includes cell survival and growth after extravasation and the vascularisation for outgrowth.

The increased motility and invasiveness of cancer cells in the first phase of metastasis are reminiscent of the epithelial to mesenchymal transition (EMT) that takes place during embryonic development. Figure I.2 shows a summary of the metastasis.

The EMT is the process by which cells undergo morphological changes from epithelial and polarized phenotype to mesenchymal, fibroblastoid phenotype, acquiring mobility properties.

This process occurs in normal conditions in embryonic cells during development. Moreover, in the adult there are cells that undergo EMT in several processes such as tubulogenesis of mammary cells or in tissue reorganization after an injury.

In addition, the EMT is an essential process in several pathologies such as chronic inflammations, fibrosis and cancer. Epithelial cells of benign tumours show epithelial polarity alteration, but the progression to a more dedifferentiated state and worse prognosis involves changes in migration that includes EMT [81].

A variety of extracellular signals have been shown to trigger transition of epithelial cells into mesenchymal or mesenchymal-like cells during embryogenesis and in tumorigenesis. Transforming growth factor-β (Tgf-β), epidermal growth factor (EGF) family members, fibroblast growth factors (FGF), hepatocyte growth factor (HGF), and insulin like growth factor (IGF) have all been shown to induce EMT in an autocrine or paracrine manner [82].

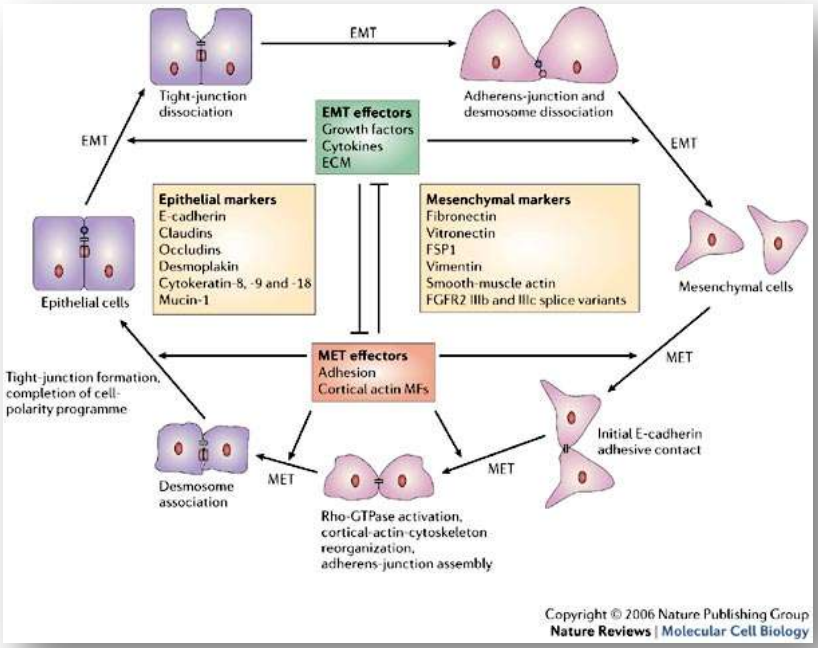


Figure I.3 Diagram showing the events that happens in the transition between epithelial to mesenchymal cell and vice versa. *Figure obtained from [83]*

Crucial changes are needed to occur in epithelial cells to make them able to invade the surrounding tissue. These cells have to alter the response and interaction among them and with the stroma, and also lose cell polarity and acquire motility and

invasiveness abilities. Many factors are able to induce this process. When these changes are phenotipically evident but there is no genetic reprogramming of epithelial and mesenchymal marker expression, the process is called *scattering*. During scattering, epithelial markers are redistributed, but not lost and mesenchymal markers are not induced. In contrast, EMT involves loss of E-cadherin, β_4 Integrins and ZO-1 (epithelial markers), and *de novo* expression of mesenchymal markers such as Vimentin [83]. A summary of the changes that happens in the EMT and the reverse process MET are showed in figure I.3.

2.7.1 Hgf and scattering

The Hgf was identified as a mitogen for hepatocytes [84], but subsequently it was shown to be identical to the scatter factor (SF), a ligand with a dramatically different activity of inducing epithelial cell dissociation or scattering [85].

This molecule acts through the receptor c-Met. Upon Hgf binding, c-Met undergoes autophosphorylation on several tyrosine residues and constitutes a unique multisubstrate docking site for SH2-containing adaptor proteins. Recruitment of these molecules results in the activation of several downstream signalling cascades, such as phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK). Finally all the activated pathways produce cellular changes including proliferation, migration, invasion and anchorage-independent growth [86].

Both Hgf and c-Met are essentials for normal embryonic development. In the normal breast, Hgf is expressed primarily by

stroma cells, while epithelial cells express c-Met, but not Hgf, creating a tightly controlled paracrine mechanism where localized expression of Hgf regulates mammary ductal growth and differentiation [87].

It has been shown also that, in contrast to what occur in normal epithelium, Hgf and c-Met are frequently overexpressed in invasive human breast carcinoma [88].

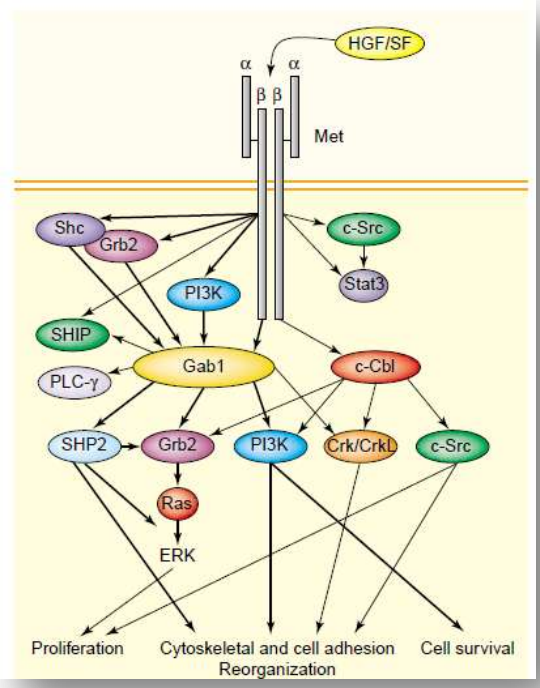


Figure I.4 Hgf/c-Met signaling pathway. Activation of the c-Met receptors results in the recruitment of numerous signal transducers to the receptor. These transducers are enable to control proliferation, cytoskeletal and cell adhesion reorganization and cell survival. *Adapted from [90].*

Binding of active Hgf to c-Met, results in autophosphorylation of c-Met. As with other receptor tyrosine kinases, these

phosphorylation events lead to full receptor activation and create sites that allow the recruitment of numerous signalling mediators. The signalling mediators recruited to c-Met include enzymes such as the Src tyrosine kinase and the PI3K, as well as several adaptor proteins such as Grb2, Gab, Shc and c-Cbl [89]. This pathway is summarized in figure I.4.

PI3K is activated downstream of c-Met and is required for Met-induced tubulogenesis and cellular motility [90]. PI3K can be recruited to the receptor complex through a variety of different mechanisms that serve to reinforce the signal; for instance, the p85 regulatory subunit of PI3K can interact directly with the Met multisubstrate docking site or with Cab1 or c-Cbl [90].

The PI3K pathway might also be important for the stimulation of anchorage-independent growth and cytoeskeletal reorganization. In this regard, PI3K is necessary for the activation of the Rho family of GTPases, which regulate Actin polymerization and cellular adhesion. Both Rac1 and Cdc42, two Rho family members, are activated downstream of Hgf and are required for Hgf-stimulated adherens junction reorganization, lamellipodia formation, cell spreading and cellular motility [91].

The Ras-ERK MAP kinase pathway downstream of Met is required for Met-induced adherens junction disassembly, cell motility and proliferation. This pathway is activated through the recruitment of the Ras GDP-GTP exchange factor (GEF) Sos to the receptor complex through association with the adaptor Grb2[92].

In summary, stimulation of Met by Hgf results in the reorganization of cytoskeletal structures and in the modulation of both cell-cell and cell-matrix adhesive contacts. Cadherin proteins form the core of adherens junctions but become relocalized and randomly distributed during Hgf cell stimulation[93]. Of note, Cadherins mediate interaction with neighbouring cell through their extracellular domains and are required for establishment of cell polarity.

2.7.2 Tgf-β and epithelial to mesenchymal transition.

Tgf-β is a very well known factor that inhibits the growth of normal cells. This inhibition has been demonstrate that is mediated by Smad proteins and the activation of cyclin kinase inhibitors (CKIs) [94]. The activity of these two signals results in a major inhibition of the cyclin dependent kinase (CDK) activities associated with the early G1 phase progression, thus locking the cell cycle prior to the G1 restriction point.

However, this response is cell context-dependent, and TGF-β can contribute to tumoral progression if cells acquire resistance to the growth-inhibitory effects of TGF-β[80]. It has been shown that in order to avoid the apoptotic effect of TGF-β, it is necessary the hyperactivation of the Ras/Raf pathway. This hyperactivation is usually achieved by using Egf, which works through its own receptor (Egf/Tgf-α receptor) to active the Ras/Raf pathway [95].

The multifunctional effects of the Tgf-β are elicited through an oligomeric complex between the type I (TβRI) and type II (TβRII)

serine-threonine kinase receptor. In response to TGF-β, TβRII phosphorylates TβRI. TβRI then activates Smad2 and Smad3, which associate with Smad4 and translocates to the nucleus, where they modulate the transcription of Tgf-β target genes. Additionally, Tgf-β can induce non-Smad pathways including p38MAPK, ERK, JNK and Rho, which are important for pro-oncogenic activities [96].

2.7.2.1 EMT transcription factors of the smad pathway

The changes in epithelial and mesenchymal markers mediated by the Smad pathway involve three families of transcription factors: Snail, Zeb and HLH families [97].

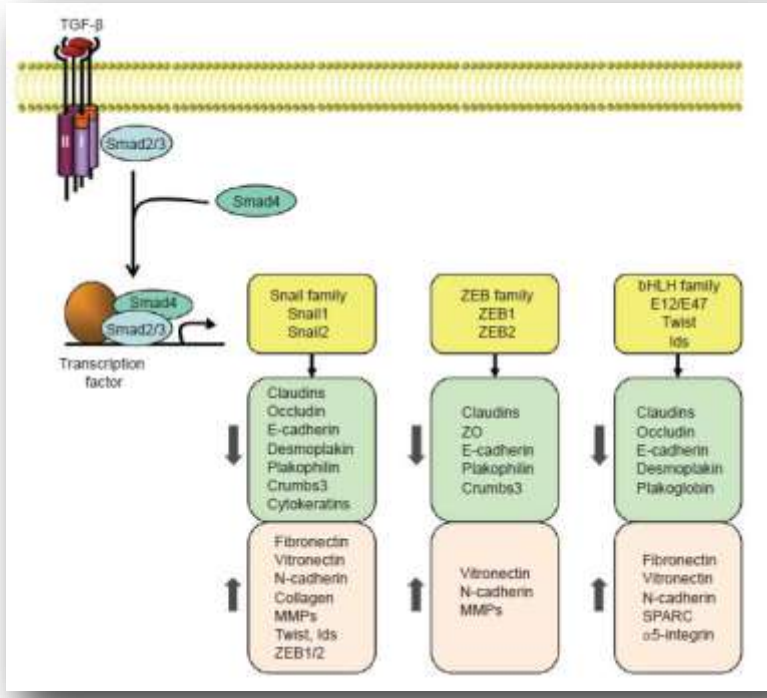


Figure I.5. Diagram showing the main transcription factors that mediate Tgf-β Smad-dependent pathway. In the figure are summarized the knowing role of Snail, Zeb and HLH family in regulating epithelial to mesenchymal transition markers. Figure obtained from [97].

2.7.2.1.1 Snail family

The transcription factors of the Snail family share extensive structural similarity, containing a characteristic C-terminal domain with four to six zinc fingers that mediates sequence specific DNA binding to E-boxes elements [98]. There are three Snail family proteins in vertebrates: Snail1, Snail2 (also known as Slug) and Snail3. These proteins function as transcription repressors through their zinc finger domains and an N-terminal SNAG domain. Snail1 upregulation is a common fact in EMT induction [98] and Snail1 levels have been correlated with more invasive tumor types [99].

Snail1 expression in Tgf-β–induced EMT could be mediated by Smad3, which directly binds to the Snail promoter and activates its transcription [100]. In the Tgf-β model, the Smad pathway can also cooperate with Ras, Notch and Wnt signaling in order to induce Snail expression in development and tumor [101].

Snail1 and Snail2 repress the expression of the epithelial marker E-cadherin. The expression of Snail proteins appears to inversely correlate with E-cadherin expression, and silencing of the Snail1 gene can restore E-cadherin levels [99], but exogenous expression of E-cadherin is not able to prevent Snail1 induced EMT ([102], suggesting additional Snail target genes in EMT.

2.7.2.1.2 ZEB family

There are two members in the ZEB family in vertebrates: ZEB1 (also δEF1, AREB6) and ZEB2 (also SIP1). These proteins have two zinc fingers that interact with regulatory DNA sequences.

Tgf-β induces the expression of ZEB proteins during EMT through an indirect mechanism mediated in part by Ets-1 [103]. Then ZEB proteins interact with Smad3 and directly repress the expression of epithelial markers genes possibly by recruiting the co-repressor CTBP [104]. On the other hand, ZEB proteins could be activated by Ras-MAPK pathway and Wnt/β-catenin signaling [105].

The induction of the ZEB proteins is necessary for the downregulation of E-cadherin expression and the promotion of cell migration, and ZEB proteins are also able to repress E-cadherin independently of Snail proteins [106].

2.7.2.1.3 Helix-loop-helix family factors.

The basic structure of this family includes two parallels α-helix linked by a loop required for dimerization. The HLH family members have been classified into seven families according to their tissue distribution, dimerization capacities and DNA-binding specificities [107].

Regarding EMT, the most representative HLH proteins belong to class I, II and V. Class I proteins also known as E-proteins (such as TCF3, which isoforms are called E12 and E47). These proteins are widely expressed and act as homodimers or heterodimers with class II proteins. Class II proteins are tissue-specific and always act as heterodimers with class I proteins. Twist 1 and Twist 2 belong to this group. Finally, class V HLHs, known as Id proteins (Id1-4), lack the basic domain and are therefore unable to bind DNA. Ids act as class I and II dominant negative factors, because of their high affinity to class I factors [105].

2.7.2.2 Non-Smad pathway

Among the non-Smad signaling responses, activation of p38 MAP kinase, Rho GTPases and the PI3K/Akt pathway in response to Tgf-β has been linked to Tgf-β-induced EMT through their regulation of distinct processes, such as cytoskeleton organization, cell growth, survival, migration and invasion [108].

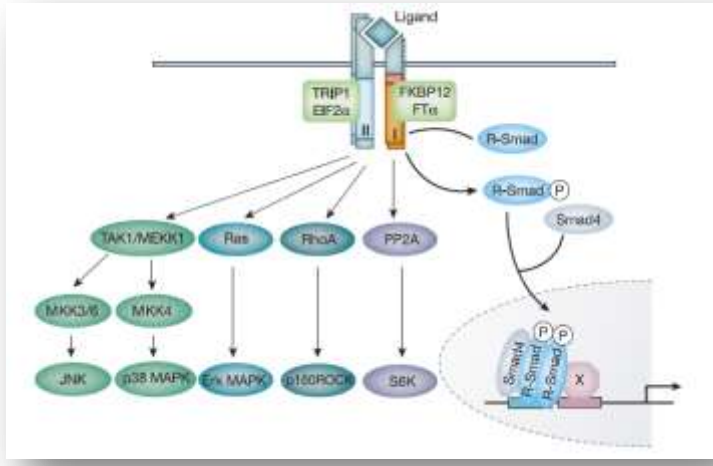


Figure I.6 Smad-independent pathway. *Adapted from [96]*

2.7.2.2.1 p38 MAP kinase

It has been describe that p38 MAPK can be activated in two different ways by Tgf-β, one mediated by Smad-dependent transcription, and another faster, that is independent on transcription [109]. The latest mechanism is poorly understood, but it has been suggested that Tgf-β activates p38 through a type I receptor-mediated mechanism, independently of Smad pathway and that activation could be mediated by Tgf-β activate kinase I

(TAK1) and MAPK/Erk kinase 1 (MEKK1) [96]. It has been shown that p38 MAP kinase pathway is required for several but not all the mechanisms in which Tgf-β is implicated; for instance, p38 MAPK is required for Tgf-β-induced apoptosis and EMT but not for growth arrest [110]. Despite this, p38 MAPK is required but no sufficient to induce EMT [111].

2.7.2.2.2 Rho GTPAses and PI3K

Depending on the cell line Tgf-β can activate Rho GTPases, including RhoA, RhoB, Rac and Cdc42 directly or through Ras activation [96]. Rac and Cdc42 can regulate p38 MAPK, whereas Rho, Rac and Cdc42 affect the cytoskeleton organization [112]. Also, activation of Rac1, RhoA and p38 MAPK, an effector of Cdc42, are required for rapid membrane ruffling and lamellipodia formation in response to Tgf-β [113]. In addition, Tgf- β can also activate the PI3K pathway by phosphorylation of its effector Akt [114, 115], and it has been shown that inhibition of PI3K activity reduces Tgf-β-induced EMT [114].

2.8 Epigenetics

The human body consists of at least 200 different cell types. Despite having identical genomes, cells can respond in markedly different ways to the same stimulus, causing cells to differentiate, migrate, proliferate, arrest or enter into apoptosis. It has become clear that maintenance of cell identity is controlled by epigenetic events.

The concept epigenetic refers to changes in phenotype or gene expression caused by mechanism other than underlying DNA sequence.

Recent studies have revealed epigenetic mechanisms involving histone modifications (acetylation, methylation, phosphorylation, ADP-ribosylation and ubiquitination) [116], DNA methylation[117, 118] and non-conding RNAs.

Thirty-four years ago, based on the knowledge of cytosine methylation in higher organisms and the newly discovered bacterial adenine methyltransferase, Riggs and Holliday, and Pugh [117, 118] independently proposed that the covalent modification of DNA by methylation might serve as a way to propagate heritable expression states in eukaryotes.

On the other hand, the post-translational modifications of the core histones were discovered nearly 40 years ago. These modifications, which include acetylation, methylation, phosphorylation, ADP-ribosylation and ubiquitination, are now known to be important mechanisms for the precise regulation of chromatin structure.

The importance of non-coding RNAs has been increasingly recognized within the last several years, particularly with the identification of new classes of small RNAs, such as microRNAs (miRNAs). These non-coding RNAs play important roles for instance in neural development and can be involved in neuronal translation control (miRNAs) or transcription regulation (small modulatory RNAs in the fate specification of adult neural stem cells), and can be pathogenic (non-coding repeats in neurodegeneration).

The mechanistic relationship among these epigenetic marks is beginning to be elucidated, and the demonstration that Polycomb proteins control DNA methylation provides compelling evidence that the mechanisms are intimately connected [119].

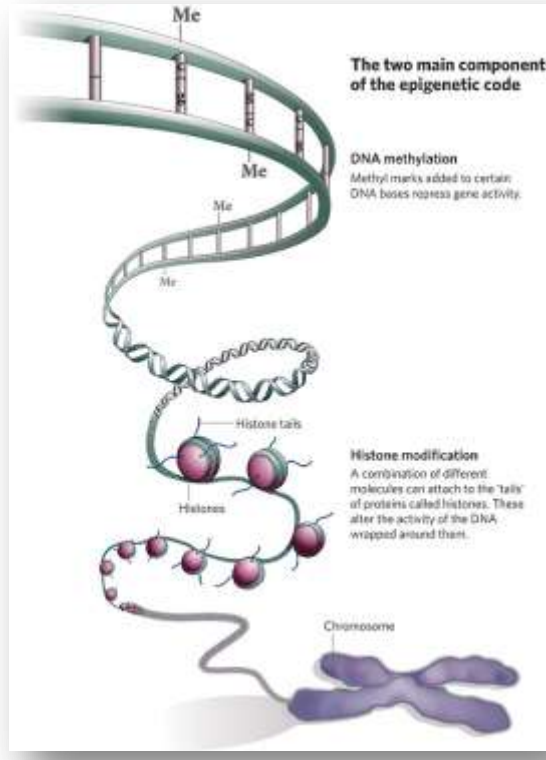


Figure I.7 Summary of the two main epigenetic mechanisms. Histone modifications and DNA methylation are the better known epigenetic mechanism, *Adapted from [131].*

2.8.1 DNA methylation

In vertebrates, DNA methylation occurs almost exclusively in the context of CpG islands. CpG islands are GC-rich regions that possess high relative densities of C-G and are positioned at the 5' ends of many human genes. Early studies estimated that approximately 60% of the human genome is associated with CpG islands, of which the great majority are unmethylated at all stages of development and in all tissue types [120].

A small but significant proportion of all CpG islands become methylated during development, and when this happens the associated promoter is stably silent. *De novo* methylation events occur in germ cells or in the early embryo, suggesting that *de novo* methylation is particularly active at these stages. There is evidence, however, that *de novo* methylation can also occur in adult somatic cells. A significant fraction of all human CpG islands are prone to progressive methylation in certain tissues during aging or in abnormal cells such as cancers cells[121].

The maintaining of the methylation patterns along consecutive cell generations is a semiconservative event, and the DNMT1 is the enzyme responsible for this process. This enzyme prefers to methylates those new CpGs whose partners on the parental strand already carry a methyl group. Thus a pattern of methylated and non-methylated CpGs along a DNA strands tends to be copied, and this provides a way of passing epigenetic information between cell generations [122, 123].

There are also *de novo* DNA methyltransferases: DNMT3A and DNMT3B. These two enzymes are highly expressed in early embryonic cells, and it is at this stage that most programmed *de novo* methylation events occur [124].

Mutations in DNMTs genes could have profound limitation on specific DNA methylation patterns. All DNA methyltransferases have been found to be overexpressed in human tumours although to moderate levels only [125]. DNA methylation has a dual role in cancer: whereas oncogenesis is promoted by local hypermethylation of tumor suppressor genes, global genomic hypomethylation affects oncogene expression and genomic instability [126].

2.8.2 Histone modifications

The core of histones that constitute the nucleosome is composed from two subunits of each histone, H2A, H2B, H3 and H4. Subsequent nucleosome cores are linked by histone H1, facilitating further compactation [127]. The N-terminal tails of the histones are projected out from the core and are marked by epigenetic modifiers affecting the compactation degree of the chromatin. Covalent modification of histones includes acetylation of lysines, methylation of lysines and arginines, phosphorylations of serines and threonines, ADP-ribosylation of glutamic acids, and ubiquitination and sumoylation of lysine residues [128]. The pattern of these marks composes what initially was called “histone code” [129]

The main regulators of histone modifications are Trithorax and Polycomb group proteins.

2.8.2.1 Polycomb group proteins

Polycomb group (PcG) proteins are highly conserved regulatory factors that are best known for their role in maintaining silent expression states of Hox genes during development, but are also involved in the regulation of normal cell proliferation, and their mutations have been linked to defects in stem cell fates and to cancer [130].

PcG proteins form multimeric complexes that exert their functions by modifying chromatin structure and by regulating the deposition and recognition of multiple post-translational histone modification [131].

Two major complexes have been described: Polycomb repressive complex 1 and 2 (PRC1 and PRC2).

PRC2 is composed in humans by proteins Enhancer of zeste (Ezh1, Ezh2), Suppressor of zeste 12 (SUZ12) and Embryonic Ectoderm Devopment (EED). The catalytic activity of PRC2 relies in EZH2, which trimethylates lysine 27 of histone H3 and also has been shown to be able to recruit DNA methyltransferases [132].

PRC1 complex is more diverse than PCR2. The core of the PRC1 is composed by Human Polycomb (HPC), Human Polyhomeotic (HPH), BMI polycomb ring finger oncogene (BMI1) and Ring1B or Rnf2. The PRC1 recognizes the trimethylation mark set by PRC2 via the chromodomain of the Polycomb (PC) protein, that have high

affinity for histone H3 tails methylated at lysine 27 [133]. Pc can interact with several PRC1 proteins *in vitro*, and it has been suggested that it is implicated in the recruitment of the other PRC1 proteins [134]. Then, Ring1b, an E3 ligase, can ubiquitinate histone H2A, and this action has been implicated in transcriptional repression [135].

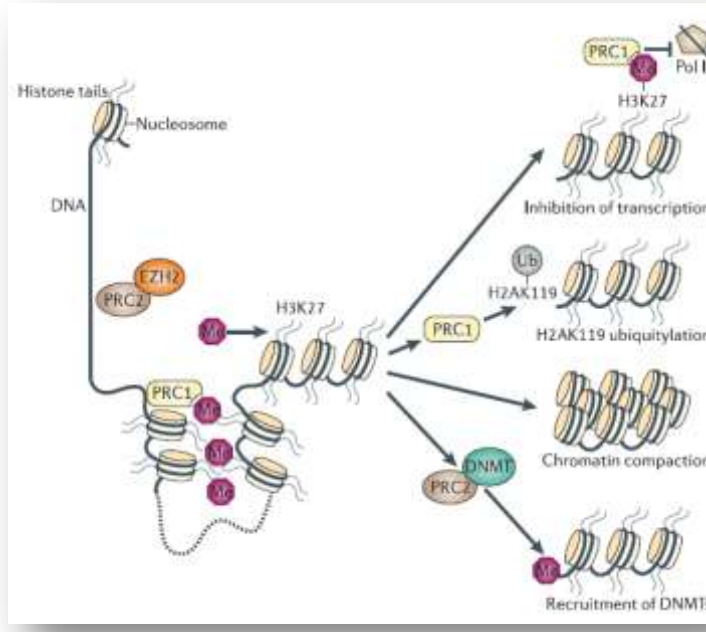


Figure I.8 Epigenetic gene silence by PcG proteins. Ezh2 trimethylates lysine 27 of histone H3. This mark is recognized by PCR1 complex and Ring1B is able to ubiquitinated histone H2A at lysine 119, establishing a second level of repression. Moreover, PcG group proteins are able to recruit DNA metyltransferases (DNMT). *Figure obtained from [140]*

Despite Polycomb group proteins were identified in Drosophila as repressors of Hox genes, recent results have shown also that Polycomb proteins are implicated in the stem cell pluripotency maintenance by repressing several genes that are necessary to the differentiation in several tissues [136]. This function of Polycomb

can be related to its transforming potential, and there are several works that try to establish a link between PcG proteins and tumor progression and malignancy.

Protein	Cancer type
EZH2	B-cell non-Hodgkin lymphoma
	Bladder
	Breast
	Colon
	Hodgkin lymphoma
	Liver
	Mantle cell lymphoma
	Melanoma
	Prostate
SUZ12	Breast
	Colon
	Liver
BMI1	B-cell non-Hodgkin lymphoma
	Leukaemia
	Mantle cell lymphoma
	Medulloblastoma
	Neuroblastoma
	Non-small cell lung cancer
PCL3	Colon
	Skin
	Lung
	Rectal
	Cervical
	Uterus
	Liver
RAE28	Acute lymphoblastic leukaemia

Figure I.9 PcG proteins and cancer. The figure shows a summary of the polycomb group proteins that have been shown to be upregulated in cancer. *Figure adapted from [140]*

POLYCOMB AND CANCER: INSIGHTS IN BREAST CANCER.

The first link between cancer and Polycomb was made in 1991, when Bmi1 was found to promote cancer by inhibiting the Cdkn2a locus [137, 138]. This locus encodes two proteins: INK4A, a cyclin-

dependent kinase inhibitor and ARF, that induces p53 by inhibiting MDM2 [139].

Subsequently, the Cdkn2a locus was found to be target of other Polycomb group proteins such as CBX7, MEL18 and RNF2, suggesting that this locus is important for the oncogenic potential of PcG proteins. Despite this, other Polycomb proteins have been shown to exert their role in cancer in a Cdkn2a-independent manner [140].

In most of the studies that link Polycomb with cancer, upregulation of PcG proteins is related with poor prognosis (summarized in figure I.9)

Varambally *et al.* showed that there is a correlation between EZH2 expression and prostate cancer prognosis [141] and one year later the same group showed similar evidences in breast cancer [142]. They demonstrated that EZH2 is significantly overexpressed in invasive breast cancer and metastatic breast cancer relative to normal tissue. These data suggest a link between EZH2 activity and the process of cellular transformation or the induction of a metastatic phenotype, but does not constitute definitive evidence, since EZH2 overexpression could be a side effect produced by the deregulation of the cell cycle that commonly occurs in metastatic tumours. In this respect it has been shown that EZH2 is regulated by E2F, a transcription factor whose expression is cell cycle-dependent [143].

Thus, it is necessary to determine whether EZH2 has transforming activity or it can confer metastatic potential. In addition, evidence

that EZH2 is targeted by specific genetic alterations, such as gene amplification, would likewise bolster the argument that EZH2 expression is functionally relevant in breast and prostate cancer.

It has been shown that the epigenetic silencing of $p16^{INK4A}$ is mediated by EZH2 and SUZ12 in normal mammary gland epithelial cells in culture [144]. To obtain these results, this group uses the HMEC (Human Mammary Epithelial Cell) model. HMEC cells show two phases of growth. In the first-phase (pre-selection), the cells grow, but after several passages, the growth ceases (selection). Then, several cells are able to escape this stage and continue into a second growth phase (post-selection). In this second phase, the cells show several preneoplastic characteristics including silencing of $p16^{INK4A}$. It has been demonstrated there is more H3K27 trimethylation in the $p16^{INK16}$ promoter, and that $p16^{INK4A}$ silencing is partly inhibited by using 5'AZA, a known inhibitor of DNA methylation, supporting the relationship between DNA methylation and PcG proteins [132].

Others links between Ezh2 and breast cancer have been recently exposed. It has been shown that Ezh2 expression is high in BRCA1-deficient tumors and that Ezh2 expression is required for BRCA1-defficient tumors survival. Despite this, the relationship between Ezh2 expression and BRCA1 was not found, because restoration of BRCA1 in BRCA1-defficient cells does not reduce EZH2 expression. [145]. Unfortunately, why Ezh2 is an advantage for BRCA1-deficient tumors remains unclear.

On the other hand, Ezh2 downregulation in ER-negative breast cancer cell lines has been shown to upregulate BRCA1 protein levels, whereas overexpression of Ezh2 decreases nuclear BRCA1. Ezh2 knockdown in ER-negative tumors decreases tumor proliferation and growth *in vivo* and *in vitro* and slows the transition between G2 and mitosis, as happens with ectopic expression of BRCA1 [146]. Despite this, the mechanism by which Ezh2 regulates BRCA1 expression is still unknown.

Regarding the role of proteins PRC1 in cancer, several works suggest that Bmi1 overexpression in cancer is associated with poor prognosis [147]. In contrast it has been recently shown that Bmi1 overexpression is associated with good prognosis, whereas EZH2 would be related to poor prognosis [147]. The explanation for this controversial data regarding Bmi1 expression in breast cancer could be the cell origin. Van Lohuizen's group suggests that, as has been previously shown, the introduction of identical transforming mutations in different mammary epithelial cell types resulted in different oncogenic potential of these cells [148]. Then, the pre-existing expression patterns or differentiation state of a cell determines how malignant that cell can become and by which mechanism.

In the context of breast cancer metastasis, PcG proteins have been less studied. Recently Bmi1 has been shown to promote brain metastasis of mammary tumoral cells, but in a context dependent manner, because Bmi1 increases the rate of proliferation and invasion, and inhibits the apoptotic response to DNA damage in the

presence of oncogenic of H-Ras, which is overexpressed in 20-30% of breast cancer [149].

The mechanisms by which cells undergo metastasis have to be further elucidated. As has been explained before, there are emerging evidences that suggest that PcG proteins could be implicated in this process. Despite this, most of the studies have been focused in PcG proteins as a markers of prognosis, but very little is known about the processes that could be regulated by these proteins. EMT, as first part of the metastasis, requires loss of cell-cell and cell-matrix adhesion, degradation of extracellular matrix, acquisition of motility, etc., but the mechanism in which PcG proteins could take place remains yet without clarification.

3 DISSERTATION RESEARCH GOALS.

The PcG ability to repress gene expression has been linked with cancer very often, because PcG protein upregulation is a common issue in many cancers, especially in poor prognosis cancers. Despite this, the mechanism that induces the deregulation of the expression of PcG proteins and the roles of these proteins in tumorigenesis remains unclear.

The main objective at the beginning of the study was to establish a cellular model of metastasis, invasion and migration of epithelial cells in order to study the possible implication of PcG proteins, and in this model analyze the pattern of expression of PcG proteins.

The next goal was to establish the possible role of the deregulated PcG proteins in our metastasis model, to study the effect of the knockdown of these proteins in the metastatic ability of the cell, to dissect the pathways regulated by PcG to affect this ability, and finally to study the expression of these proteins in human tumors.

4 MATERIALS AND METHODS

4.1.1 Cell culture and cytokines

NMuMG, MCF7 and MDA-MB-231 cells culture.

Human mammary epithelial MDA-MB-231 and MCF7, and mouse mammary epithelial NMuMG cells were grown in Dulbecco's modified Eagle Medium (D-MEM) (Gibco) with 100 U/ml penicillin, 100ug/ml streptomycin and 2mM L-glutamine. MDA-MB-231 cells were supplemented with 15% FBS, MCF7 with 10% FBS and NMuMG with 10% FBS and 10ug/ml of insulin (Sigma). The cells were tripsinized before arrive to confluence and maintained at 37^{o}C in humid atmosphere of 5% CO_2.

Cytokines treatments.

- *Tgf-β2/Egf and Hgf/Tgf-α treatments*: 1x10^6 cells were seeded in a 60 mm plate and then treated with 10ng/ml Hgf and 100ng/ml Tgf-α or 50ng/ml Egf and 5ng/ml Tgf-β2. 48h later, cells were split and 1x10^6 cells were seeded, and then treated again with the growth factors, it was repeated until the day 6. Recombinant EGF, HGF and TGF-α were from R&D Systems and Tgf-β2 from Peprotech.
- Tgf-β3 treatments 1,5x10^6 cells were seeded in a 60 mm plate and then treated with 2 ng/ml TGF-β3 (Peprotech). 24h later, cells were split and 1,5x106 cells were seeded and re-treated. Recombinant EGF, HGF and TGF-α were from R&D Systems.

- *SIS3 treatment*. The treatment was performed as previously described[150]. Cells were splited and 10^6 cells were seeded. At the same time cells were treated with 3μM of SIS3. After one hour in the incubator, cells were treated with 2ng/ml Tgf-β3.

Oligofection.

1x 10^6 cells were seeded in 60 mm culture dish. 16-24h later the media was removed and cells were maintained during the transfection in 3 ml OPTIMEM (Gibco). Cells were transfected with 2μM of oligo siRNA (Dharmacon) and 4μl of Dharmafect 4 (Dharmacon). siRNA sequences are available on request.

When the oligofection was combined with Tgf-β treatmens, after 24 hours post oligofection, cells were splited and treated as explained in the previous section.

Δ-p63-α or empty vector transfection.

$2 \cdot 10^6$ MCF7 or MDA-MB-231 cells were seeded in 60mm plates and were maintained in the incubator O.N. The next day the medium was changed by 3ml OPTIMEM. Cells were transfected using lipofectamine 2000 (4μl/plate) and 10 μg of each plasmid. Cells were maintained with the lipofectamine-plasmid mix for four hours, and then 15% FBS medium was added to the plate. The next day the media was removed and cells were maintained in the incubator with fresh media until the next day.

4.1.2 Immunocytochemistry

Cells were grown in Lab-Tek II Chamber Slide System (Nalgene) overnight. Then they were fixed in 4% paraformaldehyde and permeabilized in 0.2% Triton X-100 in phosphate-buffered saline (PBS). Subsequently, cells were incubated in blocking solution (1% BSA and 0,03% Tween-20 in PBS) for 30 minutes before incubating with primary antibody. Cells were then washed in 0.02% Tween 20/PBS and incubated with the secondary antibody. Next, cells were washed with 0.02% Tween 20-PBS, stained with 4'-6-diamidino-2-phenylindole (DAPI, Invitrogen), and mounted with Fluoromount (Southern Biotech).

4.1.3 Protein extraction and western blot analysis

Total protein extraction.

Cells were washed twice with PBS and total protein extraction was obtained by lysing the cells using RIPA buffer (150 mM NaCl, 50 mM Tris-HCl pH 8, 1% NP40, 1% sodium deoxycholate, 0.5% sodium dodecyl sulfate, 2mM EDTA, 1 mM DTT and 1 mM PMSF). Cells were incubated with RIPA buffer during 30' and after centrifugation 15' at 16000, the supernatant was kept as total protein extracts.

Nuclear protein extraction.

To perform nuclear extracts, cells were washed with PBS and recovered by scraping. After centrifugation, buffer A (10 mM Hepes-KOH pH 7.5/ 10mM NaCl/ 3mM $CaCl_2$/ 0,25M sucrose /1 mM DTT/ 1mM PMSF) was added to isolate the nuclei. Nuclei were

lysed using RIPA buffer (150 mM NaCl, 50 mM Tris-HCl pH 8, 1% NP40, 1% sodium deoxycholate, 0.5% sodium dodecyl sulfate, 2mM EDTA, 1 mM DTT and 1 mM PMSF).

Histone isolation.

Cells were washed with PBS and collected by scraping in PBS. After spinning, lysis buffer was added to the cells (containing: Tris, NaHSO3, triton X-100, MgCl2 and sucrose. pH 6,5) to break the cellular membrane. Then, samples were washed three times with lysis buffer. After suspension of the nuclei with Tris-EDTA solution, H_2SO_4 was added to the pellet and vortexed, because acid pH lead DNA rapidly to be released from the nucleosome, and then histones do not precipitate with the DNA. Samples were incubated at 4^{o}C for more than 1h and the supernatant was collected after high-speed microcentrifugarion. Acetone was added to the supernatant.

After overnight incubation at 20^{o}C, collect by microcentrifugation, wash the acetone and dry. Dissolve the histones in 25mM Tris-Cl pH=8.8, 0,1% SDS. The pH was adjusted to 7 with NaOH.

Western blot analysis.

Protein extracts were quantified using the RD DC protein assay kit (BIORAD), and after following the manufacturer's instruction, proteins were quantified in the spectrophotometer at 750nm. Then equal amounts of proteins in each condition were separated on SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes. Membranes were immunoblotted with the indicated

primary antibodies and subsequently with HRP-conjugated antibodies, and visualized using the ECL Western blotting system (Amersham Pharmacia Biotech).

Antibodies list.

Antibody	Origin	Antibody
E-cadherin	BD	Mouse
Zonula.Occludens	BD	Mouse
Vimentin	BD	Mouse
Lamin B1	Abcam	Rabbit
Ring1B	Koseki [151]	Mouse
Ring1B	MBL	Mouse
Ub-H2A	Upstate	Mouse
3-met-H3K27	Abcam	mouse
Tubulin	Sigma-aldrich	Mouse
SIM2	Abcam	Rabbit
FAK	Millipore	Rabbit
p381-FAK	Biosource	Rabbit
P63	Millipore	Mouse
GFP	Clontech	Rabbit
P63	Santa Cruz	Rabbit

4.1.4 Quantitative RT-PCR

mRNA isolation.

Genelute Total Mammalian RNA Kit (Sigma) was used to perform RNA isolation, as described by manufacturer's protocol.

After isolation, RNA was quantified with Nanodrop spectophotometer (Thermo-Scientific) at 260 nm and stored at -80^{o}C.

mRNA retrotranscription into cDNA

cDNA was obtained from isolated RNA by using Transcriptor First Strand cDNA Synthesis Kit (Roche). One μg of each sample was used as template for the reaction and 60μM of random hexamer primers. The reaction was performed in two steps, one first step where the RNA and the primers were incubate 10' at 65^{o}C and a second step where the dNTPs, reverse transcriptase and RNAse inhibitor were added. This last step was performed at 10' 25^{o}C, 60' at 50^{o}C and 5' at 85^{o}C.

quantitative RT PCR analysis.

Real-time PCR assay was performed using SYBR Green PCR master mix (Applied Biosystems). The cDNA obtained as explained in the previous step was diluted in RNAse free water at 25ng/μl final concentration. the reaction was performed following manufacturer instruction in a final volume of 10 μl, including 300nM of each primer, 5ng/μl cDNA and 1X PCR master mix.

The PCR reaction was

1. 50^{o}C 2'

2. 95ºC 10''
3. 95ºC 15''
4. 60ºC 1'

Steps 3 and 4 were repeated 40 times.

Primer list.

Gene	Primers
GAPDH	AGGCCGGTGCTGAGTATGTC GGCGGAGATGATGACCCTTT
E-CADHERIN	ACAGACCCCACGACCAATGA CCTCGTTCTCCACTCTCACATG
VIMENTIN	TGCGGCTGCGAGAGAAAT GCCAGAGAAGCATTGTCAACATC
RING1B	GCAAGGATCAACAAACACAACAA GCTTTTTGCCTCGCTGTAATCT
SLUG	CAGCGAACTGGACACACATACA AGGATCCTTGGTTGTGGTACGACA
SNAIL	ACCCCAATCGGAAGCCTAAC TGGTCGTAGGGCTGCTGGAA
TWIST	CCGGAGACCTAGATGTCATTGTT TTTTAGTTATCCAGCTCCAGATCTCT
ID2	CCCTCAACACGGATATCAGCAT CCGCTTATTCAGCCACACAGT
SIM2s	GATGCGCGCCGTCTTC

	AGTGCGATCCCAGCTCCT
ΔP63	GGTTGGCAAAATCCTGGAG TCACTAAATTGAGTCTGGGCATT
FAK	TTCGACGTTTTACCTCAGCTAGTG TTCACGCCATGCATCAGTATC

Gene	Primer
Gapdh	AGGCCGGTGCTGAGTATGTC GGCGGAGATGATGACCCTTT
E-cadherin	ACAGACCCCACGACCAATGA CCTCGTTCTCCACTCTCACATG
Vimentin	TGCGGCTGCGAGAGAAAT GCCAGAGAAGCATTGTCAACATC
Ring1b	GCAAGGATCAACAAACACAACAA GCTTTTTGCCTCGCTGTAATCT
Slug	CTGGCTGCCTTCTATGGACACA TGCCCTCAGGTTTGATCTGTCT
Snail	CCCAGTCGCGGAAGATCTT CCTTCGGATCTGCATCTTCA
Twist	CTCGCACAAGCTGAGCAACATT CGCTCTGCAGGACCTGGTA
Id2	CACAAAGTCGGAGCGTGAATAT GCATCCAGTAGGCTCCTGTCAA

SIM2s	TGCTGCACACTCTTGATGGATTTG ACCTGGGACAAGCCTAAATGG
Fak	TTCGACGTTTTACCTCAGCTAGTG TTCACGCCATGCATCAGTATC

4.1.5 Funtional assays

Wound healing assay

Cells were oligofected as explained before with Ring1B or mock siRNA. 500.000 cells were seeded in twenty-four-well plate in 0,5% FBS-DMEM. The next day a wound scratch was applied with a pipette tip and healing was measured at time 0 and 24 hours in the absence or presence of TGF-β3 (2ng/ml). Wound area was measured by using ImageJ software, and wound healing was quantified by subtracting the value of the invaded area at 24 h from the value of the invaded area at time 0.

Transwell assay

Transwell permeable support of 8 μm polycarbonate membrane (Costar) was pre-treated with matrigel (BD). $4x10^4$ oligofected cells (Ring1B or mock siRNA) were suspended in D-MEM-0,5% serum and seeded in the upper part of the chamber. The same media was added to the lower chambers. Three wells of each condition were treated with 2ng/ml TGF-β3 in both upper and lower chamber and three were non-treated controls. After 48 hours, non-migratory cells were removed from the upper chamber with a cotton-tipped

swab. Chambers were washed twice with PBS, fixed with 1% glutaraldehyde and stained with crystal violet. Membranes were washed with distilled water and detached. To quantify the invasion, membranes were placed in an eppendorff with acetic acid, and after 15' of shaking; the absorbance of the solution was measured at 590nm in a fluorimeter.

4.1.6 ChIP assay

Ub-GFP cells generation

$5x10^6$ MCF7 and MDA-MB-231 cells were seeded in 140mm plates and transfected with 20 μg of Ub-GFP plasmid using lipofectamine 2000 during four hours and the 15% FBS DMEM was added. The next day cells were splited and $5x10^6$ cells were reseeded in 140 mm plates. The following day, cells were oligofected with oligofectamine an iRing1B or iMock at 2μM final concentration O.N. After that, the media was refreshed and after 24 hours incubation with fresh media, ChIP protocol was performed.

Δ-p63-α cells generation

$5x10^6$ MCF7 and MDA-MB-231 cells were seeded in 140mm plates and transfected with 20 μg of Δ-p63-α plasmid using lipofectamine 2000 during four hours and the 15% FBS DMEM was added. The next day the media was refreshed and after 24 hours incubation with fresh media, ChIP protocol was performed.

ChIP protocol

Briefly, cells were crosslinked with 1% formaldehyde in DMEM 10' in shaking and the reaction was stoped with 2ml of Glicyne buffer at 1M concentration. After two washes with cold PBS, cells were scraped and nuclei were extracted as explained in this materials and methods.

Then each condition was resuspended in 1ml SDS lysis solution and were sonicated at 40% of amplitude 8 times during 10'' on ice. The sonicated samples were centrifuged and supernatant was kept as chromatin suspension.

Chromatin were quantified with RC DC protein assay kit and two tubes with 1mg for each condition were precleaned with protein-G-agarose prebloqued with salmon sperm (Millipore). Then the precleaned chromatin was incubated with 4μg of anti-GFP antibody or 4μg of rabbit Igg at 4^{o} O.N.

After incubation, protein-G-agarose prebloqued with salmon sperm was added and incubated for 4 hours at 4^{o}C. Then protein-G was cleaned with Low Salt buffer (), High Salt buffer (), LiCl buffer () and TE buffer. Each condition was eluted with Elution buffer (20 μl SDS 10%, 40 μl of $NaHCO_3$ and 160 μl H_2O). The crosslink was reversed by 8 ml of NaCl 5M O.N at 65^{o}C.

Then samples were incubated with RNAse A, Proteinase K and DNA was extracted with GFX columns (Amersham) following manufacture instructions.

4.1.7 Xenograft experiments

Preparation of MDA-MB-231 iRing1B or iMock cells.

MDA-MB-231 cells were transfected with the ecotropic receptor by using lipofectamine 2000 (Invitrogen) and selected with 500 μg/ml G418 (Invitrogen), for two weeks. ecotropic HEK293-Phoenix cells were cultured with DMEM (Gibco) supplemented with 10% fetal bovine serum (GIBCO) and kept at 37^{o}C in a humid atmosphere of 5% CO2 in air. Cells were at 80% of confluence by the time of transfection. Transfection of HEK293-Phoenix cells was performed in 60mm petri dishes (Corning) using FuGENE (Roche), according to manufacturer's protocol. 24 hours later, medium was renewed. A day later, HEK293-Phoenix cell medium was filtered through 0.45 μm filter and added to MDA-MB-231-ecotropic and MCF7-ecotropic cells with 5 μg/ml Polybrene (Sigma). 24 hours later the medium was refreshed and the cells were selected with 2 μg/ml puromycin (Sigma).

Subcutaneous implantation and intravenously injection.

Four severe combined immunodefiecient (SCID) mice were injected with 1x10^{6} MDA-MB-231 iRing1B or iMock cells for each condition subcutaneously or intravenously, and animals were maintained until the final point criteria were achieved.

The animals were stored in isolated jails to guarantee the necessities of the immunodepresed animals. The temperature was maintained at 22 +- 2^{o}C, with light cycles of 12h and 300 lux of luminic intensity. After arrival to the final point criteria, the animals were sacrificed with CO_2.

Final point criteria.

The supervision card was used to control the animal welfare. If one animal arrives to 8 points, it is considered as final point criteria, if there were three points in two boxes or more, both boxes were increased in one additional point.

Also were considered final point criteria, animals with tumor size higher than 1cm^2 or the presence of fistulization.

Weight	normal		0		Twice a week
	10% loss		1		
	10-20% loss		2		
	20% loss		3		
Animal observation	appearance	normal	0		diary
		Bad state coat	1		
		Bad state coat and nasal or ocular secretions.	2		
		Abnormal posture.	3		
	Automutilations or extrange noises		no	0	diary
			yes	3	
Stimuli reaction	Very aggressive or comatose		no	0	diary
			yes	3	

4.1.8 Immunohistochemistry

3-μm sections obtained from formalin-fixed, paraffin embedded tissue blocks were deparafined using xylene and rehydrated using ethanol gradient battery. The antigen retrieval was performed by using citrate buffer solution at 10mM pH 7,3 one minute at 120^{o}C and endogenous peroxidase was inhibited with 4% hydrogen peroxide solution (H_2O_2) in methanol. The samples were blocked with 1% Bovine serum albumina (BSA) in phosphate buffered saline (PBS) and antibodies were incubated in blocked solution O.N. (FAK: 200, Ring1B 1:100 and p63 1:100). Envision system labeled polymer-HRP anti-mouse and anti-rabbit (Dako cytomation), and as chromogenic substrate was used diaminobenzidine tetrahidrochloride plus (DAB+). Sections were counterstained with hematoxylin, dehydrated and mounted.

Samples were visualized with Leica DM600 Digital microscope and and images were adquired with QWin software (Leica).

5 RESULTS

With the aim of establishing a model of study, first we tried different cytokines which are known to induce morphological changes in epithelial cells and different epithelial cell lines known to answer to these cytokines. Better results were obtained with Hgf/Tgf-α and Tgf-β/Egf. Hgf, as is explained in the introduction, is able to induce *scattering* that means, morphological changes in epithelial cells without gene reprogramming. On the other hand, Tgf-β known to induce complete EMT in most epithelial cell lines, under several time and concentration conditions.

5.1 Hgf/Tgf-α and TGF-β/Egf models in NMuMG cells

5.1.1 Hgf/Tgf-α and Tgf-β/Egf model characterization in NMuMG epithelial cells

Hgf/Tgf-α treatment in NMuMG cells induces loss of cell-cell contact and Tgf-β/Egf treatments in NMuMG cells induces changes at phenotypical levels from epithelial to more mesenchymal-like or less differentiated cells.

NMuMG cells are normal mammary epithelial cells and in standard conditions they show a typical epithelial phenotype in 2D culture cells and mainly group in clusters with cell-cell contacts.

In normal cells, Tgf-β and Hgf can inhibit Ras pathway and induce apoptosis. To avoid this apoptosis in our models, we treated the

cells at the same time with cytokines that binds EGFR inducing the activation of Ras and, as a consequence, inhibiting the apoptosis mediated by Tgf-β or Hgf. In the case of Tgf-β we use Egf, whereas when we treat the cells with Hgf we use Tgf-α.

As figure R.1 shows, Hgf/Tgf-α and Tgf-β/Egf treatments produce in cells several commons characteristics in phenotype, which consists in the loss of cell-to-cell adhesion, that can be observed as more refringent intercellular contacts and also increased cytoplasmic prolongations. On the other hand, Tgf-β/Egf but not Hgf/Tgf-α induces acquisition of a mesenchymal-like phenotype, that means less polygonal cells and growth of cytoplasmic prolongations that can be considered as a motility indicator.

As both the EMT and the scatter processes share some phenotype characteristics it is difficult to assess to what extend each of these processes contributes to the microscopically observed effect and then phenotypic observation is not enough to classified both models into scatter of EMT.

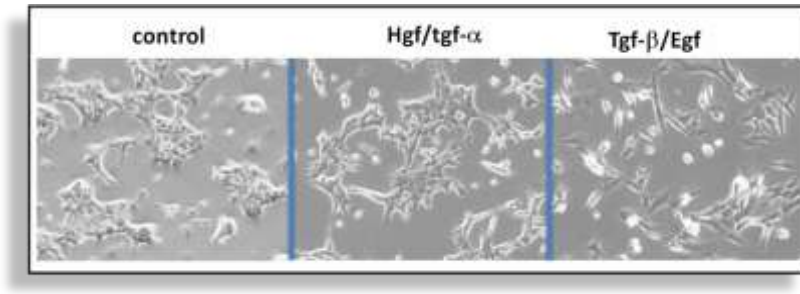


Figure R.1. NMuMG phenotype in Hgf/Tgf-α and Tgf-β2/Egf treatments. Phase contrast images of NMuMG cells treated with 10ng/ml Hgf and 100ng/ml Tgf-α or 50ng/ml Egf and 5ng/ml Tgf-β2 for 6 days.

Hgf/Tgf-α treatment in NMuMG cells induces reorganization but not loss of epithelial markers and no changes were detected in mesenchymal markers.

Based on previously reported data [85] In Hgf/Tgf-α treatments we expected to induce cell scattering. After Hgf/Tgf-α treatment, E-cadherin and Zonula Occludens, two epithelial markers disappear from cell-cell contacts and are widely distributed in the cytoplasm (figure R.2). Despite this redistribution, western blot and quantitative RT-PCR (qRT-PCR) analysis revealed that the total amount of E-cadherin did not changes (figure R.3).

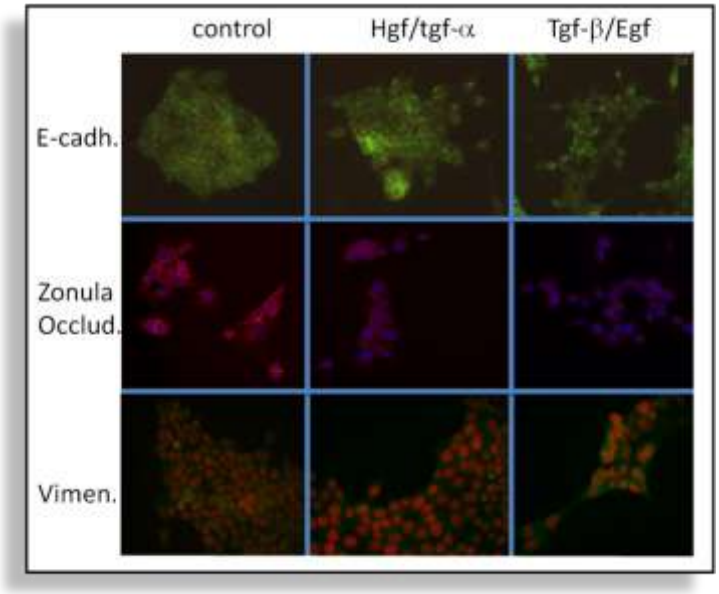


Figure R.2. Epithelial and mesenchymal markers in NMuMG cells: The panel shows the immunofluorescence analysis to detect E-cadherin (epithelial marker), Zonula occludens (localized in adherens junctions in epithelial cells but not in mesenchymal cells) and vimentin (mesenchymal marker) in NMuMG cells treated with with 10ng/ml Hgf and 100ng/ml Tgf-α or 50ng/ml Egf and 5ng/ml Tgf-β2 for 6 days

In addition, we were unable to detect Vimentin, a classical mesenchymal marker, by immunofluorescence (figure R.2) or by western blot (data not shown) neither in control nor in Hgf/Tgf-α-treated NMuMG cells, suggesting that this treatment is not enough to induce its expression in NMuMG cells. qRT-PCR supports this hypothesis, because no significant changes in Vimentin mRNA were observed in Hgf/Tgf-α-treated cells versus control cells (figure R.3).

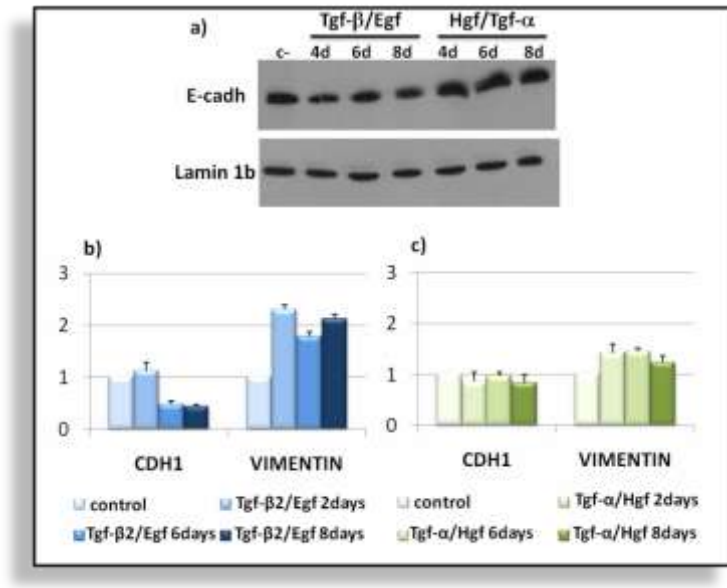


Figure R.3. Epithelial and mesenchymal markers expression in NMuMG treated cells. a) Western blot of NMuMG cells extracts to detect E-cadherin **b)** Quantitative RT-PCR to detect E-cadherin and Vimentin in Tgf-β2/Egf treated cells **c)** Quantitative RT-PCT to detect E-cadherin and vimentin in Hgf/Tgf-α treated cells.

Tgf-β2/Egf treatment induces Epithelial to Mesenchymal Transition in NMuMG cells.

To characterize the EMT in the Tgf-β2/Egf model immunofluorescence was performed in order to detect epithelial and mesenchymal markers. As it is shown in figure R.2, E-cadherin and Zonula Occludens, were lost from cell-cell contacts and Vimentin, was expressed in Tgf-β2/Egf cells, but not in untreated-control cells. These results were confirmed by western blot and qRT-PCR and are showed in figure R.3. As can also be observed in figure R.3 there is a downregulation in E-cadherin at protein level, but is lower than downregulation at mRNA level, suggesting that E-cadherin degradation system produces a slow turnover of the protein, that means a long half live of the preexisting pool of protein.

We also tested the expression of mesenchymal markers. Although we were unable to detect Vimentin expression by western blot qRT-PCR analysis indicated that there is an upregulation of Vimentin mRNA when cells are treated with Tgf-β2/Egf (figure R.3).

5.1.2 Polycomb expression in the HGF/Tgf-α and Tgf-β2/Egf treated cells.

Ring1B is upregulated in Hgf/Tgf-α and Tgf-β2/Egf treatments.

Once the experimental models were characterized at phenotypic and molecular levels, we tested whether PcG expression is regulated in these scattering and EMT processes. The expression of different PcG proteins were tested both at mRNA and protein levels and the most significant change observed was in Ring1B expression. This protein was induced in both models, suggesting

that this protein could be implicated in a pathway common to both scattering and EMT.

Interestingly, as can be observed in figure R.4, western blot detection of Ring1B by using a non-commercial antibody [151] showed that only one of the detected bands is upregulated by Hgf/Tgf-α treatment, whereas Tgf-β2/Egf treatment induces the upregulation of the three bands. Currently very little is known about Ring1B posttranslational modifications. Therefore we do not know the nature of these three bands

To confirm that there is an upregulation of Ring1B and discard the possibility that these three bands were due to the unspecific binding of the antibody, we took advantage of the availability of a commercial Ring1B antibody.

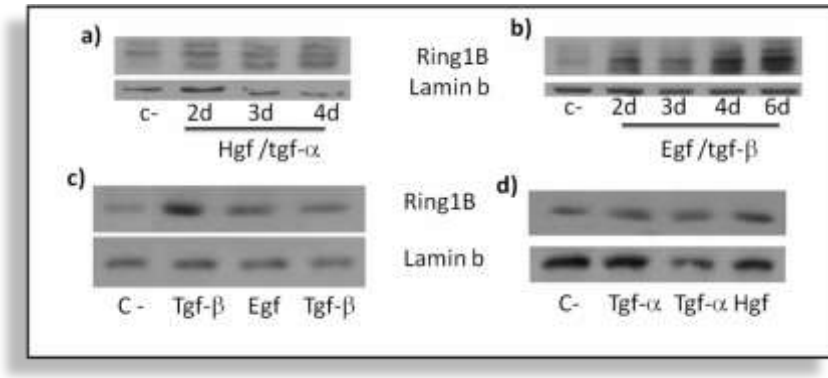


Figure R.4. Ring1B upregulation after Tgf-β2/Egf and Hgf/Tgf-α treatments in NMuMG cells. The western blot was performed with two differents antibodies. Ring1B monoclonal antibody was used to detect Ring1B in NMuMG-Tgf-β2/Egf treated cells (a) and in NMuMG-Hgf/Tgf-α treated cells (b). Ring1B commercial antibody (from MBL) was used to detect Ring1B expression in NMuMG-Tgf-β2/Egf greated for 6 days (c) or in Hgf/Tgf-α treated cells for 6 days(d).

We first confirmed the specificity of this commercial antibody by performing an immunofluorescence to detect Ring1B in U2OS BMI1-GFP cells [119], where Ring1B is expected to colocalize with BMI1 in Polycomb bodies. Once the antibody specificity was confirmed, we performed Hgf/TGF-α and Tgf-β2/Egf treatments on NMuMG cells, and we extracted the nuclear proteins. Western blot analysis of these extracts using Ring1B commercial antibody showed only one band (figure R.4). One possibility about this different band pattern displayed by both antibodies could be that the commercial one was unable to detect posttranslational modifications or that in the case of different mRNA splicing, the region of the protein recognized by the commercial antibody is not present in the spliced form. Despite these differences, Ring1B upregulation was also observed with the commercial antibody in Tgf-β/Egf, but the upregulation in Hgf/Tgf-α model is not so clear. In the same Western blot can be also observed what happens in NMuMG cells when only one cytokine is used. As can be observed, every cytokine alone is able to induce Ring1B upregulation, but the best effect is obtained when both cytokines are used at the same time.

Due to the availability of this commercial antibody, from then, all the experiments of the project were performed with this one.

Tgf-β2/Egf and Hgf/Tgf-α treatments induce histone modification in NMuMG cells.

Ring1B displays an ubiquitin ligase activity, and exerts its function by ubiquitinating histone 2A, and by interacting with other

proteins, including other histone modifiers [152]. Therefore we checked whether, besides the upregulation of Ring1B, Tgf-β2/Egf and Hgf/Tgf-α treatments resulted in an increase in ubiquitinated H2A (Ub-H2A) or alternatively, if Ring1B could be involved in EMT or scattering working only as a docking protein. To this end, we analyzed Ub-H2A by western blot performed by using the purified histones. As shown in figure R.5, there is an increase in Ub-H2A after Tgf-β2/Egf treatments, but not when the cells were treated with Hgf/Tgf-α. This difference could be attributed to the higher overexpression of Ring1B in Tgf-β2/Egf treatment when compared to the induction of Ring1B expression upon Hgf/Tgf-α treatment. Alternatively, it is also possible that as PcG proteins work as complexes, other proteins would be necessary to achieve effective H2A ubiquitination, and that upon Hgf/Tgf-α treatment, these proteins were not induced.

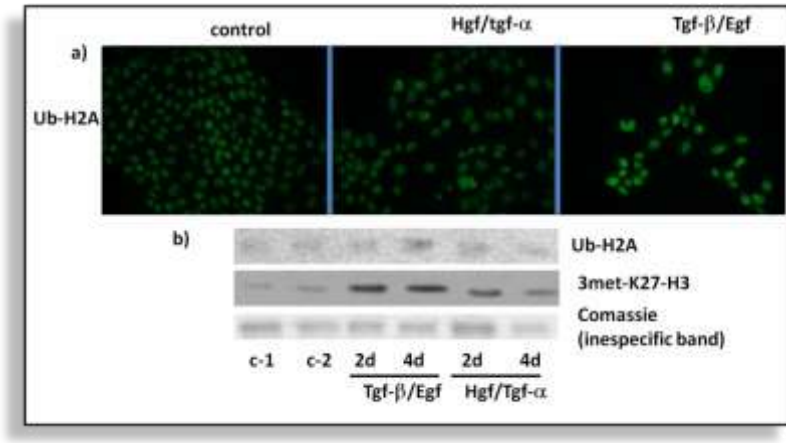


Figure R.5. Levels of ubiquitinated/H2A in Hgf/Tgf-α and Tgf-β2/Egf treatments. a) Ub-H2A immunofluorescence in NMuMG cells treated with Hgf/Tgf-α or Tgf-β2/Egf **b)** Ub-H2A and trimethylated K27H3 marks detected by western blot in NMuMG treated with Hgf/Tgf-α or Tgf-β2/Egf

It has been shown that trimethylation of lysine 27 (3met-K27H3) in histone 3 by PRC2 complex is necessary in some instances for PRC1 recruitment [133]. Therefore, we checked whether Tgf-β2/Egf and Hgf/Tgf-α treatments also increase the levels of 3met-K27H3. As is shown in figure R.5, both treatments results in an increase in the total levels of trimethylated K27H3. These results suggest that although both treatments differentially affect the catalytic activity of PRC1 complex, epigenetic events are mediating the response to these two treatments. Both marks (ub-H2A and 3met-K27H3) are repressive marks, but it has been suggested that Ub-H2A would be associated to a higher level of repression [153]. This notion could explain why scattering is easier reverted than EMT.

The key regulators of EMT change their expression with Egf/Tgf-β2 treatment, but not with Hgf/Tgf-α treatment.

Despite the fact that Hgf/Tgf-α is not supposed to induce the deregulation of mesenchymal and epithelial markers expression, it has been demonstrated that several transcription factors are regulated upon Hgf/Tgf-α treatment of NMuMG cells, and then scattering is currently considered as a partial-EMT [154].

Therefore, we analyzed the expression of Slug, Snail, Twist, Zeb1 and Zeb2 in both treatments (figure R.6), and upregulation of all these transcription factors was only obtained with Tgf-β2/Egf, whereas upon Hgf/Tgf-α treatment, only Twist was upregulated, although not statistically significant. For this reason we focused our next experiments on the Tgf-β2/Egf treatment, although the role of

Ring1B upon Hgf/Tgf-α definitively deserves more efforts to be elucidated.

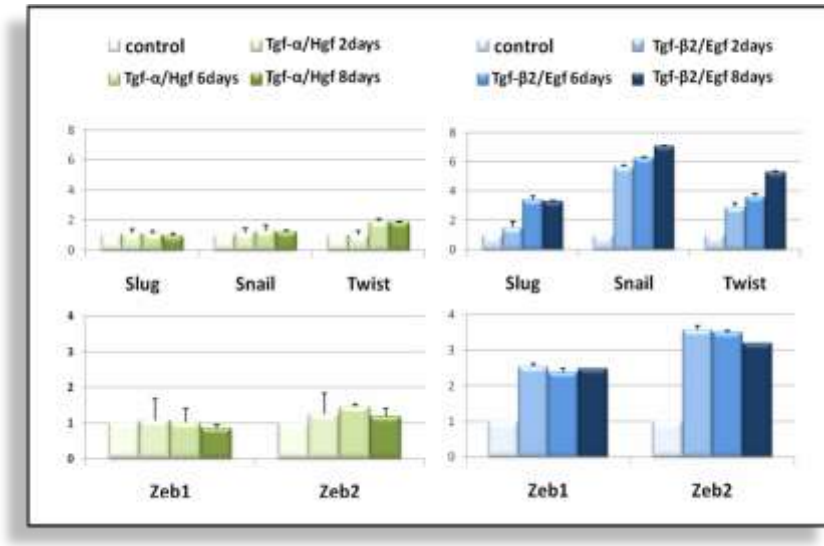


Figure R.6. EMT-related transcription factor expressions in NMuMG cells treated with Hgf/Tgf-α or Tgf-β2/Egf. Left graphs show Hgf/Tgf-α results and right panel shows the expression upon Tgf-β2/Egf treatments.

5.1.3 Implication of Ring1B in the regulation of EMT related-transcription factors in the Tgf-β2/Egf experimental model.

Slug, Id2 and Snail are good candidates to mediate the effect of Ring1B in Tgf-β2/Egf induced EMT in NMuMG cells.

Next we proceeded to knock-down Ring1B endogenous levels, in order to investigate the hypothesis that an increase in Ring1B would be required for the EMT process, by performing the experiment described in figure R.7. In summary, NMuMG cells were oligofected with an interference RNA (iRNA) for Ring1B (iRing1B) or with a control iRNA (iMOCK). Then, cells transfected with each iRNA

were treated or non-treated with Egf/Tgf-β2 and qRT-PCR was used to quantify mRNA for the main EMT transcription factors.

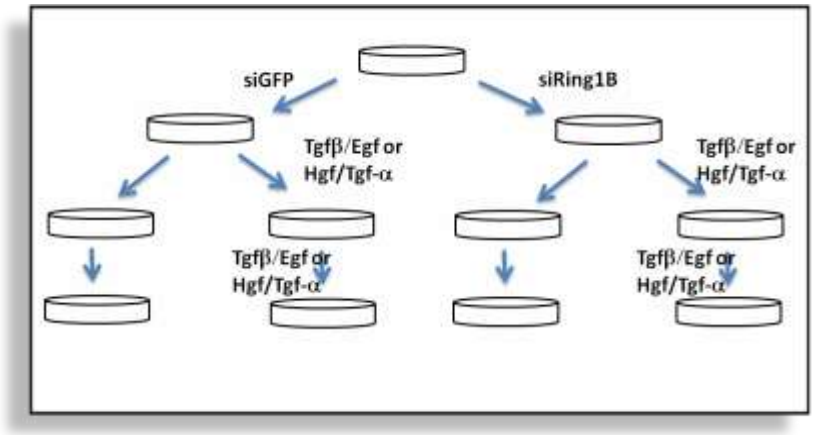


Figure R.7. Summary of the experiment performed with iMock or iRing1B oligofection and Hgf/Tgf-α or Tgf-β/Egf treatments. Cells were oligofected and the next day were reseeded and treated with the cytokines. After 2 days treatment, the cells were another time seeded and treated.

As figure R.8 shows, Slug and Snail were upregulated upon Egf/Tgf-β2 treatment whereas Id2 was downregulated, as expected. However, these changes were less pronounced in those cells oligofected with iRing1B. Despite these results are statistically significant, the increases observed were rather modest, reaching the highest differences at day 8 of the Tgf-β/Egf treatment. However, at this time point iRing1B-induced downregulation was partially lost. Therefore we were forced to work at day two, when changes in EMT related transcription factors were very subtle, or to improve our EMT model.

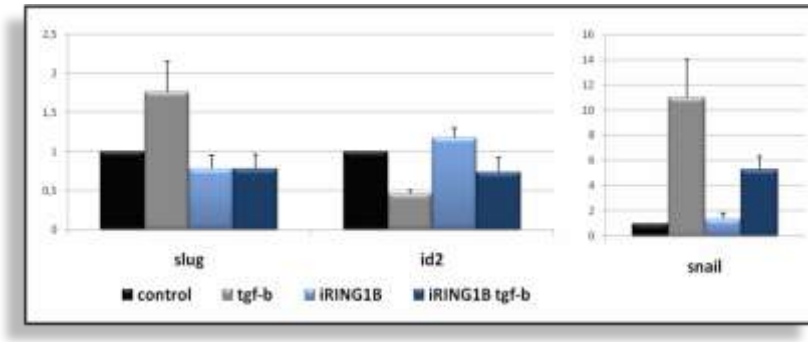


Figure R.8. Slug, id2 and Snail expression results from quantitative RT-PCR in NMuMG cells oligofected with iRing1B or iMock and treated or non-treated with Tgf-β2/Egf

Tgf-β3 induces EMT in NMuMG cells faster than Tgf-β2.

Trying to improve the model, we tested another isoform of the Tgf-β: the Tgf-β3, since some reports in the literature described that this isoform is able to trigger EMT in the same cell line [155]. In fact, with that Tgf-β isoform we got better results in a shorter period of time. Importantly, the increase in Ring1B expression was also higher than with the previously tested cocktail of Egf/Tgf-β2. The highest upregulation in Ring1B levels was obtained two days after treating the cells with 2-4 ng/ml of Tgf-β3 (figure R.9).

We also characterized the EMT triggered by Tgf-β3 in our cells. As shown in figure R.9, Tgf-β3 induces the downregulation of E-cadherin and Zonula Occludens, as detected by western blot. In addition, we also observed the upregulation of the Vimentin at the mRNA level in our model.

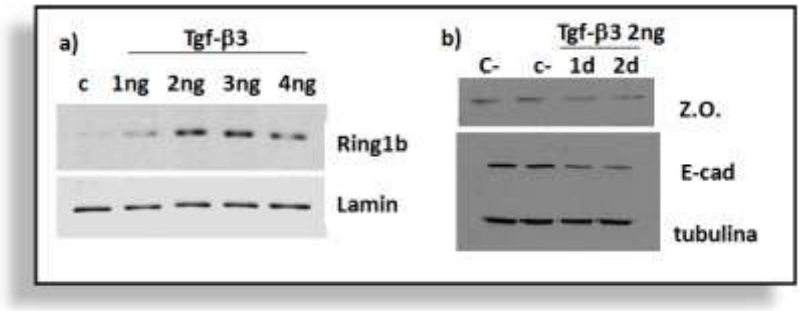


Figure R.9 Western blot analysis of Tgf-β3-induced EMT in NMuMG cells treatment. a) Ring1B upregulation in Tgf-β3 treatment at different concentrations. **b)** Epithelial markers dowregulation after Tgf-β3 treatment in NMuMG cells.

Ring1B downregulation impairs the expression of Slug, but not Id2, triggered by Tgf-β3.

Next, we determined the expression of EMT-related transcription factors upon Tgf-β3 in NMuMG cells previously oligofected with iMOCK or iRING1G, as previously performed for Egf/Tgf-β2 treated cells, by qRT-PCR. Strikingly, Ring1B knock-down was not effective in restoring Id2 levels, contrary to the data obtained upon Tgf-b2 treatment (figure R.10). A possible explanation for this discrepancy could be that Id2 downregulation by Tgf-β3 is stronger than with Tgf-β2 and then a better Ring1B knock down would be required to counteract this effect.

In contrast, Slug was upregulated two folds more than the upregulation induced by Tgf-β2. Furthermore, although the knockdown of Ring1B is not able to completely abolish the TGF-β3-induced Slug upregulation, it significantly impairs it (figure R.10).

Until now, nobody has shown a positive regulation directly mediated by Ring1B, meaning that Ring1B is a repressor. Therefore, the regulation of Slug by Ring1B could not be ascribed to a direct activation of Slug expression. Consequently, the identification of the link between Ring1B and Slug in Tgf-β3 induced EMT was our next goal.

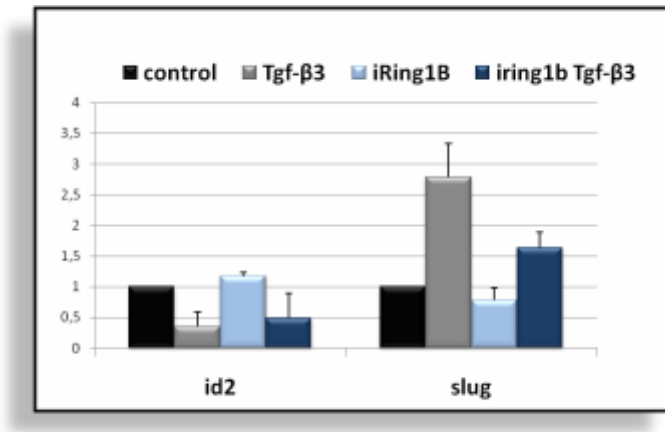


Figure R.10 id2 and Slug expression by quantitative RT-PCR in NMuMG cells oligofected with iRing1B or iMock and treated or non-treated with Tgf-β3/Egf

SIM2, a regulator of Slug, is downregulated by Tgf-β3 in NMuMG cells, but this downregulation is impaired by iRing1B

Sim2 is a member of the bHLH/PAS (helix-loop-helix/Per-Arnt-SIM) family of transcription factors [156], and differs from the members of its family by functioning as a repressor [156, 157]. Sim2 is located at chromosome 21, the chromosome whose trisomy is responsible for the Down syndrome [157]. Interestingly, people with Down Syndrome are more susceptible to childhood leukemias and germ cell cancers, but they are less likely to develop solid

tumors, and the most interesting feature in this respect is the decrease in breast cancer incidence and mortality [158]. A splice variant of human and mouse SIM2 designated SIM2 short (SIM2s), has also been identified [159] and it has been proposed that this isoform plays a role in cancer progression [160].

It has been also shown that SIM2 is expressed at relative high levels in non transformed and non-invasive breast cancer cells in comparison with the more invasive breast cancer-derived-cells. Immunohistochemical analyses of normal human breast and breast tumor tissue sections indicated that SIM2s is downregulated in the majority of human breast cancers. These results suggest that SIM2s is a breast tumor suppressor gene [161].

The knockdown of SIM2s MCF7 cells results in the loss of E-cadherin and keratin 18 and the gain of N-cadherin and Vimentin expression[162], suggesting that it may regulate factors involved en EMT. Supporting this notion, SIM2s has been shown to participate in the repression of Slug [162].

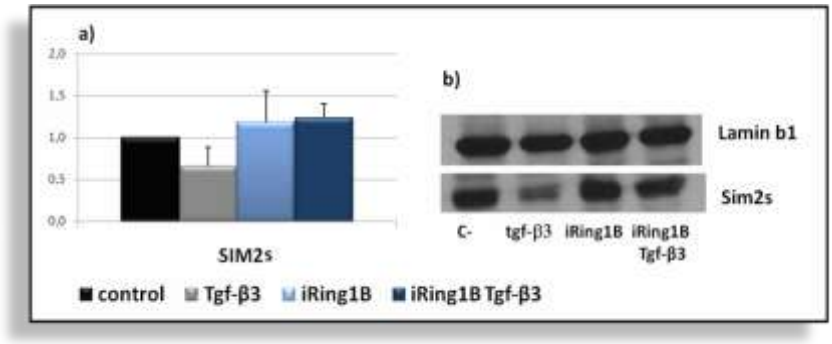


Figure R.11 SIM2s expression in NMuMG cells oligofected with iRing1B or iMock and treated or non-treated with Tgf-β3. a) SIM2s expression detected by qRT/PCR **b)** SIM2s expression by Western blot.

All these previous data led us checking whether Ring1B could be regulating SIM2s protein expression. As shown in figure R.11, SIM2s is downregulated both at mRNA and protein level in Tgf-β3 treated NMuMG cells. Moreover, when Ring1B was depleted by using interference RNA, Tgf-β3 treatment was unable to inhibit SIM2s expression.

These results regarding SIM2s and Slug suggested a direct interaction between Ring1B and SIM2s promoter, but more experiments would be required to address this issue.

The Specific Inhibitor of Smad3 protein (SIS3) is able to abolish the Tgf-β mediated Ring1B upregulation.

As explained in the introduction, the majority of the Tgf-β3 responses are mediated by the Smads pathway. Next, we tested, whether a specific inhibitor of Smad3 phosphorylation was able of inhibit Ring1B upregulation in our experimental model. As shown in figure R.12, Tgf-β3 was unable to upregulate Ring1B, when cells were treated with the Smad3 inhibitor.

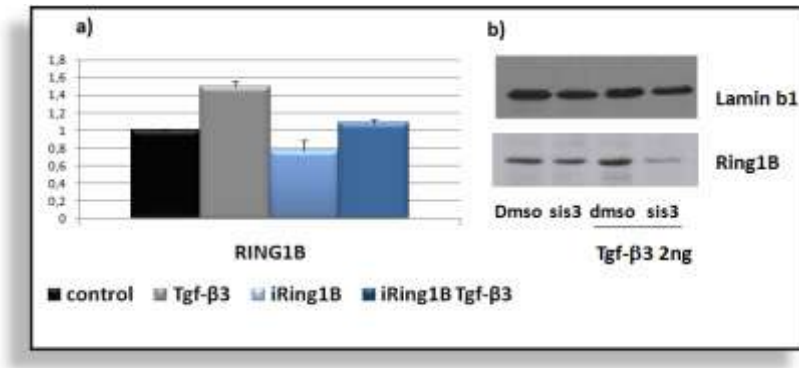


Figure R.12 Effect of the inhibition of Smad3 function on Ring1B expression. a) Quantitative RT-PCR results for Ring1B in Tgf-β3 treated NMuMG cells with or without SIS3 treatment **b)** Western blot analysis of Ring1B expression in NMuMG cells treated with Tgf-β3 and SIS3.

Additional experiments would be required to elucidate which specific pathway, downstream of Smad3, is responsible for Ring1B upregulation and the transcriptional and/or posttranscriptional mechanisms that would be involved in the regulation of Ring1B expression.

5.2 Study of Ring1B implication in other mammary epithelial cell lines upon Tgf-β3 treatment.

5.2.1 Characterization of the phenotypic changes induced by Tgf-β3 in MCF7 and MDA-MB-231 cells.

Tgf-β3 induces EMT in the MCF7 but not in MDA-MB-231 cells.

Trying to understand whether the RING1B induction in Tgf-β3 triggered EMT is a general feature, we extended our study to other two different mammary gland cell lines. MCF7 cells are a human adenocarcinoma cell line and have a reduced ability to migrate and invade in normal conditions. On the other hand, MDA-MB-231 cells are also an adenocarcinoma cell line, but they have the ability to invade and migrate. As shown in figure R.13, MCF7 cells have the phenotype of normal epithelial cells, but the MDA-MB-231 cells have lost this phenotype and are more mesenchymal or fibroblastoid.

In both cell lines we checked whether epithelial markers were downregulated and mesenchymal markers are upregulated during Tgf-β3 treatment. As shown in figure R.14, E-CADHERIN expression in MCF7 is downregulated at mRNA and protein levels upon Tgf-β3 treatment. In addition the mesenchymal marker VIMENTIN is slightly, but not significant statistically upregulated. In contrast, Tgf-β3 is not able to induce neither E-cadherin downregulation nor Vimentin upregulation in MDA-MB-231 cells at mRNA levels (figure

R.14). One explanation could be that, as MDA-MB-231 cells are in what have been called as a "metastable" stage [163], E-CADHERIN levels are too low to be affected by Tgf-β3 treatment. Accordingly, we were unable to detect E-CADHERIN protein by western blot in non-treated cells (data not shown).

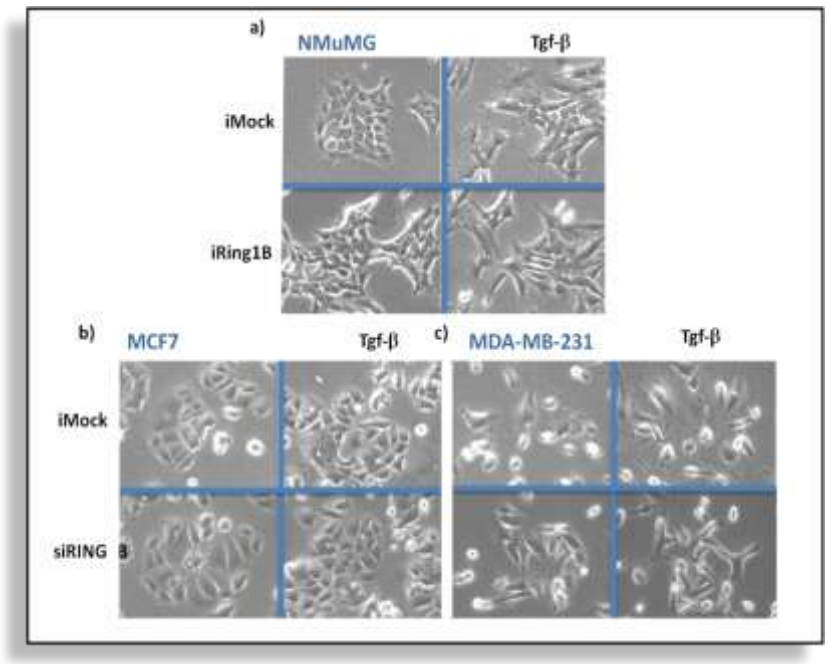


Figure R.13 Phenotype of NMuMG, MCF7 and MDA-MB-231 cells oligofected with iRing1B or iMock and treated or non-treated with 2ng/ml of Tgf-β3 for 2 days a) NMuMG cells upon Tgf-β3 treatment shows loss of epithelial phenotype, increased filopodia and lamellipodia and loss of cell-cell contact. In NMuMG cells oligofected with iRing1B and treated with Tgf-β3 this phenotype is less clear **b)** MCF7 **c)** MDA-MB-231 phenotype after iRing1B/Tgf-b3 treatment.

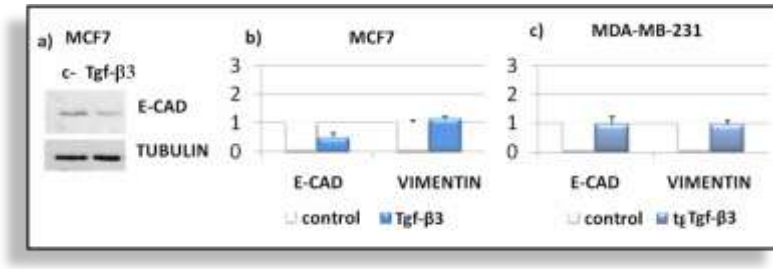


Figure R.14 Epithelial and mesenchymal markers expression in MCF7 and MDA-Mb-231 cells a) E-CADHERIN expression in MCF7 cells detected by western blot **b)** Quantitative RT-PCR analysis of E-CADHERIN and VIMENTIN in MCF7 Tgf-β3 treated cells **c)** Quantitative RT-PCR analysis of E-CADHERIN and VIMENTIN in MDA-MB-231 cells treated with Tgf-β3

5.2.2 Ring1B upregulation upon Tgf-β treatment.

Ring1B is upregulated at the protein but not at mRNA level in Tgf-β3 treated MCF7 and MDA-MB-231 cells.

MCF7 and MDA-MB-231 cells were treated with Tgf-β3 and Ring1B expression levels were analyzed by qRT-PCR and western blot (figure R.15). Despite RING1B mRNA slightly increased or did not change, Ring1B protein was upregulated in Tgf-β3-treated MCF7 and MDA-MB-231 cell. In contrast, Ring1B mRNA levels in NMuMG cells increased two times when the cells were treated with the cytokine (figure R.15). These results suggest more than one regulatory mechanism of Ring1B in Tgf-β3 treatment.

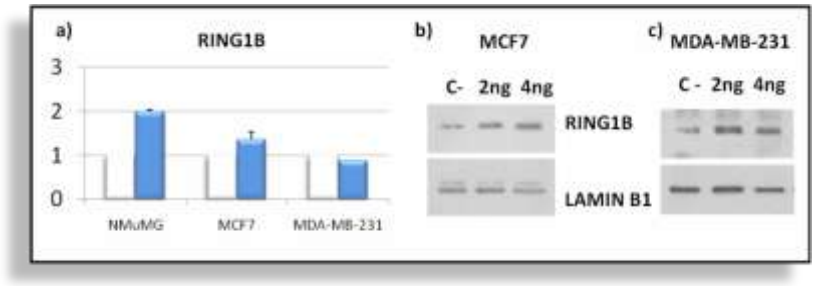


Figure R.15 Ring1B expression in three mammary epithelial cell lines a) Quantitative RT-PCR analysis to determine Ring1B expression in NMuMG, MCF7 and MDA-MB.231 cells treated with Tgf-β3 **a) and b)** RING1B expression in MCF7 (b) and MDA-MD-231 (c) cells treated with Tgf-β3 and detected by qRT-PCR

The Smad3 inhibitor SIS3 is unable to impair the induction of Ring1B overexpression in both MCF7 and MDA-MB-231 cells.

As previously performed for the Tgf-β3-treated NMuMG cells, Smad3 function was inhibited by incubation of the MCR7 and MDA-MB-231 cells with SIS3 before Tgf-β3, and RING1B expression was tested by RT-PCR and western blot. In contrast to the results obtained for Ring1B induction in NMuMG cells, this strategy in MDA-MB-231 and MCF7 was ineffective in the inhibition of RING1B induction when cells were treated with Tgf-β3 (data not shown).

These results support the hypothesis that Ring1B, in our model, is regulated, at least, by two different mechanisms. Upon Tgf-β3 treatment Ring1B mRNA regulation in NMuMG is mediated by Smad3 but, as RING1B mRNA in MCF7 or MDA-MB-231 cells did not change, Smad3 seems not to be involved in Tgf-β3-induced Ring1B mRNA induction. Additional experiments would be required to address this issue.

5.2.3 Role of RING1B in the expression of EMT-related transcription factors in Tgf-β MDA-MB-231 cells.

Ring1B does not mediate SLUG upregulation in MDA-MB-231 cells.

Next we tested the expression levels of the classical transcription factors involved in EMT (SLUG, TWIST, SNAIL, ZEB1 and ZEB2) by qRT-PCR in MCF7 and MDA-MB-231 cells previously oligofected with MOCK or RING1B interference RNA (iMOCK and iRING1B respectively). As figure R.16 shows, RING1B knockdown in MDA-MB-231 cells is not able to abolish SLUG upregulation, in contrast to what was observed when performing the same experiment in NMuMG (figure R.8). Possible explanations for this discrepancy among cell lines would be a differential regulation of Slug, which has been implicated in prolonged E-CADHERIN repression [164], in the highly invasive MDA-MB-231 cell line, in which Slug would be controlled by more than one mechanism. Alternatively, other transcription factors than SIM2s, and not sensitive to RING1B regulation, could be required for SLUG expression.

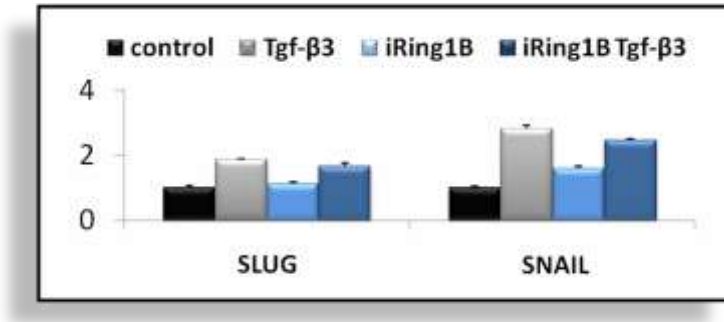


Figure R.16 SLUG and SNAIL expression in iRING1B or iMOCK oligofected MDA-MB-231 cells treated or non-treated with Tgf-β3.

5.2.4 Implication of Ring1B in migration and invasion

Ring1B is required for migration and invasion induced by Tgf-β3.

After the previous results, we tested whether Ring1B had any effect in Tgf-β3 induced migration. To this end, we used MCF7 cells, because the effects of the Tgf-β3 in these cells are optimal, as it is able to repress E-CADHERIN and to upregulate VIMENTIN (figure R.17). We performed wound healing and transwell assays, to study the migration and invasion, respectively of these cells. As shown in figure R.17, when cells are treated with Tgf-β3, they are able to invade and to migrate, whereas the non-treated control cells are not. On the other hand, when RING1B is knocked down, both migration and invasion were significantly impaired.

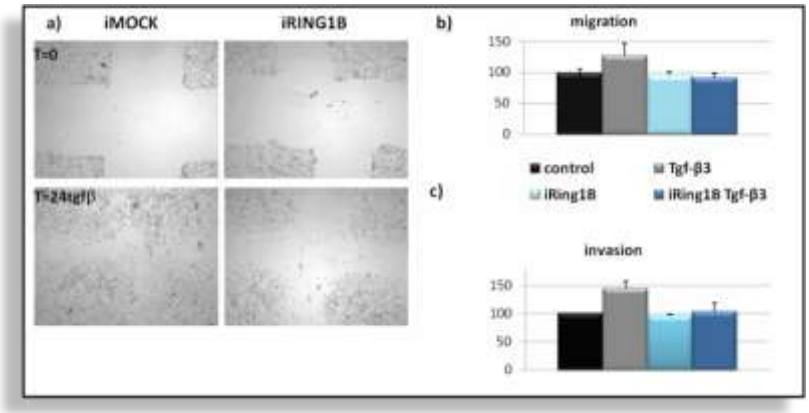


Figure R.17. Role of RING1B in MCF7 migration and invasion a) iRING1B or iMOCK MCF7 cells were seeded in 24-well plates in 0,5% FBS. The next day after seeding and a wound scratch was performed with a tip and after refresh the media, pictures were taken as time 0. Then were treated or non-treated with Tgf-β3 and incubated for 24h and the second picture was taken as time 24 hours. **b)** Quantification by ImageJ **c)** iRING1B or iMOCK MCF7 cells were seeded in the upper part of the transwell chamber previously treated with matrigel. Cells were treated or non treated with Tgf-β3 in both lower and upper chamber sides for 48 hours. Cells that pass to the bottom of the upper chamber were stained with crystal violet and quantified with spectofotometer.

Downregulation of Ring1B in MCF7 results in Vimentin repression and in the impairment of the Tgf-β3-induced E-cadherin repression.

Next we analyzed Vimentin and E-cadherin levels in MCF7 cells without or with Tgf-β and oligofected with Mock or Ring1B iRNA. As shown in figure R.18, Tgf-β3 treatment did not result in E-cadherin repression when cells had been oligofected with iRing1B. Furthermore, when Ring1B was downregulated in these cells, Vimentin was repressed at lower levels than the control.

Unfortunately, in these cells the analysis of the EMT-related transcription factors was not conclusive and therefore we do not have enough data that would allow us to hypothesize about the mechanisms implicated in this process. It is formally possible that E-cadherin repression is directly mediated by Ring1B. Alternatively, it is also possible that non-classical transcription factors are the responsible for these effects. For instance, it has recently been shown that Vimentin expression could be controlled by p63, since transfection experiments in which p63 is knockdown by iRNA results in the expression of Vimentin and other mesenchymal markers [165].

Ring1B knockdown derepresses E-cadherin expression in MDA-MB-231 cells.

Next we analyzed the levels of E-cadherin and Vimentin in MDA-MB-231 cells in similar conditions as indicated in the previous paragraph for MCF7. Importantly, E-cadherin is derrepressed when Ring1B is downregulated (figure R.18). This result, in addition to the

fact that any of the main transcription factors responsibles for E-cadherin repression were found to be upregulated by Ring1B in Tgf-β treated MDA-MB-231 cells, suggests that it is possible that in these very invasive cells E-cadherin is directly repressed by Ring1B. However additional experiments would be required to confirm this hypothesis.

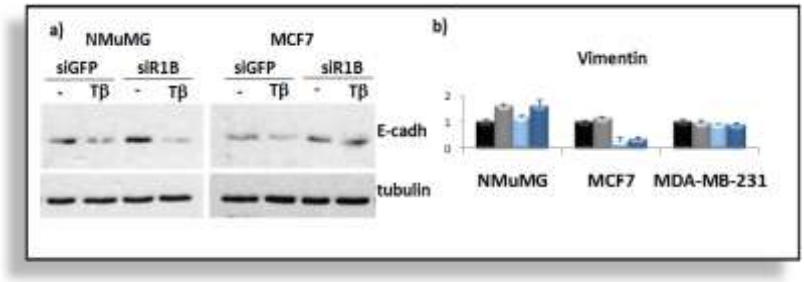


Figure R.18 Epithelial and mesenchymal expression markers in iRING1B or iMOCK oligofected cells treated or non-treated with Tgf-β3 a) E-cadherin expression in NMuMG and MCF7 cells detected by western blot **b)** Quantitative RT-PCR analysis of Vimentin in NMuMG, MCF7 and MDA-MB-231 cells.

Phenotypical changes in NMuMG, MCF7 and MDA-MB-231 depleted from Ring1B suggest the involvement of adhesion-related proteins.

Phenotypic changes in the four experimental conditions (control or Tgf-β3, with Mock or with iRing1B RNA) in the three cell lines (NMuMG, MCF7 and MDA-MB-231) have been shown above (figure R.13). In these pictures it can easily be noticed that the dark marks or dots in cytoplasm prolongations disappear when Ring1B is downregulated. It is easier to observe this effect in the MDA-MB-231 cell line, because in these cells, in control condition, these

marks can already be detected, whereas in the other two cell lines the dots appear upon Tgf-β treatment.

It is likely that these marks are related with focal contacts. Focal contacts are the sites of adhesion by which cells are attached to the underlying substrate, in this case the culture plate. These focal contacts are particularly important in migrating cells, which make adhesions at the front end and break these adhesions when they become redundant at the back.

5.2.5 Relationship between FAK and Ring1B upon Tgf-β3 treatment.

FAK protein expression is inhibited when Ring1B is downregulated in MCF7 and MDA-MB-231. In NMuMG cells there is a very slight downregulation.

The previous observation regarding the phenotype in the three cell lines tested suggests that proteins involved in cell plate anchorage could be implicated. Focal adhesion kinase (Fak) is a protein that has a central role in migration by controlling focal contacts, microtubule stabilization, stress fibers, lamelipodia, filopodia and cadherins [166].

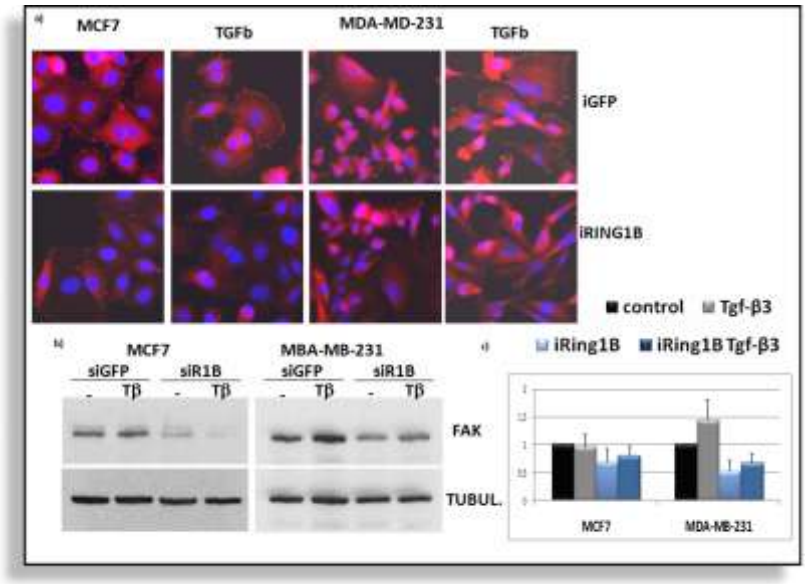


Figure R.19 FAK protein expression and mRNA levels in MCF7 and MDA-MB-231 oligofected with iRING1B or iMOCK and treated or non-treated with Tgf-β3 a) total FAK immunofluorescence in MCF7 and MDA-MB-231 detected by western blot **b)** FAK expression in MCF7 and MDA-MB-231, detected by western blot. **c)** qRT-PCR analysis of FAK expression in MCF7 and MDA-MB-231 cells.

Therefore, we checked Fak expression in the four experimental conditions by different approaches (immunofluorescence, western blot and qRT-PCR) and the results are shown in figure R.19. By performing immunofluorescence to detect Fak, we observed that when Ring1B expression is depleted, Fak expression is lost in the focal contacts. To accurately quantify Fak expression, we also performed western blot analysis and we observed that total Fak expression is lost in Ring1B knockdown cells. Therefore, Fak expression in MDA-MB-231 and MCF7 cells is downregulated when Ring1B is knocked down.

In NMuMG cells, Ring1B depletion results in a modest decrease in Fak protein and mRNA levels (data not shown)

We also checked the Fak mRNA levels in these cells, in order to investigate whether the Fak regulation was mediated by transcriptional or posttranscriptional mechanism, and in both MCF7 and MDA-MB-231 cells, Fak mRNA levels were significant decreased when Ring1B was downregulated (figure R.19).

pY861FAK is reduced in NMuMG-Tgf-β3-treated cells when Ring1B was downregulated.

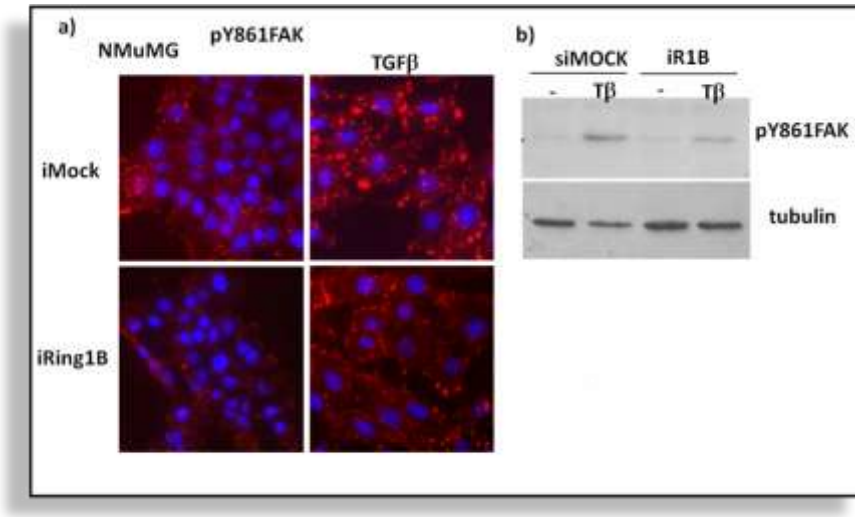


Figure R.20 Analysis of the phosphorylation status of the Fak protein in NMuMG cells oligofected with iRing1B or iMock and treated or non-treated with Tgf-β3 a) pY861Fak immynofluorescence in NMuMG cells **b)** Western blot analysis of pY861Fak in NMuMG iRing1B/Tgf-β3 treated cells

.

As Fak protein can be activated by phosphorylation at different sites and one of the residues phosphorylated in response to Tgf-β is Y861 [167], we hypothesized that differences in focal contacts and other Fak-mediated structures such as cytoplasmic prolongations

(lamelipodia and filopodia) in Tgf-β3-treated NMuMG cells with downregulated Ring1B could be ascribed to a defective phosphorylation in this residue, and not to changes at total FAK levels. As shown in figure R.20, Fak is phosphorylated in Y861 after Tgf-β3 treatment as expected, but this phosphorylation decreases when cells are oligofected with iRing1B. These results suggest that Ring1B could have a dual role controlling Fak activity: on the one hand controlling its expression and on the other hand regulating its phosphorylation.

5.2.6 Mechanisms involved in the regulation of Fak by Ring1B upon Tgf-β3 treatment.

p53 family members are good candidates to regulate the expression of Fak protein by Ring1B upon Tgf-β3 treatment.

FAK has been widely related to breast cancer. In fact Fak expression has been analyzed in ductal carcinoma *in situ* (DCIS), and its upregulation has been shown to be an early event in breast tumorigenesis [168] and to correlate with poor prognosis indicators [169].

Fak promoter contains two p53 binding sites and p53 is able to inhibit Fak transcription [170]. In addition, recent data show that FAK and p53 proteins can directly interact *in vitro* and *in vivo* [171].

Therefore, p53 was a putative candidate to mediate Fak regulation by Ring1B. In such scenario, Ring1B depletion would lead to the release of p53 expression, whose increase would result in the observed downregulation of Fak transcription. Against this

possibility is the data that only wild type, but not mutated p53 is able to bind Fak promoter [170] and MDA-MB-231 cells carry a p53 protein mutated in its DNA binding domain [172]. On the other hand, p63 and p73, the other two members of the p53 family have been described to recognize some of the promoters bound by p53, and can be good candidates to mediate Fak downregulation.

Δ-p63-α is repressed by Tgf-β and overexpressed when Ring1B is downregulated.

Therefore, we explored the hypothesis that p63 was the mediator used by Ring1B to modulate FAK expression. Figure R.21 shows, the western blot analysis of MDA-MB-231 cells when these cells were treated with Tgf-β3 and transfected with iRing1B. In this western blot the band that was more significant corresponds to Δ-p63-α .

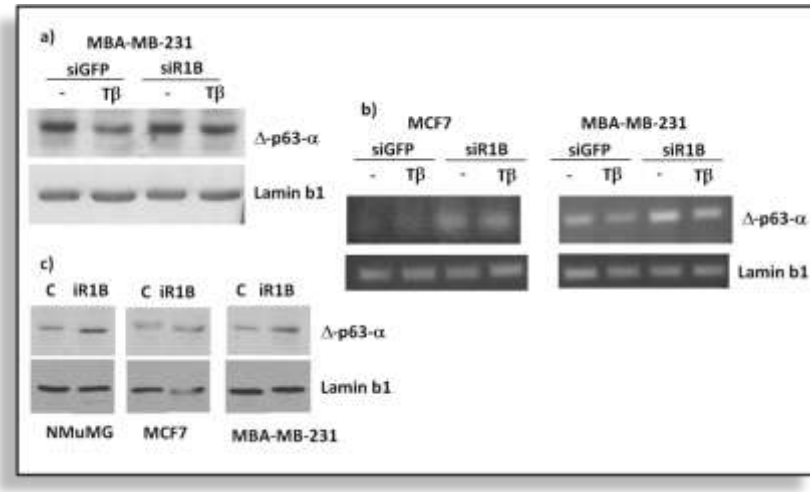


Figure R.21 Analysis of p63 expression in MCF7 and MDA-MB-231 cells oligofected with iRING1B or iMOCK and treated or non-treated with Tgf-β3 a) Western blot analysis of p63 expression in MDA-MB-231 cells. **b)** Semiquatitative RT-PCR of D-p63 expression in MCF7 and MDA-MB-231 cells. **c)** Δ-p63-α expression analysis in NMuMG, MCF7 and MDA-MB-231 cells after iRing1B oligofection by western blot.

As p63 has at least 6 different isoforms, to better confirm these results we performed independent RT-PCR with specific primers for Δ-p63 and TA-p63 isoforms in MCF7 and MDA-MB-231. As shown in figure R.21 in both cell lines Δ-p63 is upregulated when Ring1B is downregulated. In figure R.21c there is a summary of the expression of Δ-p63-α, when Ring1B is downregulated in the three different cell lines.

Δ-p63-α induces the downregulation of FAK protein in MDA-MB-231 and MCF7 cell lines.

Once we confirmed that Ring1B knockdown results in upregulation of Δ-p63-α, we investigated whether Δ-p63-α is able to repress Fak expression. To this end, MDA-MB-231 and MCF7 cells were transfected with pbabe-Δ-p63-α-HA or with an empty vector, and Fak protein levels were determinted by western blot (figure R.22). In both cell lines, when Δ-p63-α is overexpressed, Fak levels are decreased, supporting our working hypothesis that Δ-p63-α regulates FAK at least in our experimental conditions.

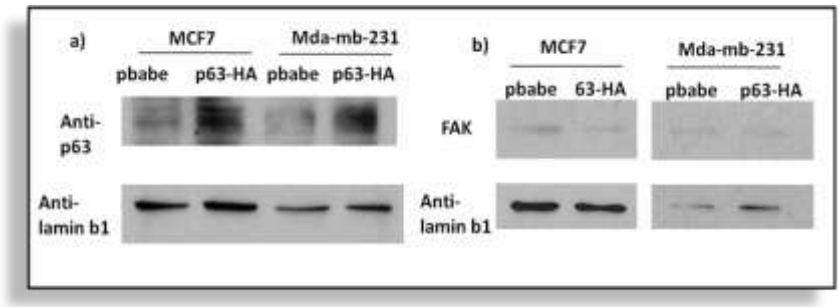


Figure R.22 Effect of Δ-p63-α overexpression on FAK expression in MCF7 and MDA-MB-231 cells a) Levels of p63 after in MCF7 and MDA-MB-231 cells transfected with empty verctor or with Δ-p63-α carrying vector. **b)** Western blot analysis of FAK expression in Δ-p63-α overexpressing in MCF7 and MDA-MB-231 cells.

Δ-p63 promoter is ubiquitinated.

Once we have identified the protein that could link Ring1B and Fak, we tried to investigate whether Δ-p63-α is a direct target of Ring1B by using as a read out the mark set by the enzymatic activity of Ring1B. Due to the lack of available Ub-H2A antibodies that properly work for chromatin immunoprecipitation (ChIP), the experimental approach was to transfect MCF7 c ells with a plasmid containing several repeats of Ubiquitin fused to the green fluorescence protein (GFP) gene. Then, anti-GFP antibody was used to perform a ChIP assay to pull down the Δ-p63 promoter. As figure R.23 shows, Ub-GFP is present at the Δ-p63 promoter, supporting the idea that Ring1B action could be mediated by Δ-p63.

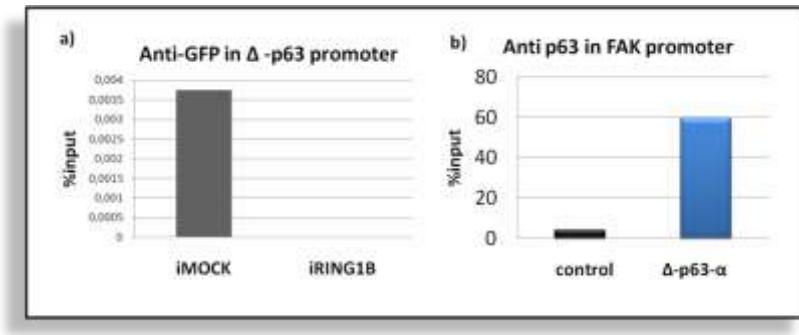


Figure R.23 Enrichment of the ubiquitin mark in Δ-p63-α promoter, and ability Δ-p63-α to be recruited to the Fak promoter. a) Levels of Ubiquitin recruited to the Δ-p63-α promoter in MCF7, determined by chromatin immunoprecipitation analysis. **b)** p63 recruitment to FAK promoter in MDA-MB-231, determined by chromatin immunoprecipitation using extracts from Δ-p63-α transfected MDA-MB-231 cells.

Δ-p63-α binds to FAK promoter.

Next we checked whether FAK regulation by Δ-p63-α is by direct binding of Δ-p63-α to the FAK promoter Therefore we performed a ChIP assay in MDA-MB-231 cells in which Δ-p63-α had been transiently overexpressed. As can be shown in figure R.23, ChIP analysis results demonstrate that Δ-p63-α is in fact able to bind to the FAK promoter.

5.3 Role of Ring1B in *in vivo* models of breast cancer metastasis. The Xenograft approach.

Downregulation of RING1B in intravenously injected MDA-MB-231 cells in SCID mice does not affect animal survival.

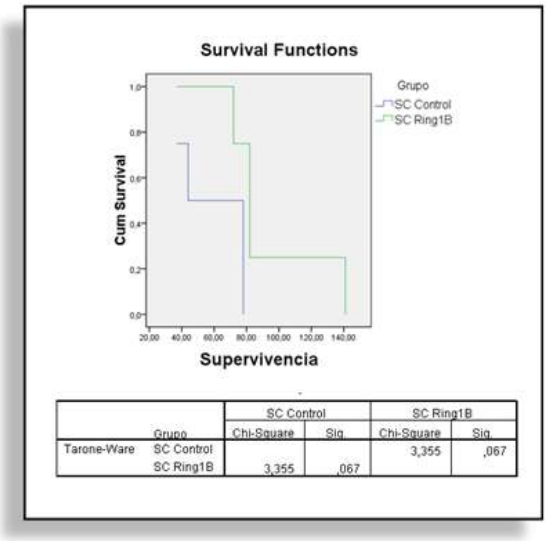
All the above exposed results suggest that RING1B protein could also play an important function in the migration and invasion of metastatic mammary epithelial cells. Then, our next goal was to analyze the role of RING1B in an *in vivo* model of metastasis.

In intravenously injected mice we wanted to study the second part of the metastasis, the stage in which cells have to survive in a non-attached manner, extravasate to a new tissue and be able to grow in this new tissue organ.

To this end, MDA-MB-231 cells were transfected with a retroviral vector carrying a short interference RNA (siRNA) for RING1B or a mock siRNA and the puromycin resistance gene, and selected with puromycin These cells were then intravenously injected into SCID mice, and these animals were maintained until final point criteria suggest the sacrifice. As figure R.24 shows, the survival curve for the animals intravenously injected with mock or with RING1B siRNA MDA-MB-231 cells did not significantly differ (data not shown). This data suggests that in the second part of the metastasis, the colonization, where cells abandon the blood and are able to growth in a different tissue, RING1B expression is not an advantage.

Downregulation of RING1B in subcutaneously injected MDA-MB-231 cells in SCID mice increases mice survival.

Then we proceeded to analyze what happens in the first part of the metastasis. By this reason we used the subcutaneously injection of MDA-MB-231 cells obtained in the same manner than as in the previous paragraph. In this model, MDA-MB-231 cells haves been shown to metastatize in normal conditions, and we want to study whether Ring1B depletion is able to reduce this metastasis.



		SC Control		SC Ring1B	
	Grupo	Chi-Square	Sig.	Chi-Square	Sig.
Tarone-Ware	SC Control SC Ring1B	3,355	,067	3,355	,067

Figure R.24 Survival experiment for subcutaneously injected mice. Four animals in each condition were injected with 10^6 MDA-MD-231 cells transfected with iRing1B or iMock. The animals were maintained until the final point criteria were achieved.

Survival curves from the mice subcutaneously injected with mock or RING1B siRNA MDA-MB-231 cells show that those mice injected with RING1B-depleted MDA-MB-231 cells display an enhanced survival (figure R.23). Moreover, most of the control animals in the necropsies show metastasis in distal organs, whereas most of the mice injected with the RING1B depleted cells have the tumor

localized only under the skin, suggesting that RING1B depletion impairs the first stages of MDA-MB-231 metastasis.

5.4 Expression pattern of p63, RING1B and FAK in ductal mammary gland cancer.

RING1B and p63 expression shows mutually exclusive pattern of expression in infiltrating ductal tumors from breast cancer patients and RING1B expression pattern correlates with FAK expression.

After the observation that RING1B is necessary to allow migration of transformed epithelial cells, likely mediated by FAK and p63, we investigated the expression of these proteins in high grade invasive ductal carcinoma, the dominant type of breast cancer (75% of cases).

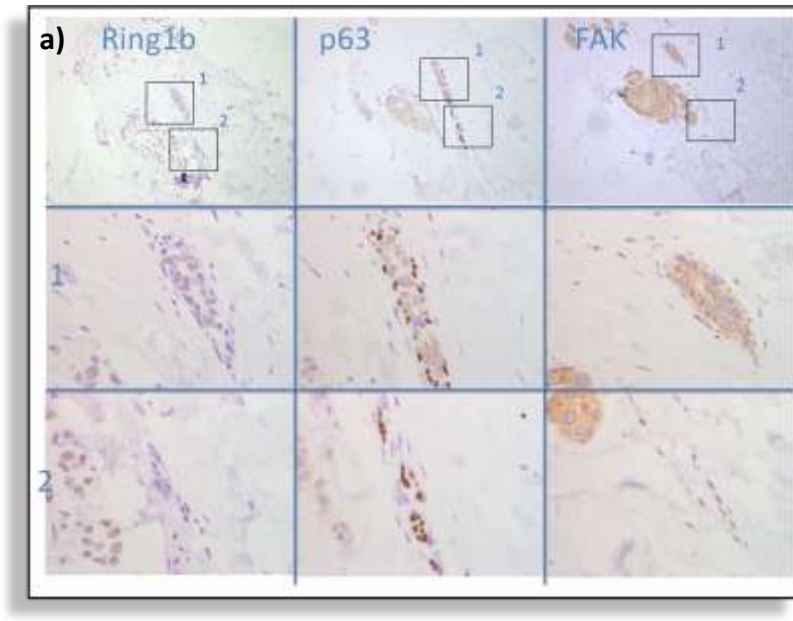
Previous studies of p63 in DCIS and infiltrating ductal carcinoma (IDC) suggest that p63 is expressed in myoepithelial cells forming a continuous peripheral border in the normal ducts, but this expression was less continuous in DCIS. Infiltrating ductal carcinoma shows occasional positive nuclear staining for p63 [173], but loss of p63 expression is the main marker of for the diagnoses of invasive breast cancer, On the other hand, presence of p63 positive cells in the most advanced grades of IDC suggest that p63 is a hallmark of very poorly differentiated cells in breast carcinoma [174].

FAK protein has been shown to be elevated in tumor tissues [175], and the underlying genetic mechanisms include copy number gains, amplification and isochromosome formation involving the FAK locus [176].

Regarding RING1B expression in DCIS and IDC, it has not been studied yet.

Our immunohistochemistry results (figure R.25) show an inverse pattern of expression of RING1B and p63 in myoepithelial cells. In normal ducts, as has been explained, p63 is expressed in all the myoepithelial cells, but RING1B expression was not detected. On the other hand, ducts that are beginning to lose the expression of p63 shows RING1B expression, suggesting that in these conditions, RING1B could be mediating the repression of p63.

In the ducts where p63 expression is lost and RING1B is expressed, FAK protein is also expressed. FAK expression correlates with RING1B expression also in those cells that are invading the surrounding fat pad. RING1B expression in these cells is very high, as well as FAK expression whereas we were unable to detect p63-positive cells.



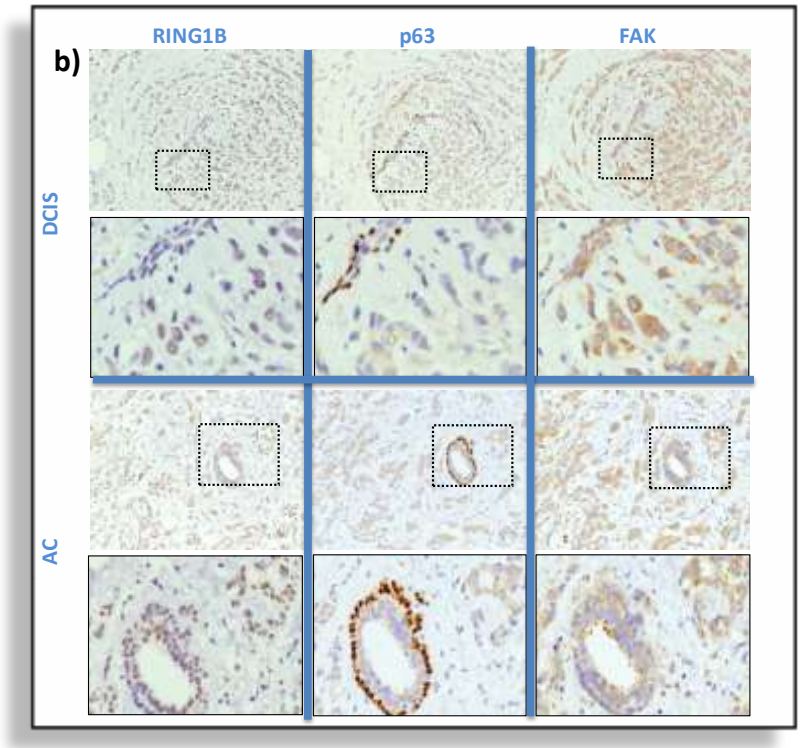


Figure R.25 Pattern of expression of RING1B, p63 and FAK protein in human DCIS.

6 DISCUSSION

From long time ago, the DNA has been extensively studied: the sequence, promoters, introns, exons, mutations, etc. But it has become clear that the sequence does not show all the information needed to understand the finely tuned gene regulation that takes place in an eukaryotic cell. Nowadays, a new vision of the DNA is emerging: it is a dynamic molecule which has to be understood in the context of the chromatin, in which the Polycomb-Trithorax equilibrium plays an important role. By this reason, epigenetic events are currently considered as important mechanisms which collaborate with genetic mutations in the human cancer initiation and progression.

The discovery of the relationship between the different epigenetic events, and the fact that they are reversible [177] may provide new targets for therapeutic intervention.

PcG proteins are implicated in cancer development [140, 178]. As it is explained in the introduction, some studies indicate that an increased expression level of some PcG proteins correlates with poor cancer prognosis, but the pathways that are involved in this upregulation and the meaning of it still remain unknown.

By this reason, we set up a cellular *in vitro* EMT model, because it is an appropriate way to follow the changes that can occur in specific stages of the tumor progression.

6.1 Ring1B regulation by Tgf-β

The main finding from our work is the upregulation of RING1B protein after Tgf-β treatment in three cell lines with different properties: the NMuMG cell line (non-transformed mammary epithelial cell line), the MCF7 cell line (mammary adenocarcinoma transformed but not invasive cell line) and the MDA-MD-231 cell line (a highly invasive mammary adenocarcinoma cell line).

In respect to the pathways involved in the modulation of Ring1B expression, our results suggest that Ring1B could be regulated in the Tgf-β model by Smad3, because inhibition of Smad3 function by Specific Inhibitor of Smad3 (SIS3) impairs Ring1B upregulation in Tgf-β3 NMuMG treated cells. However, this data could not be extrapolated to MCF7 and MDA-MB-231 cells, where SIS3 was not able to inhibit this upregulation. It is important to remark that there is an interesting difference in the regulation of RING1B among the three cell lines. In the case of NMuMG cell line, the only one that shows impaired Ring1B upregulation when Tgf-β is inhibited by SIS3, the regulation of Ring1B takes place both at mRNA as well as at protein level. In contrast, in MCF7 and MDA-MB-231 cells, Ring1B upregulation occurs only at the protein level. Then, Smad3 could be responsible for the regulation of Ring1B transcription, while additional pathways would be involved in the control of RING1B posttranscriptional mechanisms when mRNA levels are preserved. One explanation about this discrepancy between mRNA and protein levels in MCF7 and MDA-MB-231 cells treated with Tgf-β3 could be the presence of a recently discovered Internal Ribosome Entry Site (IRES) at the 5'UTR of RING1B RNA [179]. These authors suggest that this translational mechanism

happens due to the importance of proper RING1B levels, because minimal levels of Ub-H2A are necessary for cell survival. This fact suggests that Ring1B protein needs to be present under all circumstances, a requirement that could possibly be met by the presence of this IRES. However, the mechanisms that could trigger the translation of this mRNA by this means are not discussed in the cited article.

6.2 Involvement of Ring1B in epithelial and mesenchymal marker expression

At the level of epithelial/mesenchymal markers, the three cell lines show different implication of RING1B, suggesting that this protein must have different roles depending on tumor stage. In normal untransformed epithelial cells (NMuMG), where the levels of E-cadherin and Vimentin are those typically seen in epithelial cells, Ring1B does not seem to have any effect in the regulation of these markers upon Tgf-β treatment. In MCF7 cells, which are tumoral but not invasive, Ring1B is able to inhibit Vimentin expression, and opposes E-cadherin downregulation induced by Tgf-β. Finally, in MDA-MB-231, Tgf-β does not have any effect neither in E-CADHERIN nor in VIMENTING, but RING1B knock down is able to induce E-CADHERIN at mRNA level.

Since it is difficult to explain all these results together, I will try to discuss these discrepancies by focusing separately on each cell line, and trying to combine to the other cell lines when these results overlap.

6.2.1 Ring1B in Tgf-β-induced EMT in non-transformed NMuMG cells.

As mentioned above, in NMuMG cells, RING1B does not affect neither E-cadherin nor Vimentin expression. However, Slug [180], one of the transcription factor that has been shown to repress E-cadherin, seems to require Ring1B to be regulated by the Tgf-β-triggered response, whereas E-cadherin expression remains unaffected. Supporting the possibility of a Slug-independent E-cadherin regulation, Slug and E-cadherin are expressed together in several human breast cancer samples when detected by *in situ* hybridization [181]. Based on this data, it has been suggested that Slug in these tumors has a new role in tumor progression, participating in a semicohesive migration displayed by invasive structures [181]. This kind of migration is similar to keratinocyte migration during wound healing epithelialization *in vivo*, where large amounts of Slug and E-cadherin are expressed [182].

As was explained in the results, we hypothesized that the effect of Ring1B in Tgf-β induced Slug upregulation, could be mediated by Sim2s, as has been previously described [162]. Our results support this possibility, because Ring1B knock down is able to avoid the Tgf-β–induced repression of Sim2s.

To our knowledge, the binding of Ring1B to the Sim2s promoter has not been investigated. Therefore, to know if this binding effectively takes place in our model it would be interesting to perform a ChIP assay by using Ring1B or Ub-H2A antibodies, in order to detect a

direct Ring1B binding or at least an enrichment in the ubiquitination of the H2A at the Sim2 promoter, respectively.

In fact, very little is known about SIM2 regulation. It has been recently shown that SIM2 is regulated downstream of the RAS pathway and this regulation could be mediated by Notch and C/EBPβ in epithelial mammary gland cells [183], although direct binding of C/EBP or proteins of the Ras or the Notch pathways to the Sim2 promoter has not been shown, and authors also propose that other different mechanisms could be regulating Sim2 [183]. Then, one possibility is that Ring1B could be the effector of Ras pathway to repress Sim2s expression, because as it is shown in figure R.4c, Ring1B is also upregulated when the Ras is activated by Egf. However, since all of our results regarding Sim2 regulation were obtained using the Tgf-β model, an additional explanation would be required for Sim2 regulation, because it is not completely clear if Tgf-β is able to induce Ras pathway. The regulation of the Ras pathway by the Tgf-β is currently a controversial issue, likely due to differences in the experimental models used to investigate it. In addition, the relationship between Ras and Tgf-β pathways is complex because these two signalling pathways are intermingled: Tgf-β can activate RAS, and RAS can also regulate steps of the Smad signalling pathway [109], the main effector of Tgf-β. Furthermore, Tgf-β and RAS share some signalling pathways [184].

In addition, our results suggest that Ras is not the pathway responsible for the effect of Ring1B on Sim2 expression, because the results obtained in NMuMG cells could not be extrapolated to the MDA-MB-231 cells, a cell line known to have a constitutive

active Ras pathway due to a k-RAS mutation [185]. If Ras was mediating this process, then RING1B depletion in these cells should result in SIM2s repression and this is not happening, although it is possible that other proteins required for Sim2s reexpression are also deregulated in the MDA-MB-231 cell line would have to be affected, and it is not happening. Despite this, another possibility is that the mediator of Ras pathway that is implicated in SIM2s expression is also deregulated. Then, it is necessary to analyze which pathways are mediating SIM2 inhibition by Ring1B in the different cell lines. One approach could be transfecting NMuMG cells with an active form of Ras and analyzing whether the results obtained from the Tgf-β model concerning Ring1B and Sim2 are also happening by using this approach. Depending on the result obtained, specific or common pathways in Tgf-β and Ras pathways should be analyzed.

6.2.2 Ring1B in EMT and migration of transformed and non invasive MCF7 cells

In MCF7, RING1B depletion results in the downregulation of the mesenchymal marker VIMENTIN and when these RING1B-depleted cells are treated with Tgf-β, E-CADHERIN downregulation is impaired. Later on, and in order to investigate FAK regulation by RING1B, we proposed that RING1B could inhibit Δ-p63-α expression. Indeed, we demonstrate that RING1B is bound to Δ-p63-α promoter in Tgf-β treated cells, likely repressing its expression. We also show that ectopic expression of Δ-p63-α lends

to the repression of FAK that is directly target of Δ-p63-α, as shown by ChIP assay.

Taking together all these results, and since Δ-p63-α upregulation has been reported to inversely correlate with the expression of mesenchymal markers upon Tgf-β treatment [165] it is possible that Δ-p63-α–mediates the upregulation of mesenchymal markers by the Tgf-β-induced RING1B expression. On the other hand, it is also possible that FAK mediates the regulation of epithelial and mesenchymal markers in our system, since it is known that activated FAK is able to induce mesenchymal markers and to promote the degradation of E-CADHERIN [186]. Although these published data on Δ-p63-α and Fak gave new clues for understanding the regulation of proteins involved in EMT and migration, the specific mechanisms that mediate these processes have not been unveiled yet.

6.2.3 Ring1B in migration of the transformed and metastatic MDA-MB-231 cell line.

In MDA-MB-231, despite the results obtained concerning Δ-p63-α and FAK as RING1B downstream proteins are similar to that obtained in MCF7, the implication of RING1B in the regulation of the mesenchymal and epithelial markers seems to be different. Our data obtained from qRT-PCR mRNA analysis, indicates that VIMENTIN was not affected neither by RING1B downregulation nor by Tgf-β treatment. In contrast, when RING1B is depleted, E-CADHERIN expression is reactivated at the mRNA level.

At this point, it is important to emphasize that MDA-MB-231 is a cell line from a metastatic tumor and, in fact, these cells are very invasive. According with this phenotype, and as it is shown in the result section, Tgf-β does not affect the expression neither of VIMENTIN nor E-CADHERIN, suggesting that the mesenchymal phenotype is almost complete. Then, we can hypothesize that in this condition E-CADHERIN expression must be repressed by different mechanisms to that mediating E-cadherin repression in MCF7 or NMuMG cells upon Tgf-β treatment. In fact, it has been demonstrated that E-CADHERIN promoter is hypermethylated in MDA-MB-231 cells, but not in MCF7 [187]. In this respect, it has been shown that Bmi1, another member of the PRC1 complex cooperates with Dnmt1 in DNA methylation [188]. Bmi1 is able to form a complex with Dnmt1 and when Bmi1 is depleted, there is less binding of Dnmt1 to certain promoters, and interestingly there is also less Ub-H2A in this promoter. Since Ring1B is the protein responsible for the Ub-H2A mark and interacts with Bmi1 [189], these data suggest that Ring1B could be also implicated in the recruitment of Dnmt1 to these promoters. Despite our results show that E-cadherin is derepressed at mRNA level after Ring1B downregulation, the hypothesis that RING1B is important for the maintenance of the methylation status of E-cadherin promoter has not been explored yet in our laboratory, although we tested the levels of Dnmts in our iRing1B/Tgf-β model in MDA-MB-231 cells, and our preliminary results did not shown significant changes. Then, future work in this direction will be the analysis of the possible Ring1B regulation of the E-cadherin promoter in MDA-MB-

231, and to analyze the methylation status of the E-cadherin promoter after Ring1B depletion.

About the discrepancy in the regulation of the mesenchymal marker VIMENTIN between MCF7 and MDA-MB-231, we can hypothesize that at the invasive stage in which MDA-MB-231 cells are, additional mechanisms different to Δ-p63-α are required for the maintenance of the expression of the mesenchymal markers.

6.3 Ring1B regulates Fak expression

In contrast to the different regulation of the epithelial and mesenchymal markers depending on the cell line, we have obtained interesting and consistent results when Fak regulation was studied. Some of the aspects of Fak regulation have been discussed along the manuscript, but it is important to focus in this subject in more detail.

It has been previously described that Fak protein is upregulated in many different cancers including breast cancer [175, 190]. In addition, in ductal carcinoma *in situ* breast tumors it has been demonstrated that upregulation of Fak expression is an early event in breast tumorigenesis [168].

On the other hand, in Tgf-β induced EMT, Scr-mediated FAK activation is required for upregulation of mesenchymal markers and delocalization of E-cadherin [186].

Fak protein is important in **cell motility** [166, 191] and Fak overexpression is sufficient to induce cell migration [192]. The cell

migration induced by Fak requires protrusion at the leading edge of the cell, by formation of peripheral adhesions, exertion of force on these adhesions, and then the release of the adhesions at the rear of the cell [193]. In addition, Fak activation has been linked to **invasion and metastasis** signalling pathways, since inhibition of Fak in Fak-proficient invasive cancer cells prevented cell invasion and metastasis processes [194]. However Fak also plays a major role in **survival** signalling and has been linked to detachment-induced apoptosis or anoikis [195]. It has also been shown that constitutively activated forms of Fak rescued epithelial cells from anoikis, suggesting that Fak can regulate this process [195-197]. In addition Fak is able to respond to VEGF, an important factor in **angiogenesis**, promoting the formation of new blood vessels [198]. Last but not least, Fak is necessary to correct **branching morphogenesis in the mammary gland** [199].

Our functional assays show that RING1B downregulation in MCF7 cells avoids Tgf-β induced migration (wound healing) and invasion (transwell assay), and we observe that in these conditions FAK protein is downregulated. Although highly suggestive, these data do not assure that the reduction of migration and invasion is produced by FAK loss because, as it is shown, epithelial and mesenchymal markers are also regulated. To further confirm the role of FAK in these functional assays, it would be interesting to perform the same experiment, but restoring normal FAK levels.

Regarding the mechanism that RING1B would use to regulate FAK expression, we postulated the hypothesis that Ring1B would inhibit the expression of a p53 member, which in turn would repress FAK

expression. In fact, different members of the p53 family have been link to Ring1B and Fak independently.

In reference to the first part of our hypothesis, Ring1B represses a p53 member, it has been shown that Ring1B is able to bind to p73 promoter in mouse embryonic stem cells [200]. In the same study the binding of Ring1B to p63 promoter was also analyzed, but no significant results were shown. Since these data were obtained in mouse stem cells, it is very likely that the Ring1B direct targets are not the same as the Ring1B targets in other systems, whereas genes that are actively transcribed in stem cells, and therefore not bound by repressor in these cells, can be later on silenced by Ring1B binding or by other different means. In this respect, p63 has been demonstrated to be highly expressed in many stem cell from adult tissues, maintaining the pluripotency of these cells [201]. Then it is likely that p63 is expressed also in mouse embryonic stem cells, and then there is no Ring1B binding to its promoter. In that report, the expression of p63 was not analyzed.

Regarding the second part of the hypothesis, a p53 member inhibits Fak expression, it has been shown that p53 is able to bind to the Fak promoter in two different sites [170], and recently several studies about the other two memberships of p53 family, p63 and p73, show that these three proteins share functions and promoters [202]. The DNA binding specificity of p53, mediated by the DNA binding domain of the protein, is the major determinant of the spectrum of genes that p53 regulates [25, 203, 204]. Importantly, the DNA binding domain of p63 retains significant homology to that of p53, and p63 proteins can bind to p53

consensus DNA binding sites *in vitro* and *in vivo* [40, 205, 206]. However, the divergent biological roles of these two genes imply that they can also regulate a distinct subset of target genes. This paradox can be partially explained by the existence of a distinct p63 consensus DNA binding site, but this possibility remains unexplored until date.

As indicated in the results section, MDA-MB-231 cells have a mutation in the DNA binding site of p53 protein, and then this protein was not a good candidate for Fak regulation in our model. Despite this, we tested by western blot the levels of the three proteins of the p53 family (data not shown), but significant differences in the four tested conditions were obtained only for p63 expression.

Δ-p63-α is the predominant, if not the only, isoform expressed in epithelial cells [40, 207]. Previously reported data showed that Δ-p63-α could inhibit p53 or TA-p63 mediated transcription [34]. However, it was unclear whether this effect represents true transcription repression by Δ-p63-α, or if it was merely a dominant negative effect. Independent studies have found that Δ-p63-α may transactivate under certain conditions, but the majority of these studies employed ectopically expressed Δ-p63-α, and the physiologic relevance of such experimental approach is unclear [208-210].

The expression patterns of p63 have also provided important insights in p63 biological role. As determined by immunohistochemistry analysis, p63 is expressed by the cells of the

basal layers of stratified epithelial tissues, including mammary gland. [211]. This basal compartment of stratified epithelia is often considered to harbour cells of high proliferative capacity, which replenish the terminally differentiated populations in the more luminal strata [212]. Importantly, p63 is also highly expressed in cancers derived from these tissues [213].

p63 has been shown to be related with cell adhesion in mammary epithelial cells [214]. Downregulation of p63 in human myoepithelial cells or in mouse primary mammary epithelial cells induced cell detachment and subsequent apoptosis [215], similar to the effect that has been observed when Fak protein is upregulated [195]. Interestingly, this effect was specific to the downregulation of Δ-p63 isoforms. In addition, it has been reported that several genes implicated in cell adhesion processes are regulated by p63 [214].

Our results obtained by immunohistochemistry of infiltrating mammary ductal human tumors, reinforce the hypothesis of p63 being regulated by Ring1B. The expression pattern of these two proteins is mutually exclusive, suggesting a possible negative regulation between them. Despite this, additional studies are necessary to confirm the link between them and Fak. Since we have performed ChIP and western blot experiments by using p63-overexpressing cells, the results obtained indicate that p63 is able to bind and to repress FAK expression, but additional experiments must be carried out to investigate that this regulation effectively takes place under normal conditions, that is, in Tgf-β treated cells.

It could also be interesting to study this link in cells in which p63 or Ring1B are significantly expressed in standard conditions.

Finally, it is important to indicate that our group has recently linked Ring1B expression with pancreatic cancer progression [216]. Our results shows the different pattern of expression of Bmi1 and Ring1B in different stages of pancreatic cancers by immunohistochemistry and what we have demonstrated is that Bmi1 is expressed from the first stages of pancreatitis and pancreatic cancer while Ring1B is expressed in more advanced stages when pancreatic adenocarcinoma is highly metastatic.

In summary, our findings indicate that Ring1B expression is induced by Tgf-β and unveil a new role for Ring1B that is the regulation of Tgf-β-induced invasion and migration of non transformed and tumoral cells. To this end, it seems that Ring1b utilizes more than one mechanism, and the specific mechanisms regulated by Ring1B are cell type-dependent. That findings, combined with the fact that Ring1B depletion is able to inhibit the metastatic potential of MDA-MB-231, as our xenograft experiment shows, give an evidence of the function of Ring1B in metastasis that has not be shown before and establish also a possible therapeutic target.

Then, our findings give some light about the implication of a Polycomb group protein in tumor invasion and suggest that the expression of these proteins can be a useful tool in therapeutics besides their role as prognosis markers.

7 CONCLUSIONS.

- Hgf/Tgf-α treatment induces redistribution but not loss of epithelial markers in NMuMG cells.
- Tgf-β treatment induces EMT in NMuMG cells, marked by the loss of expression of epithelial markers like E-cadherin or the *de novo* expression of mesenchymal markers like Vimentin.
- Hgf/Tgf-α treatment induces a slight expression of Ring1B in NMuMG cells that does not correlate with increased enzymatic activity of Ring1B ubiquitinating K119 of the H2A.
- Tgf-β/Egf treatment induces the expression of Ring1B in NMuMG cells at mRNA and protein level. This upregulation correlates with increased K119-H2A ubiquitination.
- Both Hgf/Tgf-α and Egf/Tgf-β induces increased trimethylation of K27-H3.
- Ring1B knock down inhibits the upregulation of Slug and the downregulation of Id2 induced by Tgf-β2/Egf treatment in NMuMG cells, but the effect in Id2 is not observed when NMuMG cells are treated with Tgf-β3.
- Ring1B knock down inhibits the downregulation of SIM2s triggered by Tgf-β3 in NMuMG cells.
- Smad3 protein mediates the upregulation of Ring1B at mRNA but not at protein level.

- Ring1B is required for migration and invasion induced by Tgf-β3 in MCF7 cells.
- Tgf-β3 treatment induces de upregulation of FAK protein at mRNA and protein level in MCF7 and MDA-MB-231 cells.
- Ring1B knock down inhibits the expression of FAK protein in MCF7 and MDA-MB-231 cells.
- Ring1B knock down represses VIMENTIN in MCF7 cells.
- Ring1B knock down derepresses E-CADHERIN in MDA-MB-231 cells.
- Tgf-β3 treatment induces phosphorylation of Y861FAK in NMuMG cells.
- Ring1B knock down inhibits Y861FAK phosphorylation in NMuMG cells upon Tgf-β3 treatment.
- Δ-p63-α is repressed by Tgf-β3 and upregulated when Ring1B is depleted in MCF7 and MDA-MB-231 cells.
- Ectopic expression of Δ-p63-α induces the downregulation of FAK protein in MCF7 and MDA-MB-231 cells.
- Histones of the Δ-p63 promoter are ubiquitinated in MCF7 cells
- Δ-p63-α is able to bind to FAK promoter in MDA-MB-231 cells.
- Mice subcutaneously injected with RING1B-depleted MDA-MB-231 cells display enhanced survival than that ones with MDA-MB-231-iMOCK cells.
- RING1B depletion in MDA-MB-231 inhibits the invasive potential of these cells in subcutaneously injected mice.

- In infiltrating ductal tumors from breast cancer patients, RING1B and p63 expression are mutually exclusive and RING1B expression pattern correlates with FAK expression.

8 BIBLIOGRAPHY

1. Encyclopedia Britannica 2009 2009 [cited; Available from: http://www.britannica.com/EBchecked/topic/360922/mammary-gland.

2. Moinfar, F., *Essentials of diagnostic breast pathology: a practical approach.* 2007: Springer-Verlag. Berlin Heidelberg.

3. Fata, J.E., Z. Werb, and M.J. Bissell, *Regulation of mammary gland branching morphogenesis by the extracellular matrix and its remodeling enzymes.* Breast Cancer Res, 2004. **6**(1): p. 1-11.

4. Hebner, C., V.M. Weaver, and J. Debnath, *Modeling morphogenesis and oncogenesis in three-dimensional breast epithelial cultures.* Annu Rev Pathol, 2008. **3**: p. 313-39.

5. Wiseman, B.S. and Z. Werb, *Stromal effects on mammary gland development and breast cancer.* Science, 2002. **296**(5570): p. 1046-9.

6. Cowin, P., T.M. Rowlands, and S.J. Hatsell, *Cadherins and catenins in breast cancer.* Curr Opin Cell Biol, 2005. **17**(5): p. 499-508.

7. Sternlicht, M.D., *Key stages in mammary gland development: the cues that regulate ductal branching morphogenesis.* Breast Cancer Res, 2006. **8**(1): p. 201.

8. Sakakura, T., Y. Nishizuka, and C.J. Dawe, *Mesenchyme-dependent morphogenesis and epithelium-specific cytodifferentiation in mouse mammary gland.* Science, 1976. **194**(4272): p. 1439-41.

9. Mueller, S.O., et al., *Mammary gland development in adult mice requires epithelial and stromal estrogen receptor alpha.* Endocrinology, 2002. **143**(6): p. 2357-65.

10. Ewan, K.B., et al., *Latent transforming growth factor-beta activation in mammary gland: regulation by ovarian hormones affects ductal and alveolar proliferation.* Am J Pathol, 2002. **160**(6): p. 2081-93.

11. Silberstein, G.B., et al., *Regulation of mammary morphogenesis: evidence for extracellular matrix-mediated inhibition of ductal budding by transforming growth factor-beta 1.* Dev Biol, 1992. **152**(2): p. 354-62.

12. Niranjan, B., et al., *HGF/SF: a potent cytokine for mammary growth, morphogenesis and development.* Development, 1995. **121**(9): p. 2897-908.

13. Cheng, N., et al., *Loss of TGF-beta type II receptor in fibroblasts promotes mammary carcinoma growth and invasion through upregulation of TGF-alpha-, MSP- and HGF-mediated signaling networks.* Oncogene, 2005. **24**(32): p. 5053-68.

14. Kamalati, T., et al., *HGF/SF in mammary epithelial growth and morphogenesis: in vitro and in vivo models.* J Mammary Gland Biol Neoplasia, 1999. **4**(1): p. 69-77.

15. Citri, A. and Y. Yarden, *EGF-ERBB signalling: towards the systems level.* Nat Rev Mol Cell Biol, 2006. **7**(7): p. 505-16.

16. Yarden, Y., *The EGFR family and its ligands in human cancer. signalling mechanisms and therapeutic opportunities.* Eur J Cancer, 2001. **37 Suppl 4**: p. S3-8.

17. Luetteke, N.C., et al., *Targeted inactivation of the EGF and amphiregulin genes reveals distinct roles for EGF receptor ligands in mouse mammary gland development.* Development, 1999. **126**(12): p. 2739-50.

18. Sternlicht, M.D. and Z. Werb, *How matrix metalloproteinases regulate cell behavior.* Annu Rev Cell Dev Biol, 2001. **17**: p. 463-516.

19. Wiseman, B.S., et al., *Site-specific inductive and inhibitory activities of MMP-2 and MMP-3 orchestrate mammary gland branching morphogenesis.* J Cell Biol, 2003. **162**(6): p. 1123-33.

20. Gump, F.E., D. Kinne, and G.F. Schwartz, *Current treatment for lobular carcinoma in situ.* Ann Surg Oncol, 1998. **5**(1): p. 33-6.

21. Frykberg, E.R., *Lobular Carcinoma In Situ of the Breast.* Breast J, 1999. **5**(5): p. 296-303.

22. Tavassoli, F.A., Devilee, P., *Tumours of the Breast and Female Genital Organs*. 2003, International Agency for Research on Cancer (IARC) Press.

23. Visvader, J.E. and G.J. Lindeman, *Transcriptional regulators in mammary gland development and cancer.* Int J Biochem Cell Biol, 2003. **35**(7): p. 1034-51.

24. Lane, D.P. and L.V. Crawford, *T antigen is bound to a host protein in SV40-transformed cells.* Nature, 1979. **278**(5701): p. 261-3.

25. el-Deiry, W.S., et al., *Definition of a consensus binding site for p53.* Nat Genet, 1992. **1**(1): p. 45-9.

26. Lane, D.P., *Cancer. p53, guardian of the genome.* Nature, 1992. **358**(6381): p. 15-6.

27. Sigal, A. and V. Rotter, *Oncogenic mutations of the p53 tumor suppressor: the demons of the guardian of the genome.* Cancer Res, 2000. **60**(24): p. 6788-93.

28. Hussain, S.P. and C.C. Harris, *Molecular epidemiology and carcinogenesis: endogenous and exogenous carcinogens.* Mutat Res, 2000. **462**(2-3): p. 311-22.

29. Pharoah, P.D., N.E. Day, and C. Caldas, *Somatic mutations in the p53 gene and prognosis in breast cancer: a meta-analysis.* Br J Cancer, 1999. **80**(12): p. 1968-73.

30. Menendez, D., A. Inga, and M.A. Resnick, *The expanding universe of p53 targets.* Nat Rev Cancer, 2009. **9**(10): p. 724-37.

31. Moll, U.M., G. Riou, and A.J. Levine, *Two distinct mechanisms alter p53 in breast cancer: mutation and nuclear exclusion.* Proc Natl Acad Sci U S A, 1992. **89**(15): p. 7262-6.

32. Stankovic, T., et al., *ATM mutations and phenotypes in ataxia-telangiectasia families in the British Isles: expression of mutant ATM and the risk of leukemia, lymphoma, and breast cancer.* Am J Hum Genet, 1998. **62**(2): p. 334-45.

33. Adorno, M., et al., *A Mutant-p53/Smad complex opposes p63 to empower TGFbeta-induced metastasis.* Cell, 2009. **137**(1): p. 87-98.

34. Yang, A., et al., *p63, a p53 homolog at 3q27-29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities.* Mol Cell, 1998. **2**(3): p. 305-16.

35. Kaghad, M., et al., *Monoallelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers.* Cell, 1997. **90**(4): p. 809-19.

36. Mills, A.A., et al., *p63 is a p53 homologue required for limb and epidermal morphogenesis.* Nature, 1999. **398**(6729): p. 708-13.

37. Yang, A., et al., *p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development.* Nature, 1999. **398**(6729): p. 714-8.

38. Yang, A., et al., *p73-deficient mice have neurological, pheromonal and inflammatory defects but lack spontaneous tumours.* Nature, 2000. **404**(6773): p. 99-103.

39. Sniezek, J.C., et al., *Dominant negative p63 isoform expression in head and neck squamous cell carcinoma.* Laryngoscope, 2004. **114**(12): p. 2063-72.

40. Westfall, M.D., et al., *The Delta Np63 alpha phosphoprotein binds the p21 and 14-3-3 sigma promoters in vivo and has transcriptional repressor activity that is reduced by Hay-Wells syndrome-derived mutations.* Mol Cell Biol, 2003. **23**(7): p. 2264-76.

41. Yang, A., et al., *On the shoulders of giants: p63, p73 and the rise of p53.* Trends Genet, 2002. **18**(2): p. 90-5.

42. Flores, E.R., et al., *Tumor predisposition in mice mutant for p63 and p73: evidence for broader tumor suppressor functions for the p53 family.* Cancer Cell, 2005. **7**(4): p. 363-73.

43. Wu, G., et al., *DeltaNp63alpha and TAp63alpha regulate transcription of genes with distinct biological functions in cancer and development.* Cancer Res, 2003. **63**(10): p. 2351-7.

44. Spiesbach, K., et al., *TAp63gamma can substitute for p53 in inducing expression of the maspin tumor suppressor.* Int J Cancer, 2005. **114**(4): p. 555-62.

45. Ghioni, P., et al., *Complex transcriptional effects of p63 isoforms: identification of novel activation and repression domains.* Mol Cell Biol, 2002. **22**(24): p. 8659-68.

46. Kirschner, R.D., et al., *Transcriptional activation of the tumor suppressor and differentiation gene S100A2 by a novel p63-binding site.* Nucleic Acids Res, 2008. **36**(9): p. 2969-80.

47. Yamaguchi, K., et al., *Frequent gain of the p40/p51/p63 gene locus in primary head and neck squamous cell carcinoma.* Int J Cancer, 2000. **86**(5): p. 684-9.

48. Hu, H., et al., *Elevated expression of p63 protein in human esophageal squamous cell carcinomas.* Int J Cancer, 2002. **102**(6): p. 580-3.

49. Park, B.J., et al., *Frequent alteration of p63 expression in human primary bladder carcinomas.* Cancer Res, 2000. **60**(13): p. 3370-4.

50. Patturajan, M., et al., *DeltaNp63 induces beta-catenin nuclear accumulation and signaling.* Cancer Cell, 2002. **1**(4): p. 369-79.

51. Rocco, J.W., et al., *p63 mediates survival in squamous cell carcinoma by suppression of p73-dependent apoptosis.* Cancer Cell, 2006. **9**(1): p. 45-56.

52. Koga, F., et al., *Impaired p63 expression associates with poor prognosis and uroplakin III expression in invasive urothelial carcinoma of the bladder.* Clin Cancer Res, 2003. **9**(15): p. 5501-7.

53. Wang, X., et al., *p63 expression in normal, hyperplastic and malignant breast tissues.* Breast Cancer, 2002. **9**(3): p. 216-9.

54. Massion, P.P., et al., *Significance of p63 amplification and overexpression in lung cancer development and prognosis.* Cancer Res, 2003. **63**(21): p. 7113-21.

55. Urist, M.J., et al., *Loss of p63 expression is associated with tumor progression in bladder cancer.* Am J Pathol, 2002. **161**(4): p. 1199-206.

56. Barbieri, C.E., et al., *Loss of p63 leads to increased cell migration and up-regulation of genes involved in invasion and metastasis.* Cancer Res, 2006. **66**(15): p. 7589-97.

57. Zhang, H., et al., *BRCA1 physically associates with p53 and stimulates its transcriptional activity.* Oncogene, 1998. **16**(13): p. 1713-21.

58. Bell, D.W., et al., *Heterozygous germ line hCHK2 mutations in Li-Fraumeni syndrome.* Science, 1999. **286**(5449): p. 2528-31.

59. Greenblatt, M.S., et al., *TP53 mutations in breast cancer associated with BRCA1 or BRCA2 germ-line mutations: distinctive spectrum and structural distribution.* Cancer Res, 2001. **61**(10): p. 4092-7.

60. Turner, N.C. and J.S. Reis-Filho, *Basal-like breast cancer and the BRCA1 phenotype.* Oncogene, 2006. **25**(43): p. 5846-53.

61. Moynahan, M.E., et al., *Brca1 controls homology-directed DNA repair.* Mol Cell, 1999. **4**(4): p. 511-8.

62. Liu, S., et al., *BRCA1 regulates human mammary stem/progenitor cell fate.* Proc Natl Acad Sci U S A, 2008. **105**(5): p. 1680-5.

63. Roy, P.G. and A.M. Thompson, *Cyclin D1 and breast cancer.* Breast, 2006. **15**(6): p. 718-27.

64. Gearhart, J., E.E. Pashos, and M.K. Prasad, *Pluripotency redux--advances in stem-cell research.* N Engl J Med, 2007. **357**(15): p. 1469-72.

65. Nesbit, C.E., J.M. Tersak, and E.V. Prochownik, *MYC oncogenes and human neoplastic disease.* Oncogene, 1999. **18**(19): p. 3004-16.

66. Efstratiadis, A., M. Szabolcs, and A. Klinakis, *Notch, Myc and breast cancer.* Cell Cycle, 2007. **6**(4): p. 418-29.

67. Radtke, F. and K. Raj, *The role of Notch in tumorigenesis: oncogene or tumour suppressor?* Nat Rev Cancer, 2003. **3**(10): p. 756-67.

68. Dievart, A., N. Beaulieu, and P. Jolicoeur, *Involvement of Notch1 in the development of mouse mammary tumors.* Oncogene, 1999. **18**(44): p. 5973-81.

69. Imatani, A. and R. Callahan, *Identification of a novel NOTCH-4/INT-3 RNA species encoding an activated gene product in certain human tumor cell lines.* Oncogene, 2000. **19**(2): p. 223-31.

70. Soriano, J.V., et al., *Expression of an activated Notch4(int-3) oncoprotein disrupts morphogenesis and induces an invasive phenotype in mammary epithelial cells in vitro.* Int J Cancer, 2000. **86**(5): p. 652-9.

71. Stylianou, S., R.B. Clarke, and K. Brennan, *Aberrant activation of notch signaling in human breast cancer.* Cancer Res, 2006. **66**(3): p. 1517-25.

72. Reedijk, M., et al., *High-level coexpression of JAG1 and NOTCH1 is observed in human breast cancer and is associated with poor overall survival.* Cancer Res, 2005. **65**(18): p. 8530-7.

73. Weigelt, B., J.L. Peterse, and L.J. van 't Veer, *Breast cancer metastasis: markers and models.* Nat Rev Cancer, 2005. **5**(8): p. 591-602.

74. Morrow, M.O., C.K., *Diseases of the breast*. 3rd ed. 2004: Philadephia.

75. Pinder, S.E., et al., *Pathological prognostic factors in breast cancer. III. Vascular invasion: relationship with recurrence and survival in a large study with long-term follow-up.* Histopathology, 1994. **24**(1): p. 41-7.

76. de Mascarel, I., et al., *Obvious peritumoral emboli: an elusive prognostic factor reappraised. Multivariate analysis of 1320 node-negative breast cancers.* Eur J Cancer, 1998. **34**(1): p. 58-65.

77. McGuire, W.L. and G.M. Clark, *Prognostic factors and treatment decisions in axillary-node-negative breast cancer.* N Engl J Med, 1992. **326**(26): p. 1756-61.

78. van 't Veer, L.J., et al., *Gene expression profiling predicts clinical outcome of breast cancer.* Nature, 2002. **415**(6871): p. 530-6.

79. van de Vijver, M.J., et al., *A gene-expression signature as a predictor of survival in breast cancer.* N Engl J Med, 2002. **347**(25): p. 1999-2009.

80. Thiery, J.P., *Epithelial-mesenchymal transitions in tumour progression.* Nat Rev Cancer, 2002. **2**(6): p. 442-54.

81. Grunert, S., M. Jechlinger, and H. Beug, *Diverse cellular and molecular mechanisms contribute to epithelial plasticity and metastasis.* Nat Rev Mol Cell Biol, 2003. **4**(8): p. 657-65.

82. Huber, M.A., N. Kraut, and H. Beug, *Molecular requirements for epithelial-mesenchymal transition during tumor progression.* Curr Opin Cell Biol, 2005. **17**(5): p. 548-58.

83. Thiery, J.P. and J.P. Sleeman, *Complex networks orchestrate epithelial-mesenchymal transitions.* Nat Rev Mol Cell Biol, 2006. **7**(2): p. 131-42.

84. Nakamura, T., et al., *Molecular cloning and expression of human hepatocyte growth factor.* Nature, 1989. **342**(6248): p. 440-3.

85. Zhang, Y.W. and G.F. Vande Woude, *HGF/SF-met signaling in the control of branching morphogenesis and invasion.* J Cell Biochem, 2003. **88**(2): p. 408-17.

86. Grotegut, S., et al., *Hepatocyte growth factor induces cell scattering through MAPK/Egr-1-mediated upregulation of Snail.* Embo J, 2006. **25**(15): p. 3534-45.

87. Andermarcher, E., M.A. Surani, and E. Gherardi, *Co-expression of the HGF/SF and c-met genes during early mouse embryogenesis precedes reciprocal expression in adjacent tissues during organogenesis.* Dev Genet, 1996. **18**(3): p. 254-66.

88. Yao, Y., et al., *Scatter factor protein levels in human breast cancers: clinicopathological and biological correlations.* Am J Pathol, 1996. **149**(5): p. 1707-17.

89. Birchmeier, C. and E. Gherardi, *Developmental roles of HGF/SF and its receptor, the c-Met tyrosine kinase.* Trends Cell Biol, 1998. **8**(10): p. 404-10.

90. Rosario, M. and W. Birchmeier, *How to make tubes: signaling by the Met receptor tyrosine kinase.* Trends Cell Biol, 2003. **13**(6): p. 328-35.

91. Royal, I., et al., *Activation of cdc42, rac, PAK, and rho-kinase in response to hepatocyte growth factor differentially regulates epithelial cell colony spreading and dissociation.* Mol Biol Cell, 2000. **11**(5): p. 1709-25.

92. Ponzetto, C., et al., *A multifunctional docking site mediates signaling and transformation by the hepatocyte growth factor/scatter factor receptor family.* Cell, 1994. **77**(2): p. 261-71.

93. Pollack, A.L., R.B. Runyan, and K.E. Mostov, *Morphogenetic mechanisms of epithelial tubulogenesis: MDCK cell polarity is transiently rearranged without loss of cell-cell contact during scatter factor/hepatocyte growth factor-induced tubulogenesis.* Dev Biol, 1998. **204**(1): p. 64-79.

94. Moustakas, A., et al., *Mechanisms of TGF-beta signaling in regulation of cell growth and differentiation.* Immunol Lett, 2002. **82**(1-2): p. 85-91.

95. Wells, A., *EGF receptor.* Int J Biochem Cell Biol, 1999. **31**(6): p. 637-43.

96. Derynck, R. and Y.E. Zhang, *Smad-dependent and Smad-independent pathways in TGF-beta family signalling.* Nature, 2003. **425**(6958): p. 577-84.

97. Xu, J., S. Lamouille, and R. Derynck, *TGF-beta-induced epithelial to mesenchymal transition.* Cell Res, 2009. **19**(2): p. 156-72.

98. Nieto, M.A., *The snail superfamily of zinc-finger transcription factors.* Nat Rev Mol Cell Biol, 2002. **3**(3): p. 155-66.

99. Batlle, E., et al., *The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells.* Nat Cell Biol, 2000. **2**(2): p. 84-9.

100. Hoot, K.E., et al., *Keratinocyte-specific Smad2 ablation results in increased epithelial-mesenchymal transition during skin cancer formation and progression.* J Clin Invest, 2008. **118**(8): p. 2722-32.

101. Barrallo-Gimeno, A. and M.A. Nieto, *The Snail genes as inducers of cell movement and survival: implications in development and cancer.* Development, 2005. **132**(14): p. 3151-61.

102. Ohkubo, T. and M. Ozawa, *The transcription factor Snail downregulates the tight junction components independently of E-cadherin downregulation.* J Cell Sci, 2004. **117**(Pt 9): p. 1675-85.

103. Shirakihara, T., M. Saitoh, and K. Miyazono, *Differential regulation of epithelial and mesenchymal markers by deltaEF1 proteins in epithelial mesenchymal transition induced by TGF-beta.* Mol Biol Cell, 2007. **18**(9): p. 3533-44.

104. Postigo, A.A., et al., *Regulation of Smad signaling through a differential recruitment of coactivators and corepressors by ZEB proteins.* Embo J, 2003. **22**(10): p. 2453-62.

105. Peinado, H., D. Olmeda, and A. Cano, *Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype?* Nat Rev Cancer, 2007. **7**(6): p. 415-28.

106. Grooteclaes, M.L. and S.M. Frisch, *Evidence for a function of CtBP in epithelial gene regulation and anoikis.* Oncogene, 2000. **19**(33): p. 3823-8.

107. Massari, M.E. and C. Murre, *Helix-loop-helix proteins: regulators of transcription in eucaryotic organisms.* Mol Cell Biol, 2000. **20**(2): p. 429-40.

108. Valcourt, U., et al., *TGF-beta and the Smad signaling pathway support transcriptomic reprogramming during epithelial-mesenchymal cell transition.* Mol Biol Cell, 2005. **16**(4): p. 1987-2002.

109. Massague, J., *How cells read TGF-beta signals.* Nat Rev Mol Cell Biol, 2000. **1**(3): p. 169-78.

110. Yu, L., M.C. Hebert, and Y.E. Zhang, *TGF-beta receptor-activated p38 MAP kinase mediates Smad-independent TGF-beta responses.* Embo J, 2002. **21**(14): p. 3749-59.

111. Bhowmick, N.A., et al., *Integrin beta 1 signaling is necessary for transforming growth factor-beta activation of p38MAPK and epithelial plasticity.* J Biol Chem, 2001. **276**(50): p. 46707-13.

112. Bishop, A.L. and A. Hall, *Rho GTPases and their effector proteins.* Biochem J, 2000. **348 Pt 2**: p. 241-55.

113. Edlund, S., et al., *Transforming growth factor-beta-induced mobilization of actin cytoskeleton requires signaling by small GTPases Cdc42 and RhoA.* Mol Biol Cell, 2002. **13**(3): p. 902-14.

114. Bakin, A.V., et al., *Phosphatidylinositol 3-kinase function is required for transforming growth factor beta-mediated*

epithelial to mesenchymal transition and cell migration. J Biol Chem, 2000. **275**(47): p. 36803-10.

115. Vinals, F. and J. Pouyssegur, *Transforming growth factor beta1 (TGF-beta1) promotes endothelial cell survival during in vitro angiogenesis via an autocrine mechanism implicating TGF-alpha signaling.* Mol Cell Biol, 2001. **21**(21): p. 7218-30.

116. Allfrey, V.G., R. Faulkner, and A.E. Mirsky, *Acetylation and Methylation of Histones and Their Possible Role in the Regulation of Rna Synthesis.* Proc Natl Acad Sci U S A, 1964. **51**: p. 786-94.

117. Riggs, A.D., *X inactivation, differentiation, and DNA methylation.* Cytogenet Cell Genet, 1975. **14**(1): p. 9-25.

118. Holliday, R. and J.E. Pugh, *DNA modification mechanisms and gene activity during development.* Science, 1975. **187**(4173): p. 226-32.

119. Hernandez-Munoz, I., et al., *Association of BMI1 with polycomb bodies is dynamic and requires PRC2/EZH2 and the maintenance DNA methyltransferase DNMT1.* Mol Cell Biol, 2005. **25**(24): p. 11047-58.

120. Bird, A.P., *Functions for DNA methylation in vertebrates.* Cold Spring Harb Symp Quant Biol, 1993. **58**: p. 281-5.

121. Baylin, S.B. and J.G. Herman, *DNA hypermethylation in tumorigenesis: epigenetics joins genetics.* Trends Genet, 2000. **16**(4): p. 168-74.

122. Bestor, T.H., *Activation of mammalian DNA methyltransferase by cleavage of a Zn binding regulatory domain.* Embo J, 1992. **11**(7): p. 2611-7.

123. Pradhan, S., et al., *Recombinant human DNA (cytosine-5) methyltransferase. I. Expression, purification, and comparison of de novo and maintenance methylation.* J Biol Chem, 1999. **274**(46): p. 33002-10.

124. Okano, M., S. Xie, and E. Li, *Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransferases.* Nat Genet, 1998. **19**(3): p. 219-20.

125. Robertson, K.D., et al., *The human DNA methyltransferases (DNMTs) 1, 3a and 3b: coordinate mRNA expression in normal tissues and overexpression in tumors.* Nucleic Acids Res, 1999. **27**(11): p. 2291-8.

126. Karpf, A.R. and S. Matsui, *Genetic disruption of cytosine DNA methyltransferase enzymes induces chromosomal instability in human cancer cells.* Cancer Res, 2005. **65**(19): p. 8635-9.

127. Khorasanizadeh, S., *The nucleosome: from genomic organization to genomic regulation.* Cell, 2004. **116**(2): p. 259-72.

128. Lund, A.H. and M. van Lohuizen, *Epigenetics and cancer.* Genes Dev, 2004. **18**(19): p. 2315-35.

129. Strahl, B.D. and C.D. Allis, *The language of covalent histone modifications.* Nature, 2000. **403**(6765): p. 41-5.

130. Bantignies, F. and G. Cavalli, *Cellular memory and dynamic regulation of polycomb group proteins.* Curr Opin Cell Biol, 2006. **18**(3): p. 275-83.

131. Qiu, J., *Epigenetics: unfinished symphony.* Nature, 2006. **441**(7090): p. 143-5.

132. Vire, E., et al., *The Polycomb group protein EZH2 directly controls DNA methylation.* Nature, 2006. **439**(7078): p. 871-4.

133. Cao, R., et al., *Role of histone H3 lysine 27 methylation in Polycomb-group silencing.* Science, 2002. **298**(5595): p. 1039-43.

134. Simon, J.A. and J.W. Tamkun, *Programming off and on states in chromatin: mechanisms of Polycomb and trithorax group complexes.* Curr Opin Genet Dev, 2002. **12**(2): p. 210-8.

135. Satijn, D.P., et al., *Interference with the expression of a novel human polycomb protein, hPc2, results in cellular transformation and apoptosis.* Mol Cell Biol, 1997. **17**(10): p. 6076-86.

136. Spivakov, M. and A.G. Fisher, *Epigenetic signatures of stem-cell identity.* Nat Rev Genet, 2007. **8**(4): p. 263-271.

137. Haupt, Y., et al., *Novel zinc finger gene implicated as myc collaborator by retrovirally accelerated lymphomagenesis in E mu-myc transgenic mice.* Cell, 1991. **65**(5): p. 753-63.

138. van Lohuizen, M., et al., *Identification of cooperating oncogenes in E mu-myc transgenic mice by provirus tagging.* Cell, 1991. **65**(5): p. 737-52.

139. Sherr, C.J., *The INK4a/ARF network in tumour suppression.* Nat Rev Mol Cell Biol, 2001. **2**(10): p. 731-7.

140. Sparmann, A. and M. van Lohuizen, *Polycomb silencers control cell fate, development and cancer.* Nat Rev Cancer, 2006. **6**(11): p. 846-56.

141. Varambally, S., et al., *The polycomb group protein EZH2 is involved in progression of prostate cancer.* Nature, 2002. **419**(6907): p. 624-9.

142. Kleer, C.G., et al., *EZH2 is a marker of aggressive breast cancer and promotes neoplastic transformation of breast epithelial cells.* Proc Natl Acad Sci U S A, 2003. **100**(20): p. 11606-11.

143. Pasini, D., A.P. Bracken, and K. Helin, *Polycomb group proteins in cell cycle progression and cancer.* Cell Cycle, 2004. **3**(4): p. 396-400.

144. Reynolds, P.A., et al., *Tumor suppressor p16INK4A regulates polycomb-mediated DNA hypermethylation in human mammary epithelial cells.* J Biol Chem, 2006. **281**(34): p. 24790-802.

145. Puppe, J., et al., *BRCA1-deficient mammary tumor cells are dependent on EZH2 expression and sensitive to Polycomb*

Repressive Complex 2-inhibitor 3-deazaneplanocin A. Breast Cancer Res, 2009. **11**(4): p. R63.

146. Gonzalez, M.E., et al., *Downregulation of EZH2 decreases growth of estrogen receptor-negative invasive breast carcinoma and requires BRCA1.* Oncogene, 2009. **28**(6): p. 843-53.

147. Pietersen, A.M., et al., *EZH2 and BMI1 inversely correlate with prognosis and TP53 mutation in breast cancer.* Breast Cancer Res, 2008. **10**(6): p. R109.

148. Ince, T.A., et al., *Transformation of different human breast epithelial cell types leads to distinct tumor phenotypes.* Cancer Cell, 2007. **12**(2): p. 160-70.

149. Hoenerhoff, M.J., et al., *BMI1 cooperates with H-RAS to induce an aggressive breast cancer phenotype with brain metastases.* Oncogene, 2009. **28**(34): p. 3022-32.

150. Jinnin, M., H. Ihn, and K. Tamaki, *Characterization of SIS3, a novel specific inhibitor of Smad3, and its effect on transforming growth factor-beta1-induced extracellular matrix expression.* Mol Pharmacol, 2006. **69**(2): p. 597-607.

151. Atsuta, T., et al., *Production of monoclonal antibodies against mammalian Ring1B proteins.* Hybridoma, 2001. **20**(1): p. 43-6.

152. Sanchez, C., et al., *Proteomics analysis of Ring1B/Rnf2 interactors identifies a novel complex with the Fbxl10/Jhdm1B histone demethylase and the Bcl6 interacting corepressor.* Mol Cell Proteomics, 2007. **6**(5): p. 820-34.

153. Wang, H., et al., *Role of histone H2A ubiquitination in Polycomb silencing.* Nature, 2004. **431**(7010): p. 873-8.

154. Leroy, P. and K.E. Mostov, *Slug is required for cell survival during partial epithelial-mesenchymal transition of HGF-induced tubulogenesis.* Mol Biol Cell, 2007. **18**(5): p. 1943-52.

155. Nogai, H., et al., *Follistatin antagonizes transforming growth factor-beta3-induced epithelial-mesenchymal transition in vitro: implications for murine palatal development supported by microarray analysis.* Differentiation, 2008. **76**(4): p. 404-16.

156. Gu, Y.Z., J.B. Hogenesch, and C.A. Bradfield, *The PAS superfamily: sensors of environmental and developmental signals.* Annu Rev Pharmacol Toxicol, 2000. **40**: p. 519-61.

157. Moffett, P., et al., *Characterization of msim, a murine homologue of the Drosophila sim transcription factor.* Genomics, 1996. **35**(1): p. 144-55.

158. Boker, L.K. and J. Merrick, *Cancer incidence in persons with Down syndrome in Israel.* Downs Syndr Res Pract, 2002. **8**(1): p. 31-6.

159. Metz, R.P., et al., *Differential transcriptional regulation by mouse single-minded 2s.* J Biol Chem, 2006. **281**(16): p. 10839-48.

160. Deyoung, M.P., et al., *Down's syndrome-associated single minded gene as a novel tumor marker.* Anticancer Res, 2002. **22**(6A): p. 3149-57.

161. Kwak, H.I., et al., *Inhibition of breast cancer growth and invasion by single-minded 2s.* Carcinogenesis, 2007. **28**(2): p. 259-66.

162. Laffin, B., et al., *Loss of singleminded-2s in the mouse mammary gland induces an epithelial-mesenchymal transition associated with up-regulation of slug and matrix metalloprotease 2.* Mol Cell Biol, 2008. **28**(6): p. 1936-46.

163. Lee, J.M., et al., *The epithelial-mesenchymal transition: new insights in signaling, development, and disease.* J Cell Biol, 2006. **172**(7): p. 973-81.

164. Hajra, K.M., D.Y. Chen, and E.R. Fearon, *The SLUG zinc-finger protein represses E-cadherin in breast cancer.* Cancer Res, 2002. **62**(6): p. 1613-8.

165. Hu, M., et al., *Regulation of in situ to invasive breast carcinoma transition.* Cancer Cell, 2008. **13**(5): p. 394-406.

166. Hanks, S.K., et al., *Focal adhesion kinase signaling activities and their implications in the control of cell survival and motility.* Front Biosci, 2003. **8**: p. d982-96.

167. Calalb, M.B., T.R. Polte, and S.K. Hanks, *Tyrosine phosphorylation of focal adhesion kinase at sites in the catalytic domain regulates kinase activity: a role for Src family kinases.* Mol Cell Biol, 1995. **15**(2): p. 954-63.

168. Lightfoot, H.M., Jr., et al., *Upregulation of focal adhesion kinase (FAK) expression in ductal carcinoma in situ (DCIS) is an early event in breast tumorigenesis.* Breast Cancer Res Treat, 2004. **88**(2): p. 109-16.

169. Lark, A.L., et al., *High focal adhesion kinase expression in invasive breast carcinomas is associated with an aggressive phenotype.* Mod Pathol, 2005. **18**(10): p. 1289-94.

170. Golubovskaya, V.M., et al., *p53 regulates FAK expression in human tumor cells.* Mol Carcinog, 2008. **47**(5): p. 373-82.

171. Smith, C.S., et al., *Effect of focal adhesion kinase (FAK) downregulation with FAK antisense oligonucleotides and 5-fluorouracil on the viability of melanoma cell lines.* Melanoma Res, 2005. **15**(5): p. 357-62.

172. Olivier, M., et al., *The IARC TP53 database: new online mutation analysis and recommendations to users.* Hum Mutat, 2002. **19**(6): p. 607-14.

173. Shamloula, M.M., S.H. El-Shorbagy, and E.M. Saied, *P63 and cytokeratin8/18 expression in breast, atypical ductal hyperplasia, ductal carcinoma in situ and invasive duct carcinoma.* J Egypt Natl Canc Inst, 2007. **19**(3): p. 202-10.

174. Ribeiro-Silva, A., et al., *The relationship between p63 and p53 expression in normal and neoplastic breast tissue.* Arch Pathol Lab Med, 2003. **127**(3): p. 336-40.

175. Owens, L.V., et al., *Overexpression of the focal adhesion kinase (p125FAK) in invasive human tumors.* Cancer Res, 1995. **55**(13): p. 2752-5.

176. Agochiya, M., et al., *Increased dosage and amplification of the focal adhesion kinase gene in human cancer cells.* Oncogene, 1999. **18**(41): p. 5646-53.

177. Egger, G., et al., *Epigenetics in human disease and prospects for epigenetic therapy.* Nature, 2004. **429**(6990): p. 457-63.

178. Azuara, V., et al., *Chromatin signatures of pluripotent cell lines.* Nat Cell Biol, 2006. **8**(5): p. 532-8.

179. Boutsma, E., S. Noback, and M. van Lohuizen, *The Polycomb protein and E3 ubiquitin ligase Ring1B harbors an IRES in its highly conserved 5' UTR.* PLoS ONE, 2008. **3**(6): p. e2322.

180. Cano, A., et al., *The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression.* Nat Cell Biol, 2000. **2**(2): p. 76-83.

181. Come, C., et al., *Snail and slug play distinct roles during breast carcinoma progression.* Clin Cancer Res, 2006. **12**(18): p. 5395-402.

182. Savagner, P., et al., *Developmental transcription factor slug is required for effective re-epithelialization by adult keratinocytes.* J Cell Physiol, 2005. **202**(3): p. 858-66.

183. Gustafson, T.L., et al., *Ha-Ras transformation of MCF10A cells leads to repression of Singleminded-2s through NOTCH and C/EBPbeta.* Oncogene, 2009. **28**(12): p. 1561-8.

184. Grande, M., et al., *Transforming growth factor-beta and epidermal growth factor synergistically stimulate epithelial to mesenchymal transition (EMT) through a MEK-dependent mechanism in primary cultured pig thyrocytes.* J Cell Sci, 2002. **115**(Pt 22): p. 4227-36.

185. von Lintig, F.C., et al., *Ras activation in human breast cancer.* Breast Cancer Res Treat, 2000. **62**(1): p. 51-62.

186. Cicchini, C., et al., *TGFbeta-induced EMT requires focal adhesion kinase (FAK) signaling.* Exp Cell Res, 2008. **314**(1): p. 143-52.

187. Graff, J.R., et al., *E-cadherin expression is silenced by DNA hypermethylation in human breast and prostate carcinomas.* Cancer Res, 1995. **55**(22): p. 5195-9.

188. Negishi, M., et al., *Bmi1 cooperates with Dnmt1-associated protein 1 in gene silencing.* Biochem Biophys Res Commun, 2007. **353**(4): p. 992-8.

189. Buchwald, G., et al., *Structure and E3-ligase activity of the Ring-Ring complex of polycomb proteins Bmi1 and Ring1b.* Embo J, 2006. **25**(11): p. 2465-74.

190. Cance, W.G., et al., *Immunohistochemical analyses of focal adhesion kinase expression in benign and malignant human breast and colon tissues: correlation with preinvasive and invasive phenotypes.* Clin Cancer Res, 2000. **6**(6): p. 2417-23.

191. Hauck, C.R., et al., *Inhibition of focal adhesion kinase expression or activity disrupts epidermal growth factor-stimulated signaling promoting the migration of invasive human carcinoma cells.* Cancer Res, 2001. **61**(19): p. 7079-90.

192. Hildebrand, J.D., M.D. Schaller, and J.T. Parsons, *Identification of sequences required for the efficient localization of the focal adhesion kinase, pp125FAK, to cellular focal adhesions.* J Cell Biol, 1993. **123**(4): p. 993-1005.

193. Tilghman, R.W., et al., *Focal adhesion kinase is required for the spatial organization of the leading edge in migrating cells.* J Cell Sci, 2005. **118**(Pt 12): p. 2613-23.

194. Benlimame, N., et al., *FAK signaling is critical for ErbB-2/ErbB-3 receptor cooperation for oncogenic transformation and invasion.* J Cell Biol, 2005. **171**(3): p. 505-16.

195. Frisch, S.M., et al., *Control of adhesion-dependent cell survival by focal adhesion kinase.* J Cell Biol, 1996. **134**(3): p. 793-9.

196. Frisch, S.M. and R.A. Screaton, *Anoikis mechanisms.* Curr Opin Cell Biol, 2001. **13**(5): p. 555-62.

197. Frisch, S.M., *Evidence for a function of death-receptor-related, death-domain-containing proteins in anoikis.* Curr Biol, 1999. **9**(18): p. 1047-9.

198. Abedi, H. and I. Zachary, *Vascular endothelial growth factor stimulates tyrosine phosphorylation and recruitment to new focal adhesions of focal adhesion kinase and paxillin in endothelial cells.* J Biol Chem, 1997. **272**(24): p. 15442-51.

199. van Miltenburg, M.H., et al., *Complete focal adhesion kinase deficiency in the mammary gland causes ductal dilation and aberrant branching morphogenesis through defects in Rho kinase-dependent cell contractility.* Faseb J, 2009. **23**(10): p. 3482-93.

200. van der Stoop, P., et al., *Ubiquitin E3 ligase Ring1b/Rnf2 of polycomb repressive complex 1 contributes to stable maintenance of mouse embryonic stem cells.* PLoS One, 2008. **3**(5): p. e2235.

201. McKeon, F., *p63 and the epithelial stem cell: more than status quo?* Genes Dev, 2004. **18**(5): p. 465-9.

202. Harms, K., S. Nozell, and X. Chen, *The common and distinct target genes of the p53 family transcription factors.* Cell Mol Life Sci, 2004. **61**(7-8): p. 822-42.

203. Kern, S.E., et al., *Identification of p53 as a sequence-specific DNA-binding protein.* Science, 1991. **252**(5013): p. 1708-11.

204. Pietenpol, J.A., et al., *Sequence-specific transcriptional activation is essential for growth suppression by p53.* Proc Natl Acad Sci U S A, 1994. **91**(6): p. 1998-2002.

205. Flores, E.R., et al., *p63 and p73 are required for p53-dependent apoptosis in response to DNA damage.* Nature, 2002. **416**(6880): p. 560-4.

206. Djelloul, S., et al., *Differential protein expression, DNA binding and interaction with SV40 large tumour antigen implicate the p63-family of proteins in replicative senescence.* Oncogene, 2002. **21**(7): p. 981-9.

207. Nylander, K., P.J. Coates, and P.A. Hall, *Characterization of the expression pattern of p63 alpha and delta Np63 alpha in benign and malignant oral epithelial lesions.* Int J Cancer, 2000. **87**(3): p. 368-72.

208. Dohn, M., S. Zhang, and X. Chen, *p63alpha and DeltaNp63alpha can induce cell cycle arrest and apoptosis and differentially regulate p53 target genes.* Oncogene, 2001. **20**(25): p. 3193-205.

209. Dohn, M., J. Jiang, and X. Chen, *Receptor tyrosine kinase EphA2 is regulated by p53-family proteins and induces apoptosis.* Oncogene, 2001. **20**(45): p. 6503-15.

210. Wu, G., et al., *DeltaNp63alpha up-regulates the Hsp70 gene in human cancer.* Cancer Res, 2005. **65**(3): p. 758-66.

211. Di Como, C.J., et al., *p63 expression profiles in human normal and tumor tissues.* Clin Cancer Res, 2002. **8**(2): p. 494-501.

212. Pellegrini, G., et al., *p63 identifies keratinocyte stem cells.* Proc Natl Acad Sci U S A, 2001. **98**(6): p. 3156-61.

213. Nylander, K., et al., *Differential expression of p63 isoforms in normal tissues and neoplastic cells.* J Pathol, 2002. **198**(4): p. 417-27.

214. Carroll, D.K., J.S. Brugge, and L.D. Attardi, *p63, cell adhesion and survival.* Cell Cycle, 2007. **6**(3): p. 255-61.

215. Perez, C.A., et al., *p63 consensus DNA-binding site: identification, analysis and application into a p63MH algorithm.* Oncogene, 2007. **26**(52): p. 7363-70.

216. Martinez-Romero, C., et al., *The epigenetic regulators Bmi1 and Ring1B are differentially regulated in pancreatitis and pancreatic ductal adenocarcinoma.* J Pathol, 2009. **219**(2): p. 205-13.

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