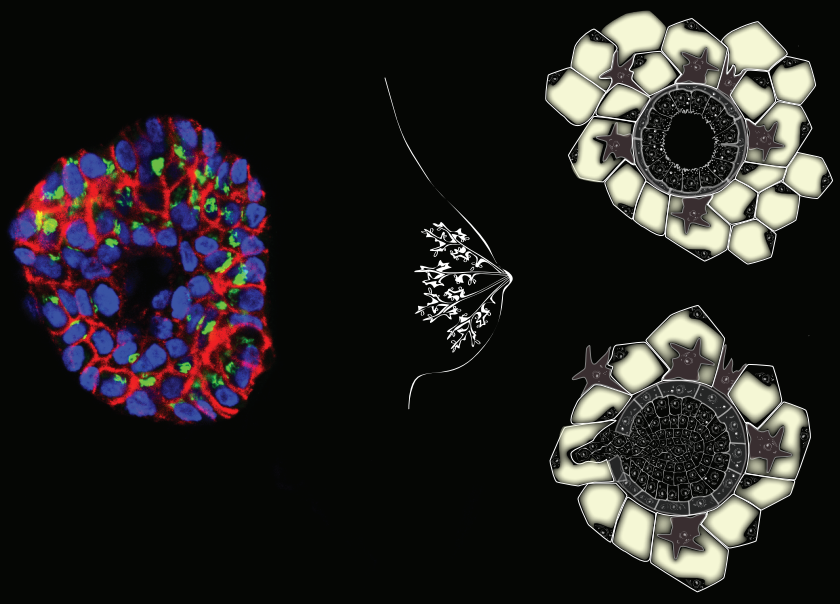
Tumor Suppressor Functions of Epithelial Integrity Genes in Breast Cancer



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Helsinki 2018

UNIVERSITY OF HELSINKI
FACULTY OF MEDICINE

Tumor Suppressor Functions of Epithelial Integrity Genes in Breast Cancer

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ACADEMIC DISSERTATION

To be publicly discussed, with the permission of the Faculty of Medicine, University of Helsinki, in Biomedicum Helsinki, Lecture Hall 3, on August 16th at 12 o'clock noon.

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"Words have no power to impress the mind

without the exquisite horror of their reality."

Edgar Alan Poe

This book is dedicated to…

My loving sister Lina Sofia de Oliveira Marques

10.8.1975 – 3.6.2008

My partner and love of my life Aarno Keimo Kristian Villa

13.6.1981 – 2.6.2018

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ORIGINAL PUBLICATIONS

The following publications are on the basis of the thesis and are cited using the roman numerals.

I – Par6G suppresses cell proliferation and is targeted by loss-of-function mutations in multiple cancers.

Marques E, Englund JI, Tervonen TA, Virkunen E, Laakso M, Myllynen M, Mäkelä A, Ahvenainen M, Lepikhova T, Monni O, Hautaniemi S, Klefström J.

Oncogene (2016) Mar; 35 (11): 1386-98

II – Myc requires RhoA/SRF to reprogram glutamine metabolism.

Haikala HM, **Marques E**, Turunen M, Klefström J.

Small GTPases. (2016) Aug; 1-9

III – Phenotype-driven identification of epithelial signaling clusters.

Marques E, Peltonen,T, Kaski S, Klefström J.

Scientific Reports (2018) Mar; 8 (1): 4034 – 4047

IV – Suppression of early hematogenous dissemination of human breast cancer cells to bone marrow by retinoic Acid-induced 2.

Werner S, Brors B, Eick J, **Marques E**, Pogenberg V, Parret A, Kemming D, Wood AW, Edgren H, Neubauer H, Streichert T, Riethdorf S, Bedi U, Baccelli I, Jücker M, Eils R, Fehm T, Trumpp A, Johnsen SA, Klefström J, Wilmanns M, Müller V, Pantel K, Wikman H.

Cancer Discovery (2015) May; 5 (5): 506-519

RELATED PUBLICATIONS

The following original publications resulted from work done during the thesis time period.

I – Faulty epithelial polarity genes and cancer.

T. Tervonen, J. Partanen, S. Saarikoski, M. Myllynen, E. Marques, K. Paasonen, A. Moilanen, P. Kovanen**, J. Klefstrom*

Advance Cancer Research (2011); 111: 97-161

II – Deregulated hepsin protease activity confers oncogenicity by concomitantly augmenting HGF/MET signalling and disrupting epithelial cohesion.

Tervonen TA, Belitškin D, Pant SM, Englund JI, Marques E, Ala-Hongisto H, Nevalaita L, Sihto H, Heikkilä P, Leidenius M, Hewitson K, Ramachandra M, Moilanen A, Joensuu H, Kovanen PE, Poso A, Klefström J.

Oncogene (2016) Apr; 35 (14): 1832-1846

III – Lentiviral shRNA Screen to Identify Epithelial Integrity Regulating Genes in MCF10A 3D Culture.

Marques E; Klefström J

Bio-protocol (2016) December; 6 (23): e2050

IV – Pharmacological reactivation of MYC-dependent apoptosis induces susceptibility to anti-PD1 immunotherapy.

Haikala HM, Anttila J*, Marques E*, Tiina Raatikainen*, Bjoern Von Eyss, Diego Balboa, Pauliina Munne, Hanna Ala-Hongisto, Vilja Eskelinen, Anni Viheriäranta, Timo Otonkoski, Julia Schüler, Teemu D. Laajala, Tero Aittokallio, Päivi Heikkilä, Marjut Leidenius, Harri Sihto, Heikki Joensuu, Panu Kovanen, Martin Eilers, Juha Klefström

Nature Communications (Under revision)

ABBREVIATIONS

α-KG	Alpha-ketoglutarate
2D	Two-dimensional
3D	Three-dimensional
AJ	Adherens junction
AKT	Protein kinase B (PKB)
ANOVA	Analysis of variance statistical test
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
aPKC	Atypical protein kinase C
ARF	Alternative reading frame
ATP	Adenosine triphosphate
bHLHZip	Basic helix-loop-helix-leucine zipper
BM	Basement membrane
BRCA1/2	Breast cancer gene 1/2
Cdc42	Cell and division cycle 42
CDK	Cyclin-dependent kinase
CRB	Crumbs polarity complex
CRIB	Cdc42 and Rac interactive domain
CtBP	Carboxyl-terminal binding protein
CTC	Circulating tumor cell
DCIS	Ductal carcinoma in situ
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTC	Disseminated tumor cell
E-box	Enhancer box
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial-to-mesenchymal transition
ER	Estrogen receptor
ERK	Extracellular signal-regulated kinase
ERB2	HER2 coding gene
FAK	Focal adhesion kinase
FDR	False discovery rate
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FOXO3a	Forkhead box 3a
G0	Cell cycle resting phase
G1	Cell cycle Gap 1
G2	Cell cycle Gap 2
GEMM	Genetically engineered mouse model
GLUT1	Glucose transporter 1
GLUL	Glutamine synthetase
GLS	Glutaminase

GH	Growth hormone
GSK3β	Glycogen synthase kinase 3β
GTP	Guanine triphosphate
HER2	Human epidermal growth factor receptor 2
HSCs	Hematopoietic stem cells
IGF	Insulin-like growth factor
ILK	Integrin-linked kinase
JAM	Junctional adhesion molecules
LKB1/STK11	Liver kinase B1/Serine-threonine kinase 11
LOH	Loss of heterozygosity
MAP	Microtubule-associated protein
MaSC	common mammary stem cell
MARK	Microtubule affinity regulated kinase
MDCK	Madin-Darby canine kidney cell
Mdm2	Mouse double minute-2
MMD	Maximum mean discrepancy statistical test
mTOR	Mammalian target of rapamycin
MSG	Metastasis-suppressor gene
MUPP	Multi PDZ-domain protein
NF-kB	Nuclear factor-kappa B
PALS1	Protein associated with lin 7 1
PAR	Partitioning defective
PATJ	PALS1-associated tight junction protein
PB1	Phox-Bem1p domain
PCR	Polymerase chain reaction
PDZ	PSD-95, Discs large, ZO-1 domain
PI	Propidium Iodide
PI3K	Phosphatidyl-inositol 3 kinase
PIP	Phosphatidyl-inositol
PR	Progesterone receptor
Prl	Prolactin
PTEN	Phosphatase and tensin homolog
RAI-2	Retinoic Acid-Induced 2
RKHS	Reproducing kernel Hilbert space
RhoA	Ras homolog gene family member A (RhoA)
ROCK	RhoA kinase (Rho-associated protein kinase)
SCRIB	Scribble polarity complex
Stat5a	Signal transducer and activator of transcription 5a
STRAD	Ste20-related adaptor protein
TEB	Terminal end bud
TGF-β1	Transforming growth factor β1
TJ	Tight junction
TNF	Tumor necrosis factor
TRAIL	TNF-related apoptosis inducing ligand
TSG	Tumor suppressor gene
ZO	Zona occludens

ABSTRACT

Epithelial integrity defines the well-established positioning and communication of the epithelial cells within the tissue architecture, which is necessary for the epithelium to flawlessly perform its functions. Loss of tissue integrity is a hallmark of practically all advanced solid cancers. Tissue integrity can be lost by altered cell-cell, cell-matrix connections, and/or mislocalization of the proteins responsible for the establishment of cell polarity. It has been postulated that the abnormal proliferative behavior observed in cancer cells is not only a consequence but also requires loss of epithelial integrity. Early studies in *Caenorhabditis elegans* and *Drosophila melanogaster* have found evidence of the tumor suppression role of polarity regulating genes. In vertebrates, the role of polarity genes in cancer development is less clear, with only few polarity genes convincingly linked to tumorigenesis in animal cancer models. The general aim of the study was to clarify the role of epithelial integrity regulating genes in breast cancer development. The study primarily focused on cell cycle and epithelial architecture alterations induced by the shRNA-mediated silencing of putative epithelial integrity regulating genes. The targeted genes were selected based on available *Drosophila* genes knowledgebases. By exploiting the advantages of the three-dimensional (3D) cell cultures, which mimic the natural cell–cell and cell–extracellular matrix interactions, combined with genes knockdown and *MYC* oncogene activation, the study was able to identify novel putative tumor suppressor functions of genes regulating epithelial integrity. Specifically, the study unraveled a *PARD6G* tumor suppressor function linked to the modulation of the AKT signaling pathway, critical for cancer development. This study also exposed an unexpected synthetic lethality between loss of *RHOA* (small GTPase) and active *MYC*. The performed experiments indicate that the observed apoptosis is a consequence of an altered glutamine metabolism, important for the survival of cell with active *MYC*. Furthermore, a novel statistical framework was developed to efficiently analyze phenotypic data resulting from genetic screens performed in 3D cultures. Finally, the genetic profiling of patients' derived breast cancer samples resulted in the identification of a genetic signature for tumors with disseminated tumor cells (DTC). Amongst this genetic signature, low *RAI-2* expression was also correlated to patients' poor prognosis. Further *in vitro* studies exposed a *RAI-2* link to epithelial integrity regulation.

In conclusion, this study successfully identified novel human epithelial integrity regulators in breast cancer, thereby opening potential therapeutic avenues for *MYC*-driven tumors and revealing a novel biomarker for early metastasis in breast cancer.

REVIEW OF THE LITERATURE

MAMMARY EPITHELIUM

The mammary epithelium dramatically differs from other epithelial tissues due to its phased developmental process, which include several hormone-driven micro- and macro-anatomy remodeling processes occurring throughout mammals' adult life. Most of this tissue development occurs after puberty reaching full maturation only upon pregnancy and subsequent lactation. Once lactation is no further sustained, tissue structure regresses to the pre-pregnancy resting state. Mammary gland plasticity implies a tight hormonal regulation of proliferation, apoptosis and of the overall epithelial architecture, paramount for a healthy (tumor free) and functional gland.

The overall mammary gland structure, developmental stages and signaling networks are highly conserved amongst mammals (Parmar and Cunha 2004, Visvader 2009). A significant amount of information regarding the mammary gland normal development and neoplastic conversion has been acquired from natural or induced mutations in mice (genetically engineered mouse models, GEMM). These studies in mice have exposed several key molecules and signaling networks that coordinate mammary gland plasticity (Hennighausen and Robinson 2005). It is worth to mention that some differences exist between mouse and human mammary gland structure. Mouse mammary gland are richer in adipocytes and present less fibrous connective tissue when compared to the human mammary gland. This particular difference is crucial when studying how the tissue density affects tumorigenesis related events (Boyd et al. 2010, Howard and Lu 2014, Sung et al. 2018). Another important difference, is that in a nulliparous (never pregnant) situation the human mammary gland, contrary to mouse, already includes glandular tissue forming lobular structures. These lobules are formed predominantly by estrogen receptor (ER) and progesterone receptor (PR) negative cells combined with few isolated ER and PR positive luminal cells. Noteworthy, rare lobules found exclusively in human, can be predominantly formed by ER+ and PR+ luminal cells (Figure 1). This indicates that even though most signaling networks are conserved between mouse and human, different responses to hormonal signaling may occur and should be taken into account when translating experimental data from mouse to humans (Visvader 2009, Carroll et al. 2017).

Structure and cellular composition

The mammary epithelium is by definition an apocrine gland, since it is formed by glandular epithelium responsible for the excretion of milk. The tissue is completed by the presence of an extracellular matrix (ECM), which mainly consists in adipocytes and stromal cells surrounding the secretory cells (Richert et al. 2000, Polyak and Kalluri 2010). The overall structure of the mammary gland can be described as a network of ducts that lead to the nipple, allowing the secretion of milk by the end of pregnancy. The increasing complexity of the ductal network is a key characteristic of the different developmental stages, which are primarily regulated by hormones (Figure 1).

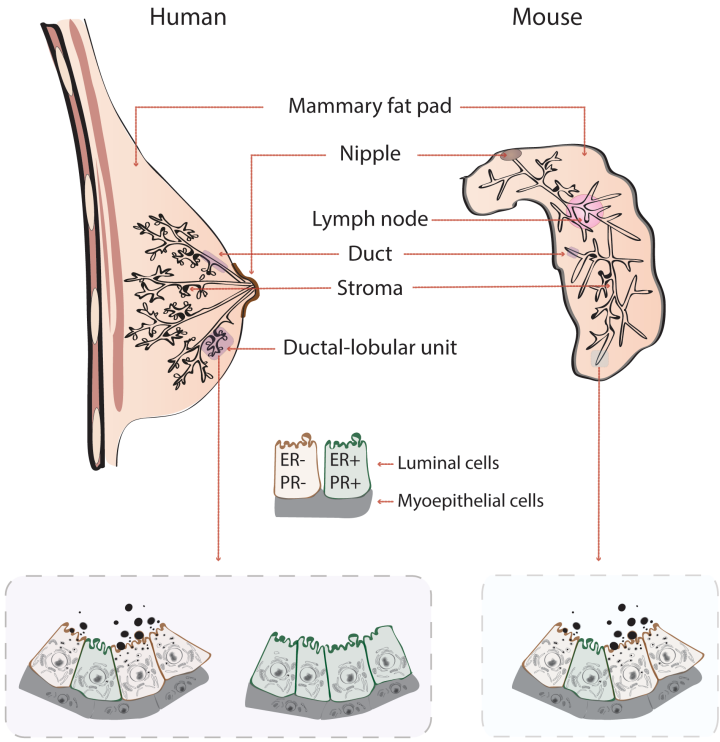

Figure 1 – Mammary gland structure is highly conserved amongst species. Representation of one of the main differences between human and mouse gland: the lack of ductal-lobular units in a nulliparous situation and the absence of ER+/PR+ alveoli units in mice. Note that, upon pregnancy, ER-/PR- cells stimulated by ER+ paracrine signals, are responsible for the production of milk (Visvader 2009, Carroll et al. 2017).

During the embryonic development, embryo ectoderm forms the mammary line and placodes that define the localization of nipples and mammary gland. Placodes are formed by multilayered clusters of columnar-shaped cells, which develop via cell migration and not by cell proliferation (Hinck and Silberstein 2005). Epithelial and mesenchymal cells interact and signal the placodes to migrate under the mesenchyme and initiate the formation

of the rudimentary ductal network (Macias and Hinck 2012). Wnt signaling is present in both the ectoderm and mesenchyme (*Wnt10b*) since a very early embryonic developmental stage and it is currently accepted as a major initiator of the mammary lines specifications (Chu et al. 2004). The parathyroid hormone-related protein (PTHLH) localized at the epithelium signals the mesenchyme to increase the expression of bone morphogenetic protein 4 (BMP4), that signals the developing nipple sheath in order to prevent the formation of hair follicle, and instead the rudimentary mammary tree develops inside the fat pad (Macias and Hinck 2012). Moreover, Lef1 knockout mice or the artificial expression of Dkk1, both Wnt pathway modulators, resulted in the arrest of the mammary gland development at the bud stage (Chu et al. 2004, Boras-Granic et al. 2006). In addition to Wnt signaling, fibroblast growth factor (FGF) has been also proven essential in the early stages of the mammary gland development (Kleinberg 1997, Hennighausen and Robinson 2001, Veltmaat et al. 2003, Hens and Wysolmerski 2005). Interestingly, the mammary gland of ER and PR knockout mice develop normally till puberty, thus highlighting a less relevant role of hormonal control in the early embryonic developmental stages (Mulac-Jericevic et al. 2003).

When reaching puberty, pluripotent stem cells will give rise to the terminal end bud (TEB), highly proliferative structures responsible for the mammary ducts elongation (Figure 2; green frame). While the early embryonic stage of the mammary gland development is mainly independent of hormonal regulation, TEB formation is stimulated by the elevation of ovarian estrogen levels occurring in puberty, marking the beginning of adult reproductive life. These structures present a club-like shape and are formed by highly proliferative cells. The inner layer is formed by the body cells and the outer layer by the cap cells, which will, respectively, develop into the mammary ductal luminal and basal cells. Cap cells are able to grow through the thinner basement membrane and extracellular matrix surrounding them, allowing the newly formed ducts to spread into the fat pad of the mammary gland. Interaction with the fat pad supports the ductal outgrowth and epithelial branching throughout the developmental stages (Paine and Lewis 2017).

During each ovarian cycle, estrogen and progesterone are responsible for regulating cell proliferation and cell turnover to promote ductal branching and formation of lobular-alveolar structures. Thus, preparing the tissue for a possible pregnancy and eventual need to produce and release milk. In case pregnancy does not occur by the end of the menstrual cycle, the high levels of estrogen and progesterone produced during the luteal phase will dramatically drop and consequently provoke the mammary epithelium to regress into a resting state (Atashgaran et al. 2016) (Figure 2).

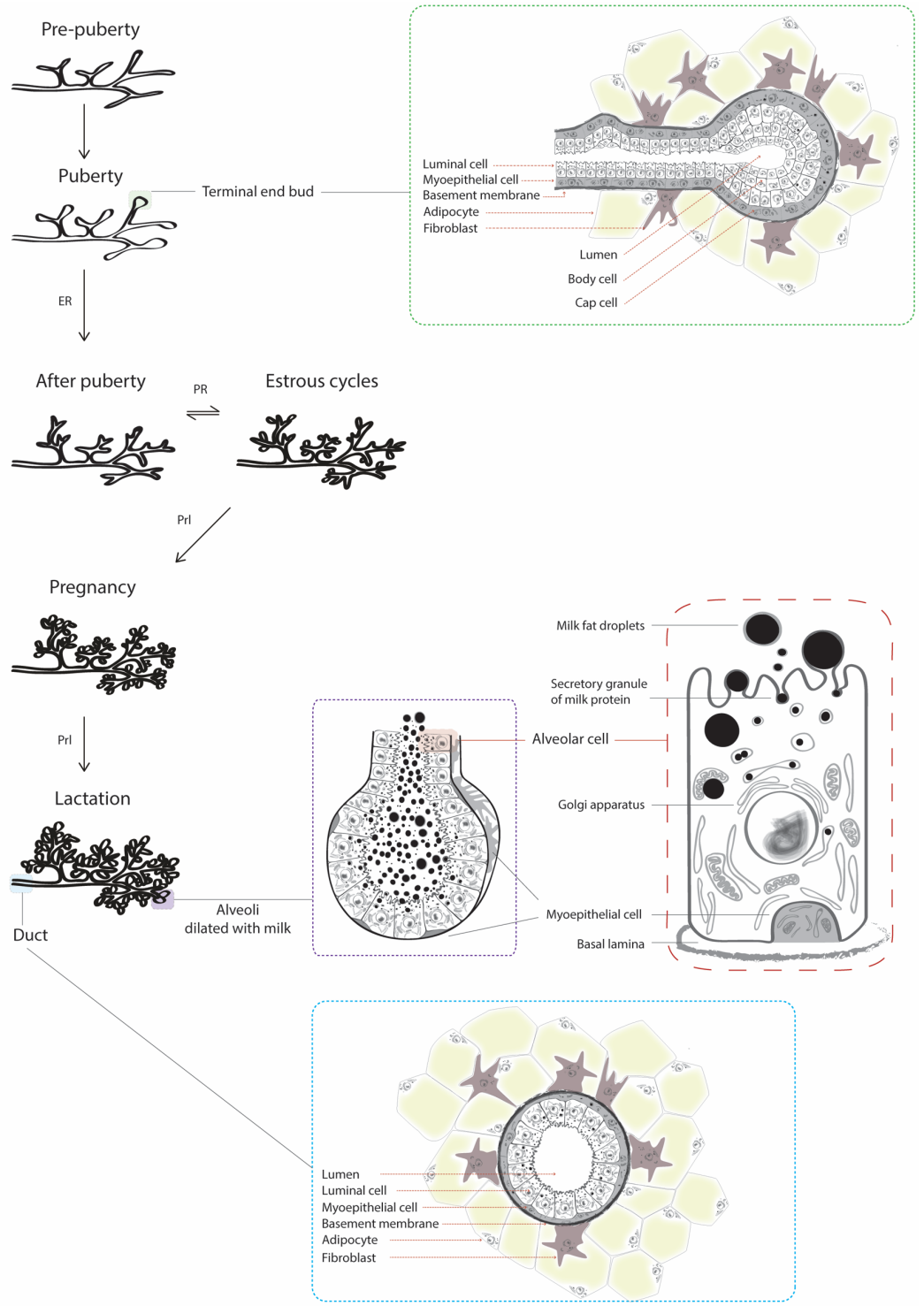

Figure 2 – Overview of the mammary gland structure in the different developmental stages. Left side represents the branching and alveologenesis stages. In the right side, representation of a terminal end bud (green frame), alveoli producing milk (purple frame) and a mammary duct (blue frame).

Upon pregnancy, prolactin and placental lactogens are essential for further proliferation and maturation of the lobular-alveolar structures. In early stages of pregnancy, prolactin is responsible for maintaining the *corpus luteum* and, consequently, the high levels of estrogen and progesterone necessary for ductal and alveolar development. When breastfeeding, the infants suckling mechanism is known to stimulate pituitary gland to release prolactin (Prl) and growth hormone (GH) that further induce milk production (Whitworth and Grosvenor 1984, Bodnar et al. 2002, Chilton and Hewetson 2005). Prolactin (Prl) and growth hormone (GH) modulate their target genes acting systemically as hormones or locally as cytokines. Alveologenesis studies in mice identified Jak2/Stat5 pathway as the main acting signaling in this developmental process (Wagner et al. 2004, Dong et al. 2010, Schmidt et al. 2014). Interestingly, Jak2 is implicated in the modulation of alternative signal transduction pathways like MAP kinase and PI3K/AKT pathways (Winston and Hunter 1995, Carter-Su et al. 2000, Thirone et al. 2006, Britschgi et al. 2012).

The fundamental function of the mammary gland, milk production, occurs in the mature terminal ductal lobular units. These are formed by several secretory alveoli facing the collecting ducts that direct the milk to the nipple opening. Both alveoli and ducts are bilayer cell structures surrounded by a basement membrane. Alveolar and ductal cells are tightly organized and polarized to form a dual layer of cells surrounding a lumen (Figure 2). In the alveoli, the inner luminal cells are responsible for the production and release of milk constituents, such as fat and milk protein, into the lumen. The surrounding myoepithelial cells not only provide structural support to the luminal cells but are also essential for the milk transportation in the ducts towards the nipple. Myoepithelial cells are able to stimulate the contraction of the ducts in response to oxytocin, released from the posterior pituitary upon infants' suckling, leading to the movement of the milk inside the ductal network (Reversi et al. 2005).

As soon as the lactation period ceases, the ductal networks and lobulo-alveolar structures will enter an involution program that remodels the mammary gland structure to a pre-pregnancy state. This process implies the collapse of the alveoli, massive ECM remodeling, adipocytes regeneration, cell death and immune cells infiltration. The lack of suckling stimuli (drop in lactogenic hormones) and the build-up pressure caused by the cessation of milk removal are considered the initiation stimuli for the involution process (Richert et al. 2000, Watson and Kreuzaler 2011).

Cell types in the mammary epithelium

The full segregation of cell lineages and the multipotency of progenitor lineages has been a debated topic and active research field with still many answered questions (Visvader and Stingl 2014, Wuidart et al. 2018). Several studies have shown that the different cell types in the mammary gland originate from a common mammary stem cell (MaSC). Early studies believed that cell lineages would be segregated upon MaSC differentiation and would be unable to give rise to cells from a different lineage. As shown in Figure 3, current studies indicate that these stem cells are able to replenish their stem cell pool and also able to differentiate into both luminal and myoepithelial progenitors. These progenitors will differentiate into mature myoepithelial or luminal cells. Luminal cells have the capacity to further differentiate into ductal and alveolar cells. Different lineage models have been hypothesized, but all agree that MaSC are able to originate more stem cells and originate the epithelial precursor cells (EPCs). In terms of signaling pathways relevant to the mammary gland development, canonical Wnt signaling pathway has been shown to be the responsible for MaSC self-renewal (Zeng and Nusse 2010).

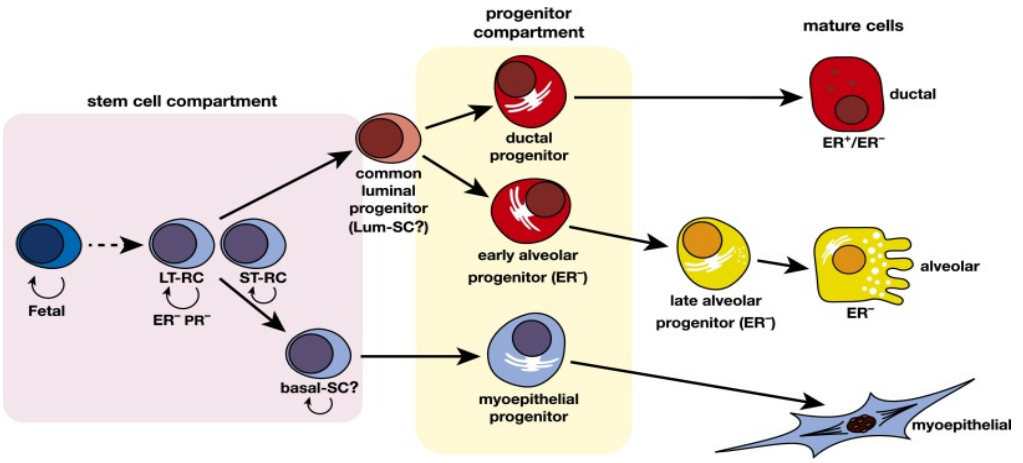

Figure 3 - Cell lineages in the mammary epithelium. (Visvader 2009, Visvader and Stingl 2014).

Some models suggested that the different mammary cell types were mainly classified based on the mammary gland developmental stage. Previously, EPC were considered to originate either from the ductal precursors, originating luminal and myoepithelial cells during puberty, or from the alveolar precursors, originating the alveoli luminal and myoepithelial cells during pregnancy (Hennighausen and Robinson 2005). Recent models, present a different view of the breast cell lineages where a common luminal progenitor is the progenitor for ductal and early alveolar progenitor cells. Myoepithelial progenitor cells are thought to originate from a basal stem cell lineage. Interestingly, the alveolar progenitors during pregnancy can also derive from the luminal progenitors and give rise not only to

alveolar cells but also to the myoepithelial progenitor cell (Rios et al. 2014, Visvader and Stingl 2014).

Essential hormonal regulators in mammary gland development

In mammals, many features that indicate the individual sexual maturation are regulated by estrogen and progesterone pleiotropic effects in the uterus, ovaries and hypothalamic-pituitary axis. Estrogen can bind to estrogen receptor α (ERα) or estrogen receptor β (ERβ), which are encoded by different genes. ER belong to the nuclear receptors family, acting as a transcription factor when bound to steroid hormones. Interestingly, in mammary development, studies showed that ERβ has no involvement in ductal and alveolar development (Forster et al. 2002). On the contrary, both stromal and epithelial ERα, are essential for ductal branching and elongation during puberty but dispensable for alveolar formation and expansion (Bocchinfuso et al. 2000, Mueller et al. 2002). Of note, ERα positive cells are able to induce proliferation in ERα negative cells via paracrine signaling. Current evidence implicates amphiregulin as ERα paracrine regulator (Kenney et al. 1996, Luetteke et al. 1999, Ciarloni et al. 2007). Further evidence shows that estrogen activation of the epidermal growth factor (EGF) family receptors results in similar effects regarding ductal elongation during mammary gland development (Kenney et al. 2003).

Progesterone can bind to two different progesterone receptor (PR) isoforms (A and B), derived from the same gene, implicated in different actions as transcription factors. In mammary development, only PR-B isoform has been proven essential in alveolar formation processes, suggesting that PR-B is the isoform responsible for the progesterone-induced proliferation in the mammary gland (Lydon et al. 1995, Mulac-Jericevic et al. 2000, Mulac-Jericevic et al. 2003). Studies on the human mammary gland development and several mouse studies have provided evidence for a paracrine proliferation regulation. PR positive cells can be found adjacent to the alveolar forming proliferative cells, both in normal situation and in experimental induced alveolar differentiation (Zeps et al. 1999, Grimm et al. 2002). Interestingly, PR-negative cells, when in contact with wild-type cells can also participate in the development of alveoli (Brisken et al. 1998). Amongst the currently known paracrine mediator of progesterone is the receptor activator of nuclear factor-kappa B (NF-kB)-ligand (RANKL), which upon overexpression mimics progesterone stimulation and rescues PR- phenotype in alveologenesis process (Fernandez-Valdivia and Lydon 2012). Wnt4 has also been suggested as a PR paracrine mediator since its upregulation is a consequence of progesterone activation in primary mammary epithelial cells (Brisken and O'Malley 2010). Cyclin D1 is involved in the cell proliferation induced by progesterone, since mice lacking Cyclin D1 presented similar impairment in alveoli formation as the PR

null mice models (Fantl et al. 1995, Sicinski et al. 1995). In brief, current literature implicates PR as essential in the formation and expansion of the alveoli structures, but its' role in ductal elongation proved to be minor.

EPITHELIAL TISSUE ARCHITECTURE

A normal epithelial architecture can be defined as the maintenance of a tightly organized sheet of polarized cells. At tissue level, polarization can be observed in glandular structures where secretory cells are orientated in order to secret their products into ducts (alveoli in breast) or directly to the exterior of the tissue (sebaceous glands in skin). The proper localization and orientation of the cells is maintained by the physical contact between epithelial cells and the extracellular matrix surrounding the basal side of the cells (Bryant and Mostov 2008).

Polarity regulating factors mediate the interactions between the cells and guide their organization inside the tissues. Planar polarity organizes the cells at their posterior-anterior axis (horizontal polarity) and apico-basal polarity organizes the cytoskeleton and multiple organelles perpendicular to the basal domain (vertical polarity) (Goldstein and Macara 2007, Simons and Mlodzik 2008). Planar polarization is responsible for the proper localization of microtubules and centrosomes, essential for cell division and migration. Furthermore, planar polarity is responsible for the proper growth guidance of axons and dendrites, hair follicle, inner ear development (Wang and Nathans 2007). Apico-basal polarity can be observed by the asymmetric distribution of organelles and macromolecules in the cells that will define cell behavior and localization. The maintenance of epithelial integrity is imperative for the dynamic tissue developmental processes, allowing a tight control over proliferation, apoptosis and number of signaling networks. Loss of epithelial integrity is typical in tumorigenesis process and a hallmark of advanced solid cancers (Humar and Guilford 2009, Polyak and Kalluri 2010, Hurst and Welch 2011, Ko et al. 2016, Williams et al. 2017).

Apico-basal cell polarity regulators

Apico-basal polarity is a characteristic feature of eukaryotic epithelial cells. Cells display an apical and basal domain where the cell's organelles and macromolecules asymmetrically distribute themselves. Cell domains are established by the well-defined positioning of the lateral cell-cell contacts and by the polarized distribution of the protein complexes responsible for the formation and maintenance of those junctions. Proteins involved in the establishment of cell polarity have not only specialized functions in the polarity protein

complexes but are also known to interact with conserved cell signaling pathways, for example, TGF beta, integrin and Wnt signaling.

Epithelial junctions and basement membrane interactions

Cell polarity is primarily defined and maintained by a set of junctions differently distributed along the basolateral side of the epithelial cells: Tight Junctions, Adherent Junctions, Gap Junctions, Desmosomes and Hemidesmosomes. These junctional systems maintain the cohesive layer of epithelial cells by connecting cells to their neighboring cells or to basement membrane. These connections are established via different transmembrane proteins, which in addition to anchoring function also mediate intracellular cell signaling (Figure 4).

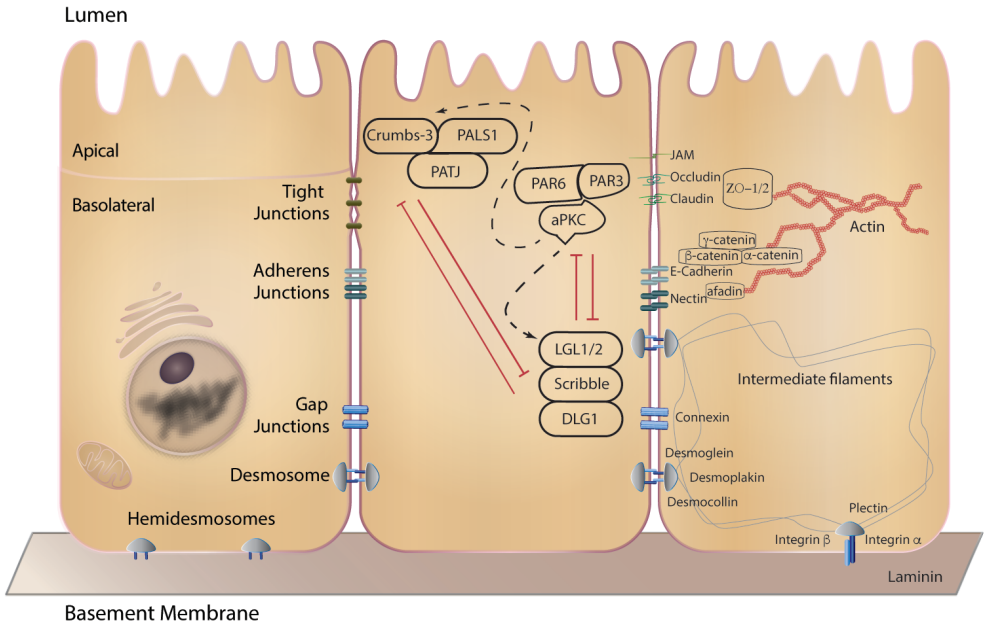

Figure 4 – Overview of the apico-basal polarity elements in the cells. 1st cell: Schematic overview of the polarized cell and cell domains; 2nd cell: Simplified representation of the protein complexes localization and their mutual interactions; 3rd cell: Overview of the cell junctions' main components and interactions with the intracellular components.

Tight Junctions (TJ)

Tight junctions (TJs), also known as zonula occludens, provide a tight connection between membranes of the neighboring cells and segregate the apical and basal domain of the cell. Located at the most apical side of the epithelial cells, their main role is to seal the luminal space. This prevents the passage of substances (including their own secretions) from the

lumen compartment to the basal side (Pitelka et al. 1973, Shin et al. 2006, Capaldo et al. 2017). It is noteworthy, that in *Drosophila*, TJs are represented by a region with similar function called sub-apical region (SAR). The physical connection between neighboring cells is formed by three major components: junctional adhesion molecules (JAMs), claudins and occludins (Shin et al. 2006, Tsukita et al. 2008). Inside the cells the main connection components interact with the zonula occludens (ZO) -1, -2 and -3 scaffold proteins. The N-terminal Post synaptic density-95, Discs large and Zonula occludens-1 (PDZ) domains of the ZO proteins interact with the transmembrane proteins (claudins and occludins), while their ZO proteins C-terminal domain binds to the actin cytoskeleton (Fanning et al. 2002). The establishment and maintenance of TJs has been intensively studied and several studies showed evidence of ZO-1 and -2 critical role in the formation of these connections (Adachi et al. 2006, Umeda et al. 2006) (Figure 4). Non-PDZ protein cingulin is amongst the many proteins that have the ability to communicate with the TJ connective proteins and has been implicated in the inhibition of RhoA and modulation of cell proliferation (Bazzoni et al. 2000, Aijaz et al. 2005, Maiers et al. 2013). Tight junctions have been proved critical not only in the cell adhesion process, but also in cell growth and polarity maintenance (Tsukita et al. 2008). In the intracellular domain, TJ's form a complex network of direct and indirect protein interactions, which connect TJs to the other junctional complexes (Gonzalez-Mariscal et al. 2003, Shin et al. 2006, Maiers et al. 2013).

Adherent Junctions (AJ)

Adherent junctions, also called as zonula adherens, connect neighboring cell membranes less tightly than TJs. In vertebrate's epithelial cells, these junctions are localized subapically below the TJs. At AJs the neighboring cells are connected via homotypic interactions of the transmembrane proteins E-cadherin (calcium-dependent adhesion) and Nectin (calcium independent) (Niessen and Gottardi 2008). The principal intracellular AJ connectors are gamma-, beta- and alpha-catenins, which connect the transmembrane E-cadherin to the actin cytoskeleton (Drees et al. 2005). At the calcium-independent AJ, afadin binds the transmembrane nectin to α-catenin and actin cytoskeleton (Mandai et al. 1997, Takahashi et al. 1999) (Figure 4). Tissue remodeling, particularly during developmental processes, require a tight maintenance of the cell and tissue polarity. This is partly accomplished by the E-cadherin mediated contacts that indirectly guide the actin cytoskeleton organization (Cavey and Lecuit 2009). Adherent junctions play a central role in several developmental processes, including epithelial-to-mesenchymal transition (EMT), which is an important cell transformation for invasive and metastatic behavior of cancer (Lamouille et al. 2014, Stocker and Chenn 2015).

Gap Junctions

Gap junctions are pore-like connections between the neighboring cells, localized in the lower portion of the lateral membranes. They directly connect the cytoplasm of adjacent cells, allowing the passage of ions and small metabolites between neighboring cells. The gap junction is formed by two hemichannels or a connexon. Hemichannels are formed by hexameric oligomerization of connexin proteins, homomeric (single type connexin) or heteromeric (multiple type connexin). The hemichannels originating from two neighboring cells can form either homotypic channels, if identical homomeric hemichannels form the junction, or heterotypic channels, when different homomeric hemichannels form the junction (Herve et al. 2007, McLachlan et al. 2007). On the intracellular side, connexins can bind, amongst others, to ZO-1, -2 and -3, occludin, claudin-1 and -5, p120-catenin and β-catenin. Since the connection pore aperture seems to be controlled by connexin phosphorylation, so other cell junctions are also able to regulate the cells communication via gap junctions (Pogoda et al. 2016). Since gap junctions primarily play a communication role between cells, their main function varies according to the tissue (Plum et al. 2000, Pernelle et al. 2018). For example, in the mammary gland the start of milk production and secretion is triggered by the signaling between luminal and myoepithelial cells, which appears to be modulated via gap junctions (Pitelka et al. 1973, El-Sabban et al. 2003). Furthermore, connexins have been implicated in breast cancer tumors, playing tumor suppressive or facilitating actions depending on the tumor stage (Banerjee 2016).

Desmosomes

Desmosomes, also called as macula adherens, are localized along the basolateral side of the cell and establish a close adhesion between the neighboring cells membranes. This connection has been shown to have calcium-dependent and calcium-independent adhesion mechanisms (Chitaev and Troyanovsky 1997, Kimura et al. 2007). The desmosomal connection between cells is formed by desmogleins and desmocollins, both belonging to the cadherin family (Kowalczyk et al. 1994, Marcozzi et al. 1998). In the internal side of the cells these proteins bind to plakoglobin (γ-catenin), plakophilin and desmoplakin and form a desmosomal plaque responsible for connecting desmosomes to the keratin intermediate filaments (Stahley et al. 2016). The presence of both types of proteins is essential to the adhesion establishment (Garrod and Chidgey 2008) (Figure 4). Desmosomes mainly confer resistance to mechanical stress in tissues like epidermis and cardiac myocytes while, in other tissues with higher need for plasticity, like in alveoli, desmosomes are found in smaller amount (Pitelka et al. 1973). Like the other cell junctions their role is not limited to cell adhesion, playing also a role in the signaling amongst their constituents. Plakoglobin can negatively modulate β-catenin, which is a transcriptional

activator mediating Wnt signal transduction (Zhurinsky et al. 2000, Miravet et al. 2002). Furthermore, the loss of desmosomes in epithelial cells has recently been implicated in initial tumorigenesis processes (Garrod and Chidgey 2008, Tervonen et al. 2016).

Hemidesmosomes

Hemidesmosomes can be visualized as half desmosomes and they are localized at the basal side of the cells connecting them to the basement membrane (BM). BM is a specialized form of ECM providing basal adhesion to epithelial cells. The connection to BM is mainly established via integrins, dystroglycan and syndecan (Barresi and Campbell 2006, Morgan et al. 2007). Integrins are the principal transmembrane proteins providing anchorage to cells (De Pascalis and Etienne-Manneville 2017). Amongst the ECM substrates they bind to collagens, fibronectin and laminins. When observed under electron microscopy, the structure of hemidesmosomes is similar to that of desmosomes, including their intracellular connection to intermediate filaments. In the internal side of the cells, the cytoplasmic domain of β4-integrins bind to plectin and hemidesmosomal plaque proteins which, in turn, bind to intermediate filaments (Schaapveld et al. 1998, Hopkinson and Jones 2000, Koster et al. 2004) (Figure 4).
The main role of hemidesmosomes is to help the tissue sustain high levels of mechanical strain, with integrin-β4 appearing to be especially important for the normal mammary gland function (Hynes 2002, Legate et al. 2009). In addition, the loss of integrin connections and ECM anchorage has been widely related to tumor invasion and migration (Huttenlocher and Horwitz 2011). Integrins are known to interact with focal adhesion kinase (FAK) and integrin-linked kinase (ILK) and hence downstream signaling pathways, such as phosphatidyl inositol-3 kinase (PI3K) and AKT pathways (Legate et al. 2009).

Apical-basal polarity complexes

Proteins are considered to form a complex if they co-localize and co-immunoprecipitate with another complex member. The complex proteins should directly interact with at least another member of the complex. Three highly conserved polarity complexes are critical for maintaining cell polarity, organization of internal signaling and for sustaining epithelial homeostasis. The localization and interaction between the components of these complexes, namely PAR, Crumbs and SCRIB, determine the apical and basal domains in epithelial cells (Figure 4).

The PAR complex

This complex, also called Par6 complex, is localized on the subapical part of the lateral membrane domain alongside TJs. The core components of this complex are PAR-3, PAR-6, atypical protein kinase C (aPKC) and CDC42 (Joberty et al. 2000). PAR proteins and aPKC were discovered in a classic genetic study using *C. elegans* zygotes (Kemphues et al. 1988). In this original study, partitioning defects (failure in asymmetric zygote cleavage patterns) were observed in the absence of PAR (partioning-defective) genes. PAR genes have proven to be highly conserved amongst species. In accordance, of the 6 genes identified in the original study only PAR-2 does not have a mammalian homologue (Goldstein and Macara 2007). The proteins belonging to the PAR family were all implicated in the establishment of cell polarity in various cell types and present different structural domains (Suzuki and Ohno 2006, Assemat et al. 2008). PAR-3 and PAR-6 are scaffold proteins with PDZ-domains (Hung and Kemphues 1999, Benton and St Johnston 2003); PAR-1 and PAR-4 are serine-threonine kinases (Guo and Kemphues 1995, Lizcano et al. 2004); PAR-5 is a 14-3-3 protein; PAR-2 is a RING-finger protein (Morton et al. 2002). Most of the PAR genes have a permanent localization in the cells: PAR-3 and PAR-6 localize at the apical side; PAR-4 and Par-5 in the membranes at the lateral side and occasionally in the cytosol (Martin and St Johnston 2003, Baas et al. 2004). The core of PAR complex is formed by PAR-6 and PAR-3, which do not possess enzymatic activity but regulate their functions via specific physical connections. Proteins interact with each other via their following protein domains: PAR-6 and PAR-3 interact via their PDZ domains; PAR6 binds to GTP-bound CDC42 by its' CRIB (central CDC42 and RAC interactive) domain; PAR6 binds to aPKC via its Phox and Bem1p (PB1) domain localized in the N-terminal (Joberty et al. 2000, Lin et al. 2000, Noda et al. 2001, Assemat et al. 2008) (Figure 5). aPKC activity has been shown to be dependent on its interaction with PAR6 (Yamanaka et al. 2001). The proper formation of the complex by physical binding of PAR-3 to PAR-6/aPKC is an essential step to establish cell polarity (Horikoshi et al. 2009). The assembly of TJs is also connected to this complex, with PAR6 negatively regulating the process and PAR3 being indispensable for TJ formation (Gao et al. 2002, Hirose et al. 2002, Hurd et al. 2003, Chen and Macara 2005). In vertebrates, PAR6 protein has three different isoforms PARD6A, PARD6B and PARD6G, which are in fact encoded by three different genes located in different chromosomes (Gao and Macara 2004, Tervonen et al. 2011).

Cell polarity is achieved by specific targeting and mutual exclusion of the polarity complexes. The exclusive localization of the proteins forming these complexes efficiently segregates the complexes to their respective cell domains. As an example, PAR complex formation can be antagonized by the SCRIB complex by competitive binding of Lethal

giant larvae (LGL) PAR-6/aPKC that prevents PAR-3 binding to its complex (Yamanaka et al. 2003). Moreover, active aPKC is able to phosphorylate LGL, leading to the release of LGL and exclusion of the SCRIB complex from the apical domain (Betschinger et al. 2003, Tian and Deng 2008) (Figure 4). In human cells, PAR-4, also called LKB-1, is cytosolic and has well-established tumor suppressor functions (Shackelford and Shaw 2009).

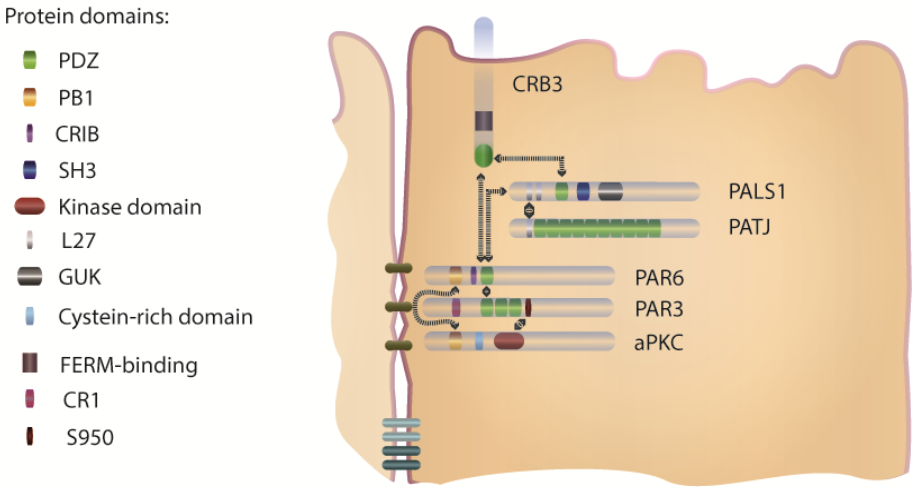

Figure 5 - Simplified diagram of the PAR and CRB 's complex protein domains. Interacting protein domains inside their complex or amongst complexes are shown (focusing on PAR6 interactions).

The Crumbs (CRB) complex

The Crumbs complex is localized above the TJ, in the most apical domain of the vertebrates' epithelial cells. The main components of this complex are Crumbs (CRB3), Protein associated with lin seven 1 (PALS1; also called MPP5), PALS1-associated tight junction protein (PATJ) and multi-PDZ domain protein MUPP1 (Assemat et al. 2008). This complex was first identified in Drosophila studies, and CRB3 was originally identified as essential in maintaining polarity in the embryonic epithelia (Tepass and Knust 1990, Tepass et al. 1990). Following studies continued gathering evidence of the conserved Crumbs complex effects in mammalian cells, including their protein domains and interactions (Knust et al. 1993, Roh et al. 2003, Shin et al. 2005). In *Drosophila, stardust* (MPP5 or PALS1 in humans) functions as the complex anchor, binding to Lin-7 and PATJ via its two L27 protein domains, and to the transmembrane protein crumbs via its PDZ domain. MPP5 has been shown to interact with PAR complex proteins PAR-6 and aPKC (Hurd et al. 2003, Wang et al. 2004, Assemat et al. 2008), and CRB3 activity appears to be regulated by its phosphorylation by aPKC (Sotillos et al. 2004). Furthermore, in drosophila, CRB3 has shown the ability to directly interact with Bazooka (PAR-3), which will prevent

the interaction between Crumbs and PAR-6 (Lemmers et al. 2004, Krahn et al. 2010). CRB3 has also been shown to induce aPKC to phosphorylate PAR-3, resulting in PAR-3 dissociation from the PAR-6/aPKC complex (Benton and St Johnston 2003, Morais-de-Sa et al. 2010).

In addition, TJ formation appears to be dependent on CRB complex activity with supporting evidence coming from studies using MCF10A cell line, where these cells regain the ability to form TJ upon overexpression of CRB3 (Lemmers et al. 2004, Fogg et al. 2005). Studies with MDCK cells lines suggested that MPP5 and PATJ are required for TJ formation (Straight et al. 2004, Shin et al. 2005, Wang et al. 2007). Furthermore, MPP5 and PATJ/MUPP acting as scaffold proteins can directly interact with claudin, occludin and ZO-3 (all TJ related proteins) (Assemat et al. 2008). The exclusion of PAR3 from PAR complex by crumbs is suggested as essential for the maintenance of the apical-basal domains (Wodarz et al. 1995, Roh et al. 2003, Lemmers et al. 2004).

The SCRIB complex

The SCRIB complex is localized in the basolateral region of the epithelial cells, below the adherent junctions (Assemat et al. 2008, Massimi et al. 2008). The main components of this complex are scribble, lethal giant larva (LGL 1-2 in mammalian cells), and discs large (DLG1-4 in mammalian cells). Similar to other complexes, also SCRIB complex was first discovered in *Drosophila* studies. In these studies, mutation of the SCRIB proteins induced hyperproliferation in the imaginal disc cells and tumorigenesis in larval and adult stages (Bilder et al. 2000). In this original study, mislocalization of the apical domain constituents into the basolateral domain was observed upon loss of *scrib*, *dlg*, or *lgl*. These studies suggested a role for SCRIB complex proteins in the maintenance of the basolateral identity (Bilder et al. 2000). Evidence for physical interaction between SCRIB proteins is scarce and molecular details of their interactions are not properly understood. Scribble has been shown to interact with Dlg in *Drosophila* synapses, while in mammalian epithelial cells scribble interacts with Lgl2 (Mathew et al. 2002, Katoh and Katoh 2004, Kallay et al. 2006). These studies indicate that even without a direct protein-protein connection to form a protein complex, the polarity maintenance is dependent on the indirect interactions between complexes. SCRIB complex maintains the basal domain identity by the previous mentioned interaction between Lgl and PAR-6/aPKC (Yamanaka et al. 2003). Interestingly, the localization of this complex, specifically of Scribble and Dlg1, seems to be modulated by AJs. Moreover, Scribble and Dlg1 co-localize with E-cadherin in human cervical epithelium and mouse intestine, implying a critical role of AJ formation in defining the basal domain of the cells (Navarro et al. 2005, Yoshihara et al. 2011).

MAMMARY TUMORIGENESIS

Breast cancer is amongst the most common malignancies affecting woman around the world, with a striking incidence of 1 in 8 women developing the disease during their lifetime. Breast cancer mortality rates have been decreasing, with good survival rates for patients where tumors are detected in a non-advanced stage. On the other hand, it is still worrying that breast cancer is the most fatal cancer amongst woman from the European countries (Ferlay et al. 2013). Interestingly, only 5%-10% of the diagnosed breast cancers are of hereditary origin, often linked to somatic mutations in BRCA1 or BRCA2 genes.

Breast cancer is a heterogenic disease that can be classified histologically and morphologically into different types and subtypes. Breast cancers are currently classified into four major subtypes according to their molecular expressions. Luminal A tumors express estrogen receptor (ER) or/and progesterone receptor (PR) and are human epidermal growth factor 2 (HER2) negative. Luminal B tumors are generally ER and/or PR positive as well as HER2 positive. HER2 tumors are HER-positive and negative for ER and PR. Basal-like cancers are triple negative breast cancers (TNBC). Claudin-low is a molecular subtype that was recently identified by gene expression profiling, and usually presents a phenotype similar to TNBC. However, only a small fraction of TNBC are claudin-low. The subtype classification has been correlated to patients' prognosis, with TNBC phenotypes correlating with the worst outcome and luminal A with the best outcome (Hon et al. 2016).

Breast cancer can have its origin either from the ducts or from the lobular-alveoli structures, resulting in ductal and lobular carcinomas, respectively (Barroso-Sousa and Metzger-Filho 2016). As previously discussed, ducts and lobular-alveoli structures are both formed by a bilayer of cells surrounding a hollow lumen. In the initial tumorigenic stages, some luminal cells acquire the ability to avoid anoikis (apoptosis induced by lack of contact with ECM) and become able to sustain a proliferative cell cycle phase (Hanahan and Weinberg 2011). These transformed cells are the result of the activation of oncogenic stimuli and/or loss of tumor suppressing regulators. Rapid proliferation of the cells results in the lumen of ducts or alveoli to be filled with transformed cells, which initially remain encapsulated by the surround myoepithelial cells and the basement membrane. This premalignant condition is called carcinoma *in situ*. Once the transformed cells manage to force themselves through the myoepithelial cell and basement membrane layers escaping into the surrounding matrix, the disease has become an invasive carcinoma with worse prognosis (Ellis 2010) (Figure 6).

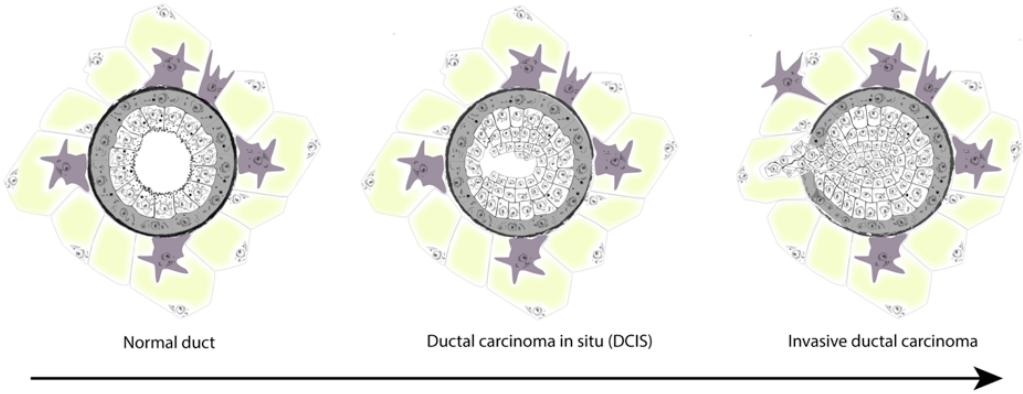

Figure 6 – Schematic representation of normal breast tissue progressing to ductal carcinoma in situ and finally to invasive ductal carcinoma.

The breast cancers are categorized according to their hormonal receptor expression (ER, PR), HER2 and proliferation status, lymph node infiltration and the overall pathologic scenario, which takes in account the histology of the cancerous lesions (Lehmann et al. 2011, Prat and Perou 2011). Nonetheless, therapy outcomes greatly vary within the currently used categories (Blows et al. 2010), implying that genome-based classification could be more suitable for designing therapeutic strategies (Curtis et al. 2012, Pereira et al. 2016). The expression profile signatures are related as follows: MSC/basal with claudin-low subtype and luminal progenitor with basal-like subtype (Shehata et al. 2012). Although the signature resemblances to luminal A, luminal B and HER2-positive subtypes in not so clear, luminal A features are more concordant with the ones of mature luminal cells. Luminal B and HER2+ could originate from a subset of cells in the luminal cells lineage, with HER2 positive tumors deriving from an amplification of the HER2 (Figure 7).

The genetic landscape of human breast cancers is diverse and recent studies have provided evidence for significant inter- and intra- tumor heterogeneity. For example, Pereira et al identified as much as 40 driver mutation that can be associated with prognosis and overall survival, linking for example PIK3CA mutations to low survival in the three subgroups of ER-positive breast cancer (Pereira et al. 2016).

The origin of the cells resulting in breast cancer are currently unclear with all cell types in the mammary epithelium potentially able to develop into tumor cells. However, cancer stem cell researchers have been debating if tumors arise from specific stem- or stem-like cells and gradually accumulate genetic alterations to develop into full-blown cancer. Emerging evidence indicates that breast cancer can originate either from a specific cell lineage stem cell progenitor or from fully differentiated cells existing in the normal tissue. In the first scenario, the cell lineage progenitor and tumor originating cell would partially define the tumor features and subtype (Figure 7). This hypothesis is supported by some

epidemiological data (hereditary breast cancer syndromes), by experimental studies that linked the ER status of tumor and normal cells (Olsson 1989), and by genetic and molecular expression correlation (Olsson 2001, Lim et al. 2009, Visvader 2009, Prat and Perou 2011).

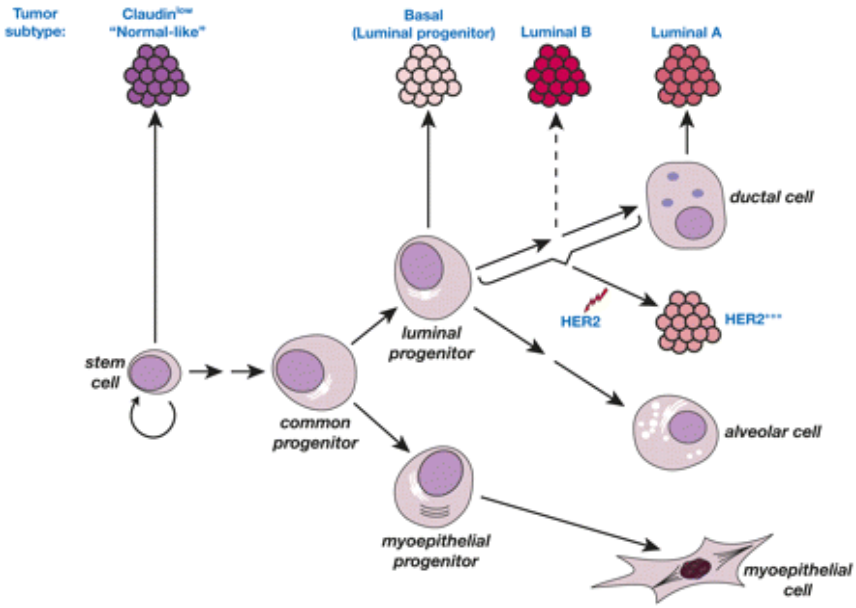

Figure 7 - Breast tumor subtypes and the potential relationships with mammary gland cell lineages (Visvader 2009).

Oncogenes

Proto-oncogenes are genes that due to their essential roles in the normal tissue, upon deregulation (mutations, gene duplication or altered DNA transcription) have the potential to become oncogenes. Oncogenes have the potential to transform normal cells into tumor cells by inducing a high proliferative status or by evading programmed cell death.
In breast cancer, studies have implicated alteration in a defined group of oncogenes, such as *ErbB2*, *PI3KCA*, *MYC* and *CCND1* (encodes cyclin D1) (Lee and Muller 2010). A chapter ahead includes a detailed description of *MYC*'s intervention in tumors cell cycle, apoptosis and metabolic transformations.

Tumor Suppressor Genes

The concept of tumor suppressor gene derived from early hybridization studies (Harris et al. 1969) where hybridization of malignant and normal cell resulted in hybrids containing DNA from both cells. These hybrid cells were normal, losing all signs of malignancy, suggesting the hybrids inherited a tumor suppressor gene from the normal cell. For tumor

cells to thrive, tumor suppressor genes need to be inactivated, which can occur via one or more of the following mechanisms: Loss of function mutations, loss of heterozygosity, gene inactivation by epigenetic mechanisms (*i.e* methylation), somatic mutations (spontaneous tumors), inherited syndrome mutations, and acquisition of the overall ability to lead malignant cells to overgrow and escape apoptosis control (Lee and Muller 2010, Hanahan and Weinberg 2011).

Epithelial Integrity as Tumor Suppressor

Interestingly, epithelial integrity by itself could be interpreted as a tumor suppressor barrier. In *Drosophila* studies tumor suppressor genes have been categorized into different classes: The neoplastic tumor suppressor genes (nTSG) and the hyperplastic tumor suppressor genes (hTSG). The knockdown or loss of hTSG will lead to a hyper-proliferative status while epithelial integrity is retained. On the contrary, nTSG show not only a hyper-proliferative status but also an overgrowth of tissue with loss of epithelial integrity that leads to tumorigenesis.

Studies in mice and human cell lines, with known oncogenes and tumor suppressor genes involved in the maintenance of epithelial integrity, suggested that an oncogenic stimulus might not be enough to drive tumorigenesis if epithelial integrity is maintained. LKB1 (STK11) has been shown to act via AMPK/mTOR signaling pathway in order to control cell growth. In mice and in vitro studies, LKB1 has been shown to maintain a proper epithelial integrity that is able to sustain *MYC*'s proliferative action (Hanahan and Weinberg 2011, Partanen et al. 2012).

Many proteins involved in the establishment and maintenance of cell polarity have been implicated in tumorigenesis mainly in *Drosophila* studies but also in mammals. For example, loss of epithelial adhesion molecules E-cadherin and ZO-1 is commonly witnessed in human cancers (Berx and Van Roy 2001). Furthermore, loss of the key components of polarity complexes have been also implicated in tumorigenesis as shown in mouse studies where loss of *CRB3* leads to the loss of contact growth-inhibition and consequently tumorigenesis (Karp et al. 2008). Scribble mutations have been identified in renal cell, breast and colon carcinomas (Sjoblom et al. 2006, Dalgliesh et al. 2010). Furthermore, *Dlg1* mutations have been observed in mammary ductal carcinoma (Fuja et al. 2004) and low *LGL* expression levels have been observed in different human tumor types (Grifoni et al. 2007). Absence or low expression of PAR3 has also been reported in several cancer types and has been suggested to modulate tumor growth and promote more aggressive tumorigenesis (Iden et al. 2012). Studies have shown that the loss of cell polarity is also linked to a misregulation of signaling pathways, such as Notch, Wnt and PI3K/AKT/mTOR signaling, that are involved in cancer proliferation and differentiation.

Metastasis

Currently most breast primary tumors, when diagnosed in an early stage, can be removed and efficiently treated allowing a good patient survival (5-years survival rate >90%). Unfortunately, the patient's survival rate drastically drops if breast cancer becomes metastatic (5-years survival rate ~30%). In breast cancer sentinel lymph nodes are frequently used to identify if tumors cells have started to disseminate via lymphatic route. Sentinel lymph nodes act as gatekeeper, preventing in a first instance the passage of tumor cells to regional lymph nodes, bone marrow and peripheral blood. Nonetheless, tumors cells can enter directly into blood vessels and bypass the blockade provided by the sentinel lymph node (Leong and Tseng 2014). Breast cancer tumor cells or cell clusters manage to travel around in the body via lymph or hematopoietic route and disseminate into distant organs, typically brain, lungs, liver and bone.

Novel studies are currently applying molecular evolutionary models to infer cancer chronograms in an attempt to address questions regarding the timing of gene mutations and their contributions to tumorigenesis and metastasis. It was previously believed that a single tumor cell lineage could originate all the metastases in the patient. In which case, all metastases would resemble more to each other than with the primary tumor from where they escaped. However, recent phylogenetic studies show that metastases tend to originate from divergent lineages of the primary tumor (Woelfle et al. 2003, Zhao et al. 2016). Since the detection of early metastasis is critical for the patient's care and survival, many studies have been attempting to identify the driver mutations that could predict the potential of tumor cells to disseminate (Zhao et al. 2016). Previous studies have successfully linked the modulation of several transcriptions factors, *i.e.* GATA3, MYC, SOX-2, OCT-4, Nanog and Lin-28A able to lead to dedifferentiation and transformation of the cells into a stem cell like state. Thus, resulting in an increased cell plasticity and potential onset of tumor dissemination (Takahashi et al. 2007, Kouros-Mehr et al. 2008, Raimondi et al. 2011, Chou et al. 2013, Riddell et al. 2014).

Circulating and Disseminated Tumor Cells

Tumor cells that manage to escape from the primary tumor can find their way into neighboring blood vessels or lymphatic blood vessel (intravasation) and become circulating tumor cells (CTCs). In breast cancer, both dissemination routes are used and, currently, different sets of genes have been implicated in lymphatic or hematogenous dissemination (Woelfle et al. 2003, Pantel and Brakenhoff 2004). CTCs are present in small numbers, which makes it difficult to harvest them, but these cells are valuable as they carry unique information of the potentially metastatic tumor cell pool. Different CTC mRNA and single-cell detection is starting to be widely used to detect the presence of CTC in the blood

stream. Even though CTC's are harder to obtain, tumor and "blood-biopsies" can be characterized using similar methods (Neves et al. 2014, Polzer et al. 2014, Kanwar et al. 2015). Molecular characterization can be performed in bulk or at single cell level, in which case highly sophisticated sorting tools are required (Autebert et al. 2012). Although several studies have succeeded in profiling CTCs for expression of the hormonal receptors (Nadal et al. 2012), HER2 (Ligthart et al. 2013), proliferation status (Paoletti et al. 2015), apoptosis (Smerage et al. 2013) and PDL1 expression (Mazel et al. 2015), the methods for obtaining information from CTCs are still far from reliable.

Epithelial to mesenchymal transition (EMT) is a process where epithelial cells change their conformation in order to provide cancer cells with the ability for invasion and migration. EMT can be stimulated by interaction with the tumor microenvironment and implicates the loss of epithelial integrity. The identification of CTC by the current analysis methods, requires a careful selection of exclusive CTC markers, since CTC and leukocytes share cell surface markers that can lead to misleading results. Previous studies have found that mesenchymal cells were highly enriched in CTCs populations and some of the normal blood cells also present mesenchymal origin (Yu et al. 2013). Furthermore, single CTC analysis is showing high discrepancy between studies making it difficult to identify a pattern to be used for all patients. As an example, analysis of breast cancer CTCs for PIK3CA mutations status has been showing different mutation patterns in different studies and in different stages of disease progression (Deng et al. 2014, Markou et al. 2014, Pestrin et al. 2015) and it is currently unclear if these discrepancies are due to the low sensitivity of the methodology or due to the actual intercellular heterogeneity (Stuelten et al. 2018). It is worth to mention, that even with all the flaws, CTC analysis can generally provide a useful read out for tumor burden, with a higher frequency in patients with metastatic tumors. In breast cancer, high CTC levels have been also observed in the non-metastatic inflammatory subtype (Hall et al. 2015), associated with a poor prognosis. Nonetheless, no clear correlation has been found between CTC count and breast cancer clinical subtypes (hormone receptor-positive, HER2-positive, triple-negative) (Pierga et al. 2012)(Bidard et al. 2016).

How the CTCs are guided into their metastatic site is still a rather unclear process. Originally it was thought that the metastatic niche preferences were purely defined by anatomical and mechanical structures in the human body (Ewing J. 1928). However, previous studies have shown that specific signaling events between the tumor cells and the host microenvironment are essential for tumor colonization (Sleeman 2012, Ghajar et al. 2013, Sosa et al. 2014). In fact, only a fraction of CTCs is capable of seeding into distant sites and persisting as disseminated tumor cells (DTCs). An even smaller fraction of DTCs, is capable of progressing toward metastases by hijacking the new niche microenvironment.

In breast cancer, metastases are mainly found in lymph nodes, lungs, brain, liver and bone with the patient's overall survival drastically dropping when tumor cells manage to spread and colonize these distant organs. The disseminated tumor cells allocated in the new tissue are not passively surviving but actively integrate the signaling in their new tissue microenvironment, manipulating it to evolve into a malignant niche (Jung et al. 2008) (Figure 8).

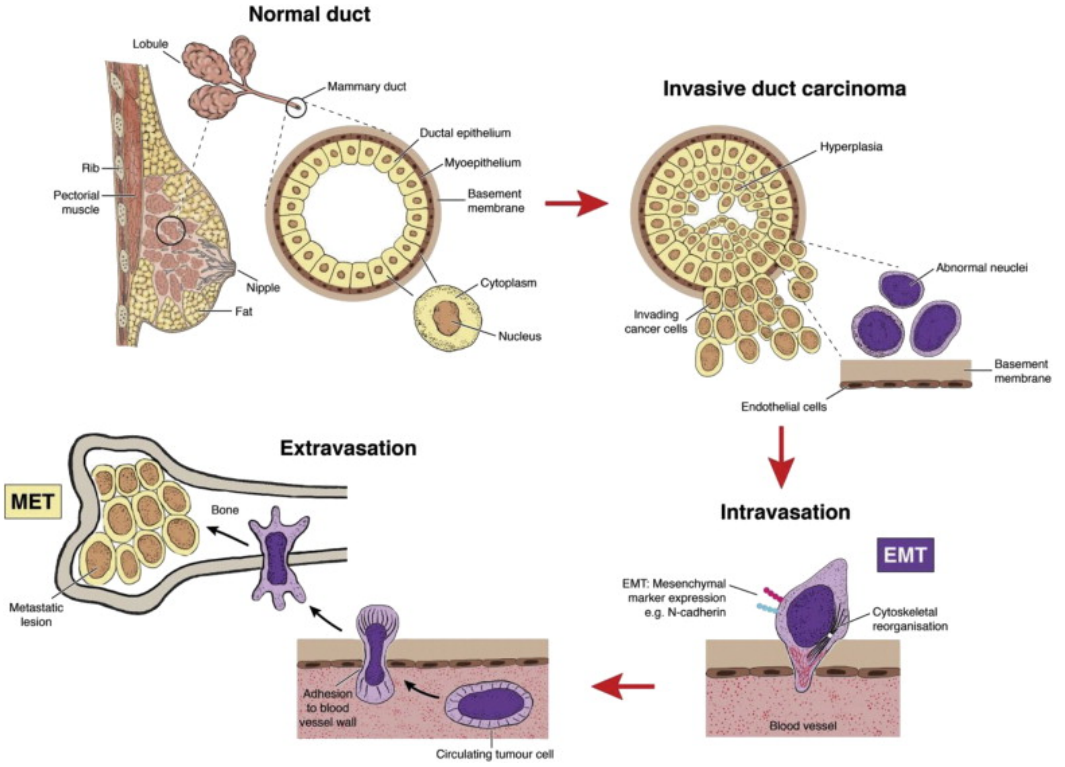

Figure 8 - Overview of the metastatic process stages, exemplified from the case of a ductal carcinoma into the bone marrow. Image from (Castle et al. 2014).

The bone marrow contains hematopoietic stem cells (HSCs), which differentiate into macrophages, osteoclasts (OCs), T-cells and other lymphocytes. In addition, the bone marrow also has mesenchymal stem cells (MSCs) that differentiate into adipocytes (A), osteoblasts (OB) and fibroblasts. DTCs that successfully disseminate into bone marrow will home into the hematopoietic stem cells (HSCs) compartment, where osteoblasts also reside. Due to DTCs localization in the bone marrow it has been suggested that they are kept dormant in the HSC compartment by the normal regulatory T-cell (Treg) inhibition of osteoclastogenesis. Nonetheless, MSC-derived cancer associated fibroblasts (CAFs), HSC-derived tumor-associated macrophages (TAMs), adipocytes and OC bone resorption are able to help DTC exit their quiescent status and to form a metastasized tumor (Shiozawa et al. 2015). Circulating tumor cells (CTCs) and disseminated tumor cells (DTCs) may have

value in disease monitoring and therapeutic intervention. Nonetheless, for a clinical application of CTC/DTC as prognostic and therapeutic targets, further optimization of sampling and analysis techniques is still required. Developing methods for harvesting and analysis of these cells will also enable better understanding of the biological mechanisms behind tumor dissemination, survival of DTCs and transition from dormancy to aggressive growth in the colonized niche (Kang and Pantel 2013).

THE C- *MYC* ONCOGENE

The Myc family of genes include *v-MYC*, *c-MYC*, *MYCN*, *L-MYC*, *S-MYC* and *B-MYC* (Dang 2012). *c-MYC* (herein mentioned as *MYC*), *MYCN* and *L-MYC* are known as proto-oncogene transcription factors. The expression of *MYCN* and *L-MYC* is generally limited to the embryonic development stages while *c-MYC* is broadly expressed during embryonic phases and in the adult proliferating tissues (Meyer and Penn 2008). Nonetheless, amplifications of *MYCN* have been observed in human neuroblastoma and correlated with patients' poor prognosis (Kohl et al. 1983, Schwab et al. 1983, Brodeur et al. 1984). In addition, amplification of *L-MYC* has been more recently associated to several cancer types including ovarian carcinoma and lung cancer (Wu et al. 2003). Furthermore, studies in lung cancer showed evidence of deregulation of one of the three *MYC* proto-oncogenes (Nau et al. 1985, Zimmerman and Alt 1990, Zajac-Kaye 2001).

In breast cancer cases, *MYC* locus is found amplified in approximately 20% of the cases (Aulmann et al. 2006, Corzo et al. 2006) and it has been shown to correlate mostly with the high-grade ductal carcinoma *in situ* (DCIS) and with invasive carcinoma (Blancato et al. 2004).

MYC binds its heterodimerization partner MAX via their helix-loop-helix leucine-zipper domains, forming a heterodimer capable to bind to E-Box sequences in DNA. Consequently, the heterodimers have the capability to modulate the transcription of target genes. Due to the highly conserved E-box regions in the genome, MYC-MAX can bind to a wide number of promoters in the genome (Blackwood and Eisenman 1991, Pelengaris et al. 2002). Nonetheless, the affinity of MYC-MAX to different promoters varies and therefore *MYC* levels can lead to different effects (Lin et al. 2012, Nie et al. 2012). The MYC-MAX complexes activate gene expression but, if these complexes involve the *MYC*-integrated factor MIZ-1, the promoter binding represses the target genes (Wiese et al. 2013, Walz et al. 2014). The interactions between MYC-MAX and MIZ-1 comprise the regulatory core of MYC's functions in normal and malignant physiology.

MYC's involvement in tumorigenesis depends on its cell cycle promoting effects, ability to reprogram cells into a de-differentiated pluripotent state (Takahashi and Yamanaka 2006,

Takahashi et al. 2007), ability to increase protein synthesis (Mateyak et al. 1997) and ribosomal biogenesis (Kim et al. 2000, Gomez-Roman et al. 2003, Meyer and Penn 2008).

MYC and Cell cycle

MYC expression is found in human embryonic stages and in highly proliferating adult tissues (Bettess et al. 2005, Shen et al. 2013). In quiescent tissues, MYC expression is low, non-detectable or non-existent. Several studies have implicated MYC in cell cycle regulation at different levels. The knockout of *MYC* in mice proved to be lethal at embryonic stage, with embryos lacking the essential proliferation, tissue growth arrests their development preventing them to survive further than day 9.5 (Baudino et al. 2002, Wilson et al. 2004). MYC's capacity to stimulate cell proliferation derives from its ability to prevent cell cycle exit and from MYC's ability to force resting cells to re-enter the G1 phase of the cell cycle. This notion is based on early findings that show how ectopic expression of *MYC* can overcome proliferation arrest due to growth factor-deprivation, pushing the cells back into an active cell cycle (Eilers et al. 1991). Furthermore, the pioneering studies of Marie Henriksson and Bernhard Luscher demonstrated that enforced *MYC* expression prevents cells to enter nutrient-deprivation induced cell cycle arrest (Henriksson and Luscher 1996). Similar results were obtained in studies using adhesion-induced cell cycle arrest, also demonstrating *MYC*'s critical role in enforcing cell cycle (Benaud and Dickson 2001). *MYC* induced metabolic changes, essential to answer the high demands of the induced proliferative state, are discussed below.

MYC interacts with a broad number of cell cycle regulators that orchestrate the progression from G0/G1 phase into S phase (Figure 9). Progression of the cell cycle is mainly regulated by sequential actions of Cyclin-Cdk complexes. Cyclin-dependent protein kinase (Cdk) is the catalytic subunit of the complex and only becomes active when bound to Cyclin (the regulatory unit of the complex). Cdks, when active, can phosphorylate different substrates, including the retinoblastoma protein (Rb), which leads to the activation of the E2F transcription factor that will activate target genes related to DNA synthesis. Several inhibitors of the Cyclin-Cdk complexes, such as p15, p16, p21 or p27, also play a critical role in control of cell cycle progression.

MYC manages to promote cell cycle progression by activating the S-phase promoting E2F, Cyclins (D1, D2, E1 and A2), Cyclin dependent kinase 4 (CDK4) (Perez-Roger et al. 1999, Hermeking et al. 2000, Meyer and Penn 2008) and cell division cycle 25A (CDC25A). *MYC* also drives cell cycle progression by repressing the transcription of Cdk inhibitors p21, p27, p15 / p16 (Staller et al. 2001) and INK4A (when interacting with Miz-

1 or FOXO3a) (Staller et al. 2001, Yang et al. 2001, Dang et al. 2006, Bretones et al. 2015).

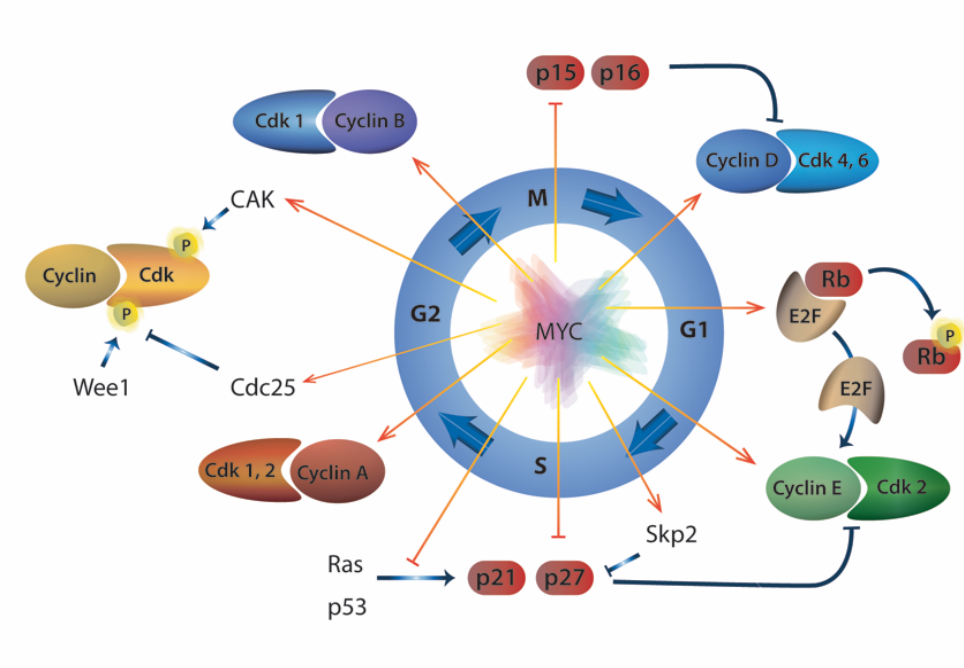

Figure 9 – Representations of the cell cycle phases and their main regulators, highlighting *MYC*'s activating or repressive effects on cell cycle regulators.

MYC role in apoptosis

Early discoveries revealed that MYC's function were not only able to sustain uncontrolled proliferation, but also to induce programmed cell death or apoptosis. Apoptosis effects seem less conserved than the proliferative effects, as they vary more with *MYC* expression levels, cell type and cells biological state (Askew et al. 1991, Evan et al. 1992, Shi et al. 1992). In mammals, apoptosis is regulated via two principal pathways, which are the cell death receptor or extrinsic apoptosis pathway and the mitochondrial regulated intrinsic pathway (Tiwari et al. 2015). In the mitochondrial pathway, the balance between anti- and pro-apoptotic Bcl-2 family proteins determines the outcome of the apoptosis program. The pro-apoptotic multi BH3-domain proteins BAK and BAX when co-localized at the mitochondrial outer-membrane are able to form oligomers. These oligomers pierce the mitochondrial outer-membrane resulting in the release of cytochrome c and other pro-apoptotic proteins into the cytosol. The mitochondrial release of pro-apoptotic effector proteins ultimately activates the caspase-dependent apoptotic process (Elmore 2007). MYC can induce BAX translocation to the mitochondrial outer-membrane, where it will oligomerize to activate the apoptosis machinery (Annis et al. 2005). In mammary epithelial

cells, BAK appears to play a dominant role in MYC-dependent sensitization to apoptosis. MYC activation leads to conformational activation of BAK that "primes" cells to apoptosis, and further death receptor binding TRAIL ligand (TNF-related apoptosis-inducing ligand) induces BAK/BAX oligomerization and cytochrome c release (Nieminen et al. 2007). Furthermore, MYC has also been shown to repress NF-kB as a sensitization mechanism (Klefstrom et al. 1997). NFkB has been implicated in the survival signaling, for example, via transcription of anti-apoptotic BCL-2 family members (PUMA) (Wang et al. 2009). These results suggest that NF-kB as a role in cancer cells evading apoptosis (Klapproth et al. 2009). Moreover, MYC is indirectly involved in p53 expression, which is a well-known tumor suppressor protein, with cell cycle and apoptosis regulating abilities. DNA damage and other cell stress signaling events induce p53 expression, which will drive cells into apoptosis. In this mechanism, *MYC* induces ARF expression that will inhibit MDM2, which normally represses p53 expression. Furthermore, the regulation between MYC and p53 seems mutual since previous studies showed that p53 can bind to *MYC* promoter decreasing its transcription (Hoffman and Liebermann 2008).

MYC and Metabolic transformation

A sufficient supply of oxygen and nutrients is paramount for cell survival. In normal cells if this premise is not fulfilled, cells exit cell cycle and upon continued stress they will undergo apoptotic or autophagy process. In healthy cells, cell cycle relies on the hydrolysis of ATP to ADP as a source of energy (Hardie et al. 2012). ATP is primarily obtained via oxidative phosphorylation (OXPHOS), by the ATP synthase located at the mitochondria electron transport chain (Chaban et al. 2014).

Warburg effect, first reported back in the 50's, describes how cancer cells switch their main metabolic process to anaerobic glycolysis even if in the presence of sufficient oxygen. At that point it was unclear what would be the advantage of switching into an anaerobic respiration as source of energy, and it was hypothesized that cancer cells presented faulty mitochondria that were unable to performed aerobic respiration. Following studies clarified that cancer cells do not present any defects in the OXPHOS and they purposefully switch to anaerobic glycolysis as energy source (Fantin et al. 2006). Although less efficient in terms of energy production, it proved to be a faster process than the OXPHOS for production of the needed biomass (Shestov et al. 2014). Due to the high proliferation rate observed in tumor cells, it is critical that they maintain a high levels of both ATP and nutrients to suffice the demands of the newly formed cells (Vander Heiden et al. 2011). Decades after the initial Warburg observation, the metabolic transformation has been accepted as a hallmark of cancer (Hanahan and Weinberg 2011).

Due to *MYC*'s universal ability to bind to promoter E-boxes, it is able to modulate the expression of several metabolic regulators in the glycolytic and glutamine metabolism, including a direct and indirect modulation of not only the transporters and enzymes involved in glycolysis and glutaminolysis, but also the ones involved in fatty acid synthesis, serine and mitochondrial metabolism (Dang et al. 2009, DeBerardinis and Chandel 2016).

Cells use glutamine metabolism in parallel to glycolysis to obtain energy and nutrients, which is performed in slightly different ways in each tissue. For example, kidneys use glutamine catabolism to maintain stable pH levels (producing ammonia) and as carbon supply for gluconeogenesis (Stumvoll et al. 1999). Lungs, skeletal muscle and adipose tissues rely on the synthesis of glutamine by glutamine synthetase (GLUL) (Hensley et al. 2013). Observing the varied ways glutamine is used in normal tissues, it is no surprise that there is not a unique tumor metabolome profile and that metabolic transformations seem to differ amongst tissues, tumor type and tumor microenvironment (Vander Heiden et al. 2011, Cluntun et al. 2017).

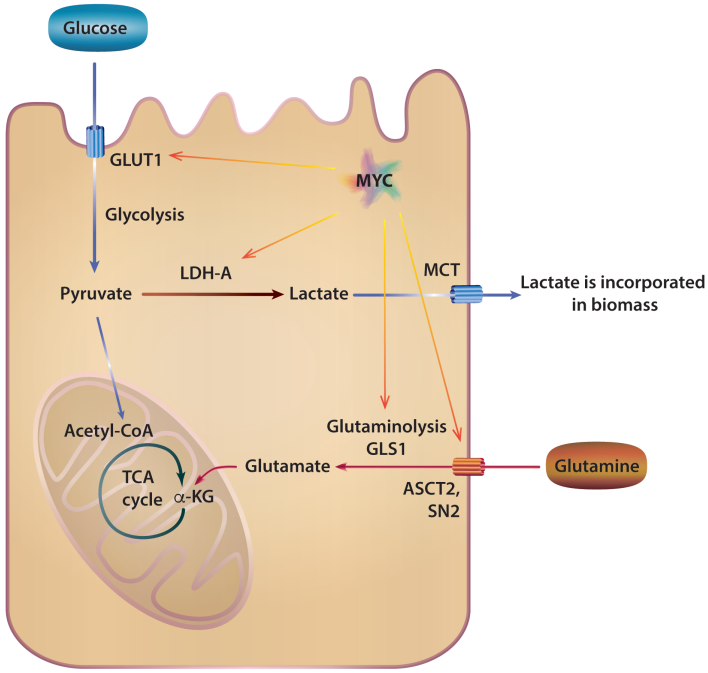

Figure 10 - Simplified diagram of *MYC*'s key actions on glycolysis and glutaminolysis. *MYC* is able to increase the uptake of glucose and glutamine by activation of their transmembrane transporters. *MYC* also modulates glycolysis and glutaminase by activation of key enzymes in both metabolic pathways.

In *MYC*-driven tumors, *MYC* has been shown to increase the intake of glucose into the cells by modulating the membrane bound glucose transporter GLUT1 (Osthus et al. 2000). Once

glucose is inside the cell, glycolysis pathway proceeds normally till the formation of pyruvate. *MYC* is then responsible for increased lactate dehydrogenase A (LDH-A) activity, resulting in a shift of the primary usage of pyruvate into the production of lactate which, after exiting the cell, integrates to the proliferating cells biomass. *MYC* has also been shown to affect glutaminolysis at two levels by modulating glutamine transporter activity, allowing a higher intake of glutamine, (Wise et al. 2008) and by increasing glutaminase (GLS) activity (DeBerardinis et al. 2007). Once glutamine is inside the cells, GLS is responsible for its transformation into glutamate that will be further transformed into α-ketoglutarate (α-KG) which will incorporate into the tricarboxylic acid (TCA) cycle, also known as Krebs cycle, in the mitochondria. GLS activity is modulated by miR23a and miR23b, which *MYC* has been shown to repress. Consequently, by suppressing the GSL repressors, *MYC* is able to increase GLS activity (Gao et al. 2009) (Figure 10).

AIMS OF THE STUDY

I. Identify crucial genes and key signaling pathways for the establishment and maintenance of a non-malignant mammary epithelial morphology in 3D mammary epithelial cell culture.

   a. Use a library of shRNAs that target *Drosophila*-informed putative human epithelial integrity regulators (hEIR) genes to unravel which genes are crucial for the establishment of a normal epithelial architecture with proliferation limiting features.
   b. Determine if hEIR co-operates with *MYC* oncogene to alter the structures epithelial architecture by altering their proliferation control.
   c. Identify the cell signaling pathway modulated by Par6 family knockdown.

II. Determine the molecular mechanisms underlying the synthetic lethal interaction observed upon combined RHOA knockdown and *MYC* activation.

III. Develop a suitable statistical framework to analyze the primary morphological data resulting from shRNA screen performed in 3D epithelial culture for phenotypic similarities. In addition, compare at network level the phenotypic similarities to proteomic level interactions for generation of novel hypotheses.

IV. Explore genetic signatures of breast cancer patients with diagnosed bone marrow disseminated cells to identify determinants of early tumor cells dissemination and loss of epithelial integrity.

MATERIAL AND METHODS

MATERIALS

Materials used in the thesis studies are listed below along with a brief description and original publication where they were fully described.

Viral vectors	Description (supplier)	Publication
pBABE MycER	Retroviral expression vector with c-Myc fused with hormone binding domain of estrogen receptor (ER), tamoxifen inducible. (Acquired from Dr. Gerald Evan)	I - II
pDSL_hpUGIH	Lentiviral shRNA vector containing GFP marker and hygromycin selection marker. (Alliance for Cellular Signaling, Berkeley, CA, USA)	I
pENTR-H1	Modified entry vector for gateway cloning. (Biomedicum Functional Genomics Unit, Helsinki, Finland)	I
pGIPZ	Lentiviral mIR-30 based shRNA vector containing GFP marker. (Open Biosystems, Fisher Scientific, Vantaa, Finland)	I
pLKO.1-puro	Lentiviral shRNA vector containing puromycin selection marker (Broad Institute TRC library, MISSION TRC-Hs 1.0 library; Sigma, St Louis, MO, USA).	I

Cell lines	Description (supplier)	Publication
MCF10A	Non-tumorigenic human mammary epithelial cell line (American type culture collection - ATCC).	I, II, IV
MCF12	Non-tumorigenic human mammary epithelial cell line (American type culture collection - ATCC)	Unpublished data related to I
MCF7	Human breast adenocarcinoma cell line (Acquired from Dr. Volker Assmann (UKE)).	IV
KPL-1	Human breast cancer cell line derived (Acquired from Dr. K. Iljin (VTT, Espoo, Finland)).	IV
CAMA-1	Luminal-type human breast cancer cell line, expressing high levels of estrogen and androgen receptors (Acquired from Dr. K. Iljin (VTT, Espoo, Finland)).	IV
HEK-293-ft	Human embryonic kidney fibroblast cell line (American type culture collection - ATCC).	I, II, IV

Note that all the respective cell culture recipes are described in the original publications

Reagents	Description (supplier)	Publication
Hoechst 33258	Cell-permeant blue dye that binds to DNA at the A-T minor groove (Sigma-Aldrich).	I, II, IV
4-Hydroxytamoxifen (4- OHT)	Selective estrogen receptor modulator and major active metabolite of tamoxifen (Sigma-Aldrich).	I, II, IV
MK-2206	AKT inhibitor; MK-2206 is an allosteric inhibitor which inhibits auto-phosphorylation of both AKT T308 and S473.	I - II
GDC-0941	PI3K inhibitor; GDC-0941 binds the ATP-binding pocket of PI3K and prevent formation of the second messenger PIP3 from PIP2, and thus blocks the signal transduction to downstream effectors of PI3K (Selleckchem).	I
BX-795	PDK1 inhibitor; BX-795 effectively blocks PDK1 activity by their ability to block phosphorylation of S6K1, Akt, PKCδ, and GSK3β (Selleckchem).	I
Rho inhibitor C3	Commonly known as botulinum neurotoxin C3 though it has no toxicity. Selectively ADP-ribosylates and thereby inactivates the effector domain of the Rho family of GTP-binding proteins (Sigma-Aldrich).	II
Rho activator II	Activator for Rho pathway; Reagent enters the cell and activates Rho GTPase isoform (CT04, Cytoskeleton)	II
ROCK inhibitor Y-27632	Selective, ATP-competitive, inhibitor of Rho-associated protein kinase (ROCK) (Sigma-Aldrich).	II
CCG-1423	RhoA/SRF inhibitor; Inhibitor against RhoA- and RhoC-mediated cellular activities by targeting signaling events downstream of Gα12/13 and RhoA/C, affecting MKL recruitment and/or post recruitment function of MKL1, but not SRF-SRE interaction or ROCK kinase activity (Sigma-Aldrich).	II
ML-7	Selective myosin light chain kinase inhibitor (Sigma-Aldrich).	II
Recombinant human TRAIL	TRAIL (TNF-related apoptosis-inducing ligand), also known as APO-2 ligand, is a type II transmembrane protein with a carboxy-terminal extracellular domain, which exhibits homology to other TNF family members. TRAIL transcripts have been shown to be constitutively expressed in a variety of human tissues (R&D systems).	II

METHODS

The relevant techniques applied during this study are listed below with the reference to the publication where they were used and fully described.

Methods	Publication
Cell culture (2D & 3D)	I, II, IV, Related publication IV
shRNA design, cloning and validation	I - II
qPCR	I
Western Blot	I - II
Lentivirus production and transduction	I - II
Immunofluorescence staining (IF)	I, II, IV
Microscopy imaging & analysis	I – IV
Cell cycle analysis	I
Cell-cycle re-entry assay	I
Flow cytometry (PI staining)	Unpublished data related to I
Caspase 3/7 activity assay	II, Related publication IV
Statistical analysis	I, III, IV, Related publication IV
*** Animal experiments (planning and execution)**	Related publication II, IV
Primary cells isolation and culture	II, IV
Fat Pad Transplantation (syngrafts and xenografts)	II, IV
Drugs administration	II, IV
Tumor formation follow up (palpations)	II, IV
Primary tumor recession without euthanasia	IV
Blood collection	II, IV
Tissues collection upon euthanasia	II, IV
Mouse colonies maintenance	II, IV
*** Patient derived 3D explants culture (PDEX)**	Related publication IV
*** Immunohistochemistry (IHC)**	Related publication II, IV

* Techniques used exclusively in the related publications are listed here only for demonstration of the broad spectrum of methods used during the thesis time period. Full description of these methods can be found in the original publications.

All the methods are thoroughly described in the original publications. A short description of the essential methods to the core publications of this thesis is presented below. The techniques exclusive to the related publications are not further mentioned below in order to keep this section concise.

Cell culture

Cell lines were stored at -180 C when not in use. Cells were cultured in their adequate culture medium at 37 ºC in a 5 % CO_2 humidified atmosphere. All used cell lines are listed in the materials section and respective culture mediums recipes can be found in the original publications.

Three-dimensional cell culture (3D) (I, II, IV)

Matrigel™ basement membrane matrix was used throughout the experiments used in this thesis. Matrigel is a solubilized basement membrane extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma. This preparation contains extracellular matrix proteins such as laminin (major component), collagen IV, heparan sulfate proteoglycans and entactin/nidogen, thus providing a suitable environment for the epithelial cells to grow and form mature acinar structures. In the related publication II, egg white was also used instead of Matrigel. The application of different materials as extracellular matrix differ in the cell seeding protocol and due to the matrices physical and chemical properties in the 3D structures maturation times and overall development. Several matrices are commercially available to perform 3D cultures. Note that all should be tested for the cell line or primary cells before endeavoring large studies.

We opted to use eight-chamber slides (Thermo Scientific) for 3D culture. This allows multiple independent treatments and staining per slide. After fixation, these slides can be imaged without the need to transfer the cultures out of the slides. Upon removal of the cultures from the slide, cells can be used for western blot or embed into paraffin blocks.

The eight chamber slides (Thermo Scientific), pipets and pipet tips were precooled and Matrigel thawed to 4 ºC. Each chamber was carefully coated at 4 ºC with 35 μl of the Matrigel™ and further moved to 37 ºC to solidify. Confluent cells in culture were detached from the culture plates with 0,05 % Trypsin EDTA (Sigma), suspended in a mix of culturing media and 2 % Matrigel. The optimal amount of 1500 cells were seeded per well and medium was refreshed every 3-4 days.

shRNA design and validation (I)

All shRNA were designed to target the first and / or longest ENSEMBL transcript of the selected genes. In order to find a functional shRNA, 1-3 shRNA target sequences were chosen per transcript. The designed shRNA oligos were cloned into pDSL_UGIH lentiviral shRNA vector as fully described in publication I supplementary data. To successfully target all the selected genes, shRNAs in pLKO lentiviral vector and in pGIPZ mir-30-based

vector were purchased. The complete list of transcripts and genes that comprise the shRNA hEIR-targeting library can be found in publication I supplementary data.

For shRNA validation, after lentiviral transduction, MCF10A cells were selected with hygromycin or puromycin depending on the lentivector, using a concentration previously titrated for MCF10A cells (data provided by the Functional Genomics Unit). Since shRNA's were lentivirally introduced into the cells, further experiments, including knockdown validation, were performed in BSL2 facilities till all the cells tested negative for Replication Competent Virus (RCV) (test was performed by the Functional Genomics Unit).

RT-qPCR (I)

Quantitative polymerase chain reaction (qPCR) was used to quantify and verify the specificity of the used shRNA knockdowns. Primers used and validation results for the shRNA used in experiments are also provided in publication I supplementary data. For our screen purposes we defined a working shRNA if a minimum of 25 % downregulation was obtained.

RNA from the cells was collected with RNeasy isolation kit (Qiagen). Tissues for RNA isolation (related publication VI) were first homogenized in the presence of ceramic beads (CK14, Bertin Technologies) in a Precellys homogenizer (Bertin Technologies). For the cDNA synthesis we utilized the DyNAmo cDNA synthesis kit (Finnzymes). All qPCR reactions done in the scope of our screen were performed using DyNAmo HS SYBR Green qPCR kit (Finnzymes), in AbiPrism 7500 Fast Real-Time PCR system (Applied Biosystems). Experiments and mRNA relative levels were analyzed by applying the $\Delta\Delta C_T$ method using housekeeping genes expression for normalization (methods and supervision by Functional Genomics Unit).

Western Blot (I – II)

Depending on the antibodies availability, Western Blot was also used, separately or in combination with qPCR, to verify the selected shRNA efficiency. This method was also used to evaluate protein expression alterations in signaling pathways induced by experimental manipulations. Detailed information regarding all antibodies used can be found in the original publications.

Cell lines were lysed in their culture plates after a PBS wash to cleanse any cell culture medium residues. ELB lysis buffer [150 mM NaCl, 50 mM HEPES pH 7,4 (Sigma), 5 mM EDTA (Sigma), Nonidet 1 % NP-40 (Fluka)] supplemented with a commercially available protease inhibitor cocktail [cOmplete EDTA-free (Roche)] was used for all cell lines.

Lysates were passed through a 20-gauge needle and kept on ice for 10 min. Finally, cell debris were discarded by centrifugation of the lysates for 15 min at 13 000 rpm, +4 °C. Protein concentrations were measured using BioRad DC™ Protein Assay Kit at 690 nm absorbance in a Multiskan Ascent (Thermo) with Ascent software.

Lentivirus production and transduction (I)

Lentiviruses were used to produce cell lines with the validated shRNA's targeting our genes of interest. Production of lentiviral particles and cell lines transductions were done according to Functional Genomics Unit standard procedures.

Lentiviral vectors are transfected into 293ft cells growing for one day in ultra-adherent plates (24-well or 6-well plates). The selected transfer plasmid (shRNA expression vector): Delta 8.9 (packaging): pCMV-VSVg (envelope) are mixed in a weight concentration ratio of 4:3:2 (tot. 10 µg; 4.44 µg, 3.33 µg, 2.22 µg) in 150 mM NaCl and incubated for 5 min at room temperature. Transfection mix consists in JetPEI and 150 mM NaCl, incubated for 5 min at room temperature. Lentiviral vector mix and transfection reagent JetPEI mix were then combined and incubated for 20 min at room temperature before being drop wise added to the cells. Transfected cells were incubated at 37 °C in 5 % CO_2 ambient, for a minimum time of 4h, before complementing the wells with fresh media. Viruses were harvested after 72h by filtering the media through a 0.45 µm filter.

Lentiviral infection was performed on cells, seeded on the previous day, which reached an estimated confluence of 50-60 %. After carefully discarding the cell culture medium and washing the cells once with PBS, the freshly harvested virus combined with the cells respective culture medium in a ratio of 1:1 was gently added on top of the cells. Polybrene was added to the cells at a ratio of 1:1.000 (8 µg/µl) and incubated for 10min at 37 °C. To improve virus transduction, plates were centrifuged at 770*x g* for 30 min at room temperature, and further incubated for a minimum of 4h. After the last incubation time, the medium containing virus particles was replaced for fresh media. 72h after infection cells were either lysed for RT-qPCR and/or Western blot analysis to evaluate the knockdown efficiency. For experiments done under biosafety level 2 conditions, these cells were place into Matrigel® for our 3D culture assays.

Immunofluorescence staining (IF) (I – II, IV)

This antibody-based fluorescent-labelling technique allows the visualization and eventually quantification of the proteins of interest under the fluorescence microscope. We applied it to verify proliferation, apoptotic and core signaling proteins activation in cells grown either in 2D or 3D cultures.

For the 2D cultures, cells were cultured on top of coverslips and upon experimental terminus they were fixed with 4 % PFA, permeabilized with 0.1 % Triton-X and sequential washes and incubation times were followed accordingly to the laboratory standard operating procedure.

The 3D grown cultures were fixed with 2 % PFA and permeabilized with 0.25 % Triton-X100. Blocking of non-specific binding sites was done for 1- 2h in IF buffer (7.7 mM NaN3, 0.1 % BSA, 0.2 % Triton X-100 (Sigma) and 0.05 % Tween20 in PBS) supplemented with 10 % normal goat serum. Primary antibodies were incubated overnight at +4 °C and afterwards washed 3 x 15min with IF buffer in gentle rocking. Appropriate Alexa Fluor secondary antibodies were incubated at room temperature for 40 – 50min and Nuclei counterstain was done for 10-15min with Hoechst33258 (1:10 000 in PBS). The eight-chamber slide wells and glue attaching the wells walls were gently removed and slides were mounted with Immu-Mount reagent.

Microscopy imaging & analysis (I – IV)

Immunofluorescent stained samples are viable for imaging after storage in the dark at +4 °C for no longer than one week. Note that antibodies targeting phosphorylated proteins in our 3D structures were in general less efficient and/or less stable in storage.

Samples for quantitative purpose were imaged and/or analyzed in Zeiss Axio Imager Z2 upright epifluorescence microscopes. Samples exclusively used to evaluate morphometric changes were imaged with Zeiss Axiovert 200 microscope equipped with Apotome system. Shape descriptors were measured with Image J software (version 1.42q) using Nuclei and E-cadherin staining to visualize the borders of the acini structures. We defined that a minimum of 30 acini structures should be imaged per experimental condition.

For detailed images we took advantage of the confocal laser scanning microscopy. Immunostained cells and structures were imaged using Zeiss LSM Meta 510 and 780 confocal microscopes equipped with argon (488), helium-neon (543 and 633) and diode (405) lasers and Plan-Neofluar 40x DIC objective (NA=1.3, oil). Z-Stack images were processed into videos and 3D images with Image J software plugin 3D de-convolution. Microscopes, training and imaging advice were kindly provided by Biomedicum Imaging Unit.

Tumor-Suppressor Predictor (TSP) (I)

In the scope of publication I, together with our collaborators, we developed a TSP algorithm. The algorithm searches evidence for loss of gene function in different cancer by using information from The Cancer Genome Atlas, Catalogue of Somatic Mutations in

Cancer and Tumorscape. Searches are focused on loss of gene expression, somatic mutation frequency and chromosomal deletions. The algorithm compiles information that supports a loss of gene function and calculates a single value that scores the total evidence of tumor-suppressor function for each human gene. The TSP algorithms, source databases and methods of data derivation are described in Supplementary Materials of publication I.

Cell cycle analysis (I)

Cell-cycle re-entry assay

We adopted a commonly used cell-cycle arrest assay to synchronize the cells in culture, to access what pathway alterations are preventing this arrest and which ones are needed to drive quiescent cells re-enter a normal cell cycle. The cells lines with validated knockdown of our genes of interest, were examined in 2D and 3D, with or without MYC activation, for a possible impairment to enter cell cycle arrest and for their ability to re-enter it. We optimized the deprivation culture media and time points for full cell cycle arrest in MCF10A and MCF12 cell lines. In 2D culture cells were driven into a reversible quiescent state upon withdrawal of nutrients and serum growth factors. Cell cycle arrest was observed at 24h for MCF10A or 48h for MCF12 in culture without EGF/INS (MCF10A) or essential nutrients (MCF12) deprived medium. Cells fully re–enter cell cycle after 24h in culture with a complete medium replenished with nutrients and growth factors. In 3D cultures, cells enter a quiescent status upon acinar structures full maturation. Using Matrigel, this should happen after 7 days in culture with complete cell culture medium. In 3D cultures the cell cycle re-entry seemed only possible upon a combination of epithelial integrity impairment and MYC activation.

Flow Cytometry (PI staining)

Propidium Iodide (PI) is a cell-impermeant red-fluorescent dye commonly used to counterstain nucleus and chromosomes. Fluorescence emission is enhanced upon its' binding to DNA (nonspecific intercalation between the bases) allowing the researcher to access the viability or cell cycle phase of the cells under analysis. This allows the differentiation between cells in G0/G1, S and G2 cell cycle phases due to their different chromosome density.

For flow cytometry analysis we collected approximately 1 million cells per experimental condition into pre-coated (2.5 % BSA in PBS) FACS tubes. After centrifugation (1600 rpm for 5 min) to get rid of the detaching agent (0.05 % Trypsin-EDTA) cells were fixed in absolute ethanol at -20°C and incubated overnight at 4 °C. After centrifugation (2500 rpm for 5 min) and one PBS washing steps, cells were treated for 30 min at 37 °C with RNase A (1 mg/ml) (Sigma-Aldrich) to avoid the unwanted binding of PI to RNA. Staining with 20

μg PI (Thermo-Fischer) was done keeping the cells protected from light for a minimum time of 30min at room temperature. Samples were analyzed at a slow flow rate (< 500 events/sec) on the Accuri C6 flow cytometer (BD Biosciences). Staining protocol and gating techniques were done according to Biomedicum Flow Cytometry Core Facility standard operating procedures.

Caspase 3/7 activity assay (II, Related publication IV)

Caspase.Glo® 3/7 assay (Promega) was used in the mentioned publications to quantify cell death induced by pathway inhibitors and drugs. This robust and straightforward assay consists on a substrate that lyses the cell and upon caspase cleavage generates a "glow-type" luminescent signal.

In the refereed studies cells were seeded on low adhesion plates (primary cells) or in normal 96-well plates (cell lines). They were cultures for 48h, allowing either the formation of mammospheres (low adhesion) or seeding into the plates (normal adhesion). After the aforementioned culture time, drug treatments were maintained for 24h. In the correspondent groups, Myc-ER was activated (using 100nM 4-OHT) after 24h of culture. Manufacture's protocol was followed and luminescence (RLU) was measured with VICTOR™ x3 plate reader (PerkinElmer).

Statistical analysis

To inspect the collected data and prove null hypothesis, statistical analysis was used in all the original publications. Descriptive analysis like average, mean, standard deviation, standard error variation, frequencies and normality analysis of the data were calculated. For the inferential analysis, which refers to testing null hypothesis, the parametric paired t-Test and two-way ANOVA were used. The non-parametric analysis Wilcoxon and MMD were used in publication III.

Descriptive statistical analysis and data wrangling was primarily done in Excel. For publications, most visual representation of the descriptive analysis was done in GraphPad Prism, complemented with frequencies analysis done in SPSS. Inferential statistics to test data normality and null hypothesis was performed in SPSS and GraphPad. In a collaboration effort, for publication III, R language was used for the MMD statistics and integration of the resulting phenotype networks with STRINGdb. Cytoscape software was used to visualize the phenotype networks obtained. All used algorithms are fully described in the original publications.

RESULTS AND DISCUSSION

LENTIVIRAL SHRNA SCREEN FOR HUMAN EPITHELIAL INTEGRITY REGULATORS UNRAVELS GENES WITH TUMOR SUPPRESSIVE ABILITY AND INTERESTING SYNERGIES WITH ONCOGENIC MYC. (I)

This study was designed to identify putative human epithelial integrity regulator (hEIR) genes and to determine their roles in oncogenic transformations. The hEIR genes are human orthologues of known epithelial integrity regulators in Drosophila, including regulators of cell polarity, cell adhesion, Wnt and Hippo pathways. To address this question, we opted for an approach based on shRNA-mediated gene silencing in 3D cultures of the non-tumorigenic mammary cell line MCF10A. This cell line presents characteristics similar to the basal mammary epithelial cells and when 3D cultured in Matrigel (solubilized basement membrane extracted from the Engelbreth-Holm-Swarm mouse sarcoma) forms acinar cysts that resemble the glandular structures present in the mammary epithelium. Due to their ability to mimic the normal epithelial architecture of the mammary gland, the 3D cultures of MCF10A cells have been extensively used in a number of studies (Bissell and Hines 2011, Qu et al. 2015, Choi et al. 2016). In normal conditions, MCF10A acinar structures are formed either from a single cell or by migration and aggregation of neighboring cells. Cells proliferate till they form a single layered round structure (acini) with a hollow lumen. Studies have shown that the pro-death mediator BIM is required for lumen formation in 3D cultures of MCF10A cells, indicating that lumen is formed by cavitation, the clearance of cells located in the center of the acini by apoptosis (Debnath et al. 2002, Reginato et al. 2005, Mailleux et al. 2008). Upon structures full maturation, occurring between day 7 and 10 in culture, the cells forming the structures enter a quiescent status (cell cycle arrest) while remaining biologically functional. We also took advantage of the retroviral expression vector Myc-ER, consisting of *MYC* fused to a hormone binding domain of estrogen receptor (ER). This allowed us to create the MCF10A-MycER cell line, where *MYC* oncogene can be conditionally activated by 4-hydroxytamoxifen (4-OHT), an active metabolite of tamoxifen and a selective estrogen receptor modulator. To create the cell lines used in the experiments, MCF10A-MycER cells were lentivirally transduced either with a shRNA control (non-target sequence) or with shRNA targeting our genes of interest.

The study was divided in two independent screens, with and without oncogenic MYC activation (I: Figure 1b; III: Figure 1b). In the first screen, shRNA transduced MCF10A-MycER cells were cultured for 10 days in order to allow the development of fully matured acinar structures. The first screen allowed the identification of genes that impair the

structures maturation, associated with a tightly controlled cell cycle arrest, and/or lead to the formation of aberrantly shaped acini. In the second screen, shRNA transduced cells were seeded as previously but *MYC* was activated either in a chronic or acute way. Chronic activation means that *MYC* activation was kept from day 1 to 10 of culture while acute activation means that *MYC* activation was performed from day 15 to 18 of culture. The screen with chronic *MYC* activation, allowed us to identify synergistic or additive effects between *MYC*'s oncogenic stimulus and loss of epithelial regulators during the development of the acinar structures. Previous studies have shown that once cells enter a quiescent state, cell cycle arrest and fully established epithelial integrity, the activation of *MYC* oncogene alone is unable to re-activate the cell cycle (Benaud and Dickson 2001, Partanen et al. 2007, Partanen et al. 2012). The screen with acute *MYC* activation, after cells entered cell cycle arrest, allowed us to identify the epithelial regulators that are critical in restraining *MYC*'s proliferative functions.

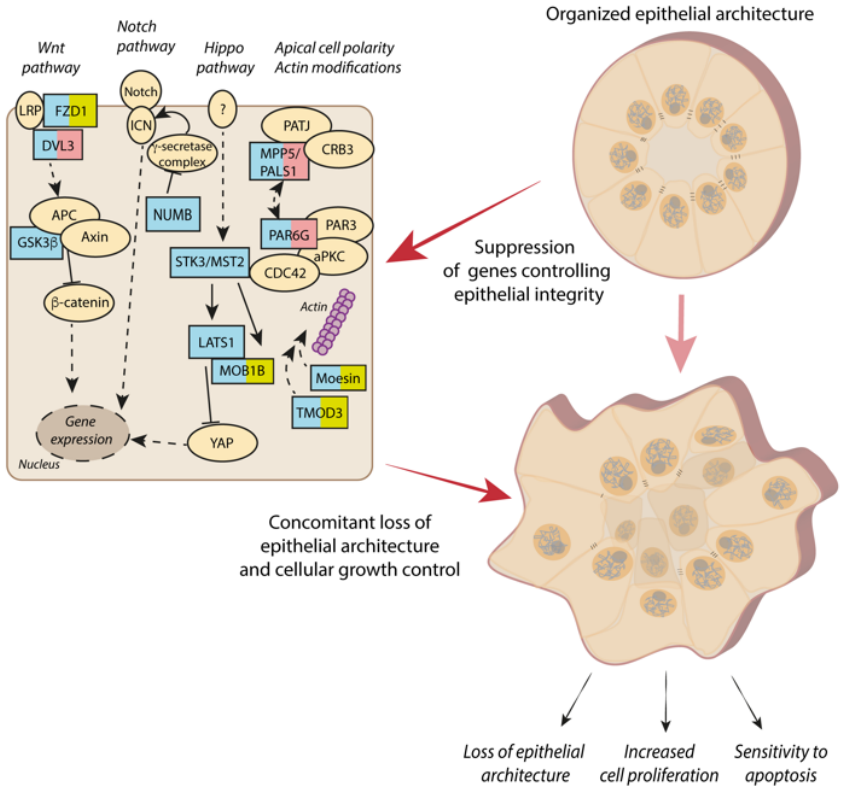

Figure 11 – Illustration of the rationale behind the study. We explored which of our genes of interest (represented on the left side) contribute to the loss of epithelial architecture, increased proliferation or sensitivity to apoptosis (represented on the right side).

Identification of genes that impair the cell cycle resulting in aberrantly shaped acinar structures.

The first part of this study aimed solely to identify which of the selected genes are involved in the development and maintenance of a normal architecture in the acinar structures. On day 10 of culture, we evaluated phenotype changes by measuring the area (size) and circularity (symmetry) of the formed structures (I: Figure 2a-d). After cautious observation of several structures, we considered a phenotype aberrant when the structures area and circularity diverged, respectively, 20% and 10% from the observed in the control structures. A total of sixteen gene knockdowns (out of the thirty-four) resulted in the formation of aberrant sized structures. From the eleven that presented abnormally larger area, five of them also presented loss of symmetry. The knockdown of DVL3 and MPP5 (PALS1) lead to the development of the most misshapen structures (I: Figure 2d-e). A detailed inspection of the structures formed under DVL3 downregulation revealed a disrupted apical polarity together with, what seemed to be, an uncontrolled branching phenotype.

To evaluate if the abnormally large structures derive from the inability to suppress proliferation upon maturation, we measured proliferation on day 18 of culture, when the majority of the structures are expected to have normally entered quiescence. Among the five genes that upon knockdown caused large and misshapen structures, the downregulation of *PARD6G*, *DVL3* and *MPP5 (PALS1)* (but not *NUMB* or *STK3*) compromised the cells ability to halt proliferation. Studies in Drosophila have identified two types of tumor suppressor genes: the hyperplastic tumor suppressor genes (hTSGs) and the neoplastic tumor suppressor genes (nTSGs). Loss of hTSGs results in altered cell cycle with no impact on the epithelial integrity, while the loss of nTSGs results in both the loss of cell cycle control and on altered tissue morphology, both features that promote metastatic potency (Bilder 2004). By analogy, *PARD6G*, *DVL3* and *MPP5 (PALS1)* genes could be considered human versions of nTSGs. It is worth of notice that all misshapen structures were also abnormally large. Altogether, these findings support the notion that the maintenance of epithelial integrity is essential for a proper cell cycle control.

Chronic MYC activation combined with downregulation of epithelial integrity regulators results in altered cell growth patterns and apoptosis.

The second part of this study focused on the potential cooperation between *MYC* oncogenic features and the loss of epithelial integrity. We primarily examined how the downregulation of hEIR would potentiate *MYC*'s proliferative effects. In the current study, the chronic activation of *MYC* was able to increase the control structures size in about 30%.

Out of the seventeen gene knockdowns that lead to the development of structures with aberrant size, nine were able to potentiate *MYC's* proliferative effects. Amongst these nine genes we identified *PARD6G*, *DVL3* and *MPP5 (PALS1),* that in the first part of the study were implicated in the formation of misshapen and large structures. On the other hand, the downregulation of *PARD6B and EZRIN* only promoted abnormally large structures when combined with chronic *MYC* activation (I: Figure 3a-d). Altogether, the results suggest that *MYC*-proliferative effects benefit from the loss of genes responsible for the maintenance of epithelial integrity and cell cycle control.

Interestingly, the downregulation of a set of genes counteracted the *MYC*-driven growth advantage (I: Figure 3b). In particular, the downregulation of *RHOA* resulted in a very particular phenotype with very small and misshapen structures. A detailed examination of these structures exposed a significant increase of active caspase-3 positive cells, indicating a widespread occurrence of apoptosis in these structures. This observation was the foundation for a follow up publication (II) where this synthetic lethality phenotype was further validated and mechanistically explored.

Epithelial integrity regulators are critical for restraining MYC-induced cell cycle re-entry.

In the second screen, we also evaluated another cooperative angle between *MYC* activation and loss of epithelial integrity. Here we focused on *MYC*'s limited ability to force cells to re-enter the cell cycle once structures had normally entered quiescence. Previous studies showed that, in mature acini, *MYC* activation was unable to induce cells to re-enter the cell cycle, while remaining sensitive to *MYC*'s apoptotic effects (Partanen et al. 2007). However, *MYC*'s ability to stimulate the cell cycle progression in mature structures is restored when epithelial integrity is disrupted, either by placing the structures into laminin-free hydrogels (impaired basement membrane connections and polarity), or by knocking down the tumor suppressor *LKB1* (*PAR4* homolog) (Partanen et al. 2007). Even though LKB1 has been mainly studied for its role in cell metabolism regulation, this protein is also able to regulate epithelial polarity and tissue architecture. Thus, we hypothesized that among our genes of interest, which included other PAR family genes, we could observe similar cooperation, between *MYC* activation and loss of polarity, enabling mature structures to re-enter cell cycle.

shRNA transduced MCF10A-MycER cells were cultured under normal conditions for 15 days, allowing them to form fully matured structures. Subsequently, we activated *MYC* (acute activation) and accessed the number of proliferative cells in the acinar structures. We observed a 20% or higher increase in the proliferative activity of the structures formed by cells lacking LLGL2, TMOD3, NUMB and PARD6B (I: Figure 4a). Moreover, concurrent with the results obtained in the first screen, the knockdown of *PARD6G* was

able to significantly increase cells proliferative activity, but since the knockdown of *PARD6G* alone already results in a very high proliferative activity (structures unable to enter quiescence), the combination with acute *MYC* activation at day 15 did not further enhance proliferation. Interestingly, the knockdown of *PARD6B* showed a cooperative effect with acute MYC activation, to significantly increase the proliferative activity in these structures. Note that the knockdown of *PARD6B* alone was unable to sustain cell proliferation but it did disrupt epithelial integrity, thus enabling MYC's ability to push cells to re-enter the cell cycle. The observed degree of synergy, between acute MYC activation and PARD6B downregulation, is comparable to the results previously reported with LKB1 (I: Figure 4 d). We conclude that both PARD6 genes included in this screen present the ability to influence epithelial cell cycle restriction.

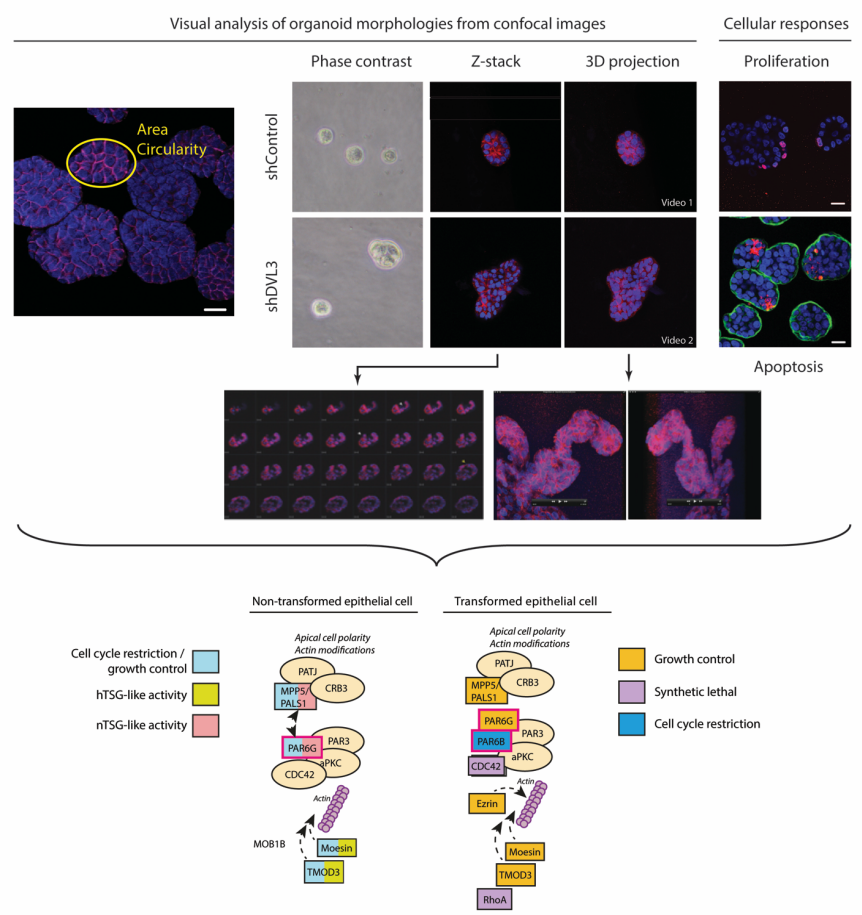

Figure 12 - Study schematics, showing the different approaches taken to evaluate the changes upon polarity genes knockdown. Upper panel shows how acini structures look under the microscope and the diverse parameters analyzed to access epithelial integrity changes. Lower panel shows how we categorized the observed phenotypes in the first (hEIR downregulation) and second screen (hEIR downregulation with chronic Myc activation).

The special case of PAR6 genes family: tampering with AKT pathway & dual roles in epithelial cancers.

The findings in this study indicate that both *PARD6B* and *PARD6G* have important roles in suppressing proliferation in epithelial cells upon maturing of the epithelial structures. To finalize this study, we wanted to explore which biochemical alteration and/or signaling pathways are implicated in the proliferation regulation by Par6 proteins. In humans, there is three Par6 proteins that are encoded by three different genes *PARD6A*, *PARD6B* and *PAR6G*. In brief, PAR6 is a multimodular scaffold protein that, together with PAR3 and aPKC, forms a conserved complex fundamental in the regulation of cell polarity (Assemat et al. 2008). Since these regulatory functions have been associated to a proper connection to active PKCz, when phosphorylated at threonine 410 (T410) (Whyte et al. 2010), we verified that both PAR6 knockdowns lead to a diminished T410/412 phosphorylation of PKCz (I: Figure 5a). Nonetheless, even in the presence of a seemingly impaired Par complex, cells were able to maintain a proper apico-basal polarity (I: Figure 5b).

Amongst the well-established proliferation related cell signaling pathways, we found that PAR6 downregulation was able to alter AKT signaling pathway. We observed a significant increase in phospho-AKT positive cells in the structures lacking *PARD6G*. Moreover, *PARD6B* knockdown leads to a noticeable but less significant increase in the number of phospho-AKT positive cells in the formed acinar structures. Interestingly, as shown previously for the proliferative activity, the increase in phospho-AKT positive cells was significantly higher when *PARD6B* knockdown was combined with acute MYC activation. Phosphorylation of ribosomal protein S6 (p-S6) by AKT, via mammalian target of rapamycin complex 1 (mTORC1) is a commonly used readout of AKT pathway's activation (Hassan et al. 2013). Similar to the observed with phospho-AKT, *PARD6G* knockdown alone and the combination of MYC activation with *PARD6B* knockdown resulted in an increased number of p-S6 cells in the acinar structures. Nonetheless, the S6 activation pattern (p-S6 positive cells increase ratio) is more sporadic than the observed with AKT activation, suggesting that the pathway activation is not linear throughout AKT pathway downstream elements.

It has been previously established that for a complete AKT activation, AKT needs to be phosphorylated at both T308 and S473 residues (Alessi et al. 1997, Cunliffe et al. 2012). In 2D experiments with control cells we were able to efficiently induce cell cycle arrest by culturing cells in a deprived cell medium. Interestingly, the knockdown of *PARD6B* and *PARD6G* were equally able to sustain AKT phosphorylation at both sites even when cultured in deprived cell medium (I: Figure 5g). Consistent with previous results, the

knockdown of *PARD6G* and to less extent of *PARD6B* was able to sustain cells proliferative activity (Ki67 positive cells) even when cultured in deprived cell medium (I: Figure 5h). The impact of these two genes in the cells cell cycle was further explored by flow cytometric cell cycle analysis in two non-transformed cells lines with conditionally active MYC (Figure 13 – unpublished data). However, the obtained results were less clear than previously, with *PARD6B* and *PARD6G* knockdown leading to only a slight increase in the number of cells found in the proliferative phases. In addition, in these experiments we did not observe a proliferative cooperation between MYC activation and *PARD6B* knockdown.

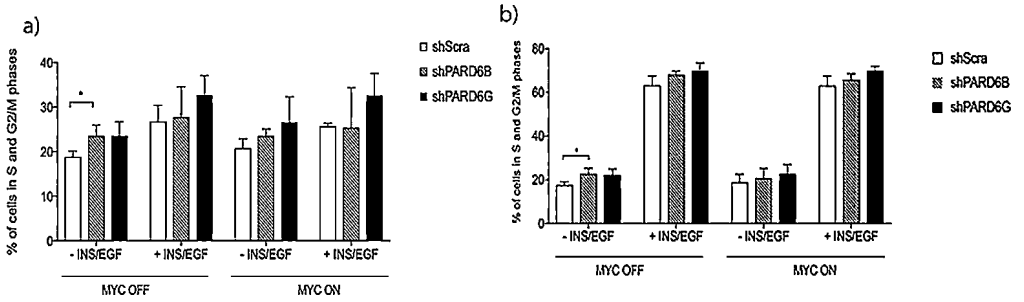

Figure 13 – Flow cytometry cell cycle analysis in a) MCF10A and b) MCF12A lacking *PARD6B* and *PARD6G*. Experiments were performed under growth factor deprivation and *MYC* activation. Data is presented as percentage of cells that were found in a proliferative phase of the cell cycle. Graphs shows mean and SEM values from three independent experiments, * $p<0,05$ (unpublished results)

We addressed the question of whether AKT activation by Par6 knockdown was due to the canonical PI3K/PDK1 pathway modulation by employing PI3K and PDK1 selective inhibitors. PI3K inhibition successfully abolished T308 AKT phosphorylation in all experimental conditions. Interestingly, the inhibition of PDK1 selectively abolished T308 phosphorylation previously induced in both PARD6B and PARD6G knockdown cells (I: Figure 5i). Furthermore, the increase in proliferation by PARD6G knockdown was partially abolished by inhibition of PDK1 (I: Figure 5j). Altogether, we found that via their inhibitory effects on PI3K/PDK1/AKT pathway, both Par6 proteins are necessary for normal cell proliferation suppression.

We finalized this study by exploring the status of *PARD6* genes in human cancers in publicly available databases, compiled in the cBioPortal database. We could clearly observe a pattern where *PARD6B* is mainly found upregulated, while *PARD6G* is found downregulated in several cancer types (I: Figure 6). We gathered information regarding gain- or loss-of-function modifications, gene amplifications or deletions, and loss of heterozygosity. The overall scenario, in epithelial cells, points out that *PARD6G* is mainly found downregulated and affected by loss of function genetic alterations,

whereas *PARD6B* is frequently found amplified, being involved in gene amplification and gain of function alterations. Of note, previous studies have convincingly reported the presence of *PARD6B* upregulation in breast cancer (Cunliffe et al. 2012), whereas other studies showed that induced *PARD6B* overexpressed results in increased proliferation via an extracellular signal-regulated kinase, mitogen-activated protein kinase (MAPK), pathway (Nolan et al. 2008). Altogether, ours and others results lead us to hypothesize that both *PARD6* genes have the potential to act via different signaling pathways depending on their expression levels (Figure 14). While *PARD6G* tumor-suppressor functions derive from its ability to repress AKT-induced proliferation, *PARD6B* oncogenic functions seem to be primarily related to induced proliferation via MAPK pathway (Figure 14). Very few studies addressed the potential differences between PAR6 proteins. In terms of their cellular localization, a study in Madin-Darby canine kidney epithelial cells, reported that PAR6A and PAR6G are mainly localized at the TJs, whereas PAR6B can be found more broadly distributed throughout the cytoplasm (Gao and Macara 2004). Nonetheless, it was previously reported that overexpression of PAR6B inhibits the formation of tight junctions (Gao et al. 2002). Other studies linked PAR6 proteins expression with the proper cell cytoskeleton distribution. In particular, PAR6G has been reported as a component of the mother centriole, controlling the centrosomal protein composition in a PAR6A-dependent way (Dormoy et al. 2013) and other study showed that PAR6B expression is required for the proper orientation of the mitotic spindle upon cell division (Durgan et al. 2011).

Altogether, considering this study results and the little information available about PAR6B and PAR6G, we can only hypothesize that the dual role of PAR6B and PAR6G in tumorigenesis is a consequence of their different ability to interact with proliferation-inducing pathways.

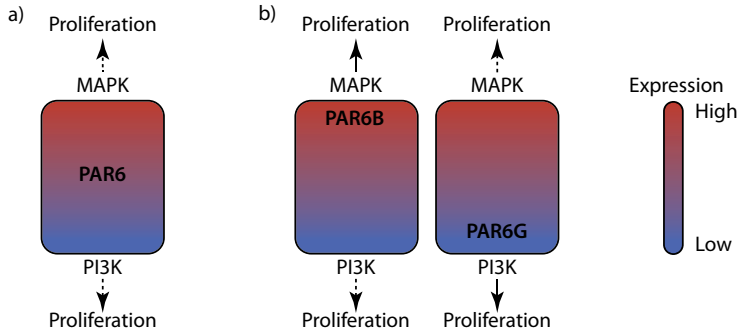

Figure 14 – Schematics of the hypothesized actions of PAR6 in proliferation regulation. a) In mammary epithelial 3D cultures, PAR6 overexpression induces MAPK pathway activation, whereas PAR6 downregulation induces PI3K/AKT pathway activation. b) Tumorigenic processes favor *PARD6B* upregulation and *PARD6G* downregulation (Image from (Marques and Klefstrom 2015).

SYNTHETIC LETHAL PHENOTYPE CAUSED BY THE PERTURBATION OF MYC-INDUCED GLUTAMINE METABOLISM VIA RHOA PATHWAY. (II)

In the previously described study, when screening for associations between MYC activation and deregulation of epithelial integrity regulators we came across an interesting synthetic lethality phenotype. The phenotype was observed when the expression of the small GTPase RHOA was downregulated in the context of chronic *MYC* activation (I). The mechanism behind this phenotype was the target of this follow up study (II). In our screen, we observed similar yet less dramatic phenotype upon chronic MYC activation and the knockdown of CDC42. Interestingly, both genes belong to the Rho GTPases family, and have been implicated in an anchorage-dependent anoikis process. In these studies, the phenotype was attributed to the loss of contact with the basement membrane and as consequence, to the loss of essential integrin connections (Weaver et al. 2002, Cheng et al. 2004, Ma et al. 2007). We further explored if the observed MYC-dependent lethality was derived from the loss of similar adhesion-dependent survival mechanisms. Of note, the knockdown of *RHOA* alone did not result in any noticeable change in the MCF10A 3D epithelial architecture. Nonetheless, chronic MYC activation (from day 1 to 10 in culture) could have impaired the establishment of critical integrin connections with the basement membrane (BM). To address this question, we primarily tested if the synergy between *RHOA* knockdown and MYC activation was also observed in the 2D context. As shown in II: Figure 1, all approaches to knockdown the *RHOA* pathway activity (siRHOA, Rho inhibitor, ROCK inhibitor) in the presence of active MYC, induced a significant increase in apoptosis (increased caspase 3/7 active levels). Even though, cells in culture can secrete basement membrane constituents, we believe that during the short culture time the cells are not able to establish a proper BM in 2D cultures. Therefore, we discarded the original hypothesis that the impaired integrin-contact with BM was the reason behind the observed lethality.

Contrary to other studies, in our MCF10A 3D cultures the downregulation of RHOA alone was unable to provoke noticeable polarity changes. Studies in canine kidney cells, MDCK, demonstrated that the blockage of β1-integrin signaling leads to an inverted polarity of the cells and the knockdown of RHOA/ROCK pathway was able to rescue this phenotype (Rogers et al. 2003, Yu et al. 2008). Moreover, others have noted an hyperactivation of RHOA accompanied by disruption of normal polarity in human epithelial cancers (Kato et al. 2012).

We next focused on the possible relationship between Rho GTPases (RhoA, RhoC and CDC42) and cancer metabolism (Wang et al. 2010). It is currently accepted that cancer cells undergo a metabolism switch, trading glucose for glutamine as their main source of

energy (ATP) (Lu et al. 2010). Several studies have observed a so-called glutamine addiction provoked by MYC and other oncogenes activation (Wise and Thompson 2010, Smith et al. 2016, Cluntun et al. 2017). A link between Rho GTPases and metabolism alterations was found during a screen searching for small molecules that could prevent key tumorigenic processes induced by Rho-GTPases (Wang et al. 2010). Note, that while Rho-GTPases are rarely found mutated in cancer, its altered regulation is suggested, since often changes in their expression and activity are observed. GTPase–dependent transformations in breast cancer models, have been efficiently reversed by a molecule that targets a specific isoform (splice variant) of the mitochondrial glutaminase enzyme (GLS1). GLS1 is part of the initial phase in the glutaminolysis process, responsible for catabolizing glutamine into glutamate (Jin et al. 2016, Lukey et al. 2016).

We hypothesized that the observed synthetic lethality would derive from overlapping effects of RHOA and MYC in glutamine metabolism. Indeed, both ectopic activation of RHOA and MYC resulted in an increase of GLS1 activity and consequently of glutaminolysis process. Furthermore, we successfully rescued the observed synthetically lethal phenotype by adding a downstream metabolite of GLS1 (alpha-ketoglutarate (α-KG)). These results indicate that the synthetic lethality observed upon RHOA knockdown with active MYC is dependent of glutamine metabolism.

In search of the mechanism behind this dependency, we based the following experiments in studies that previous implicated RHOA/ROCK pathway in the regulation of the serum response factor (SRF) transcriptional responses, which include cell survival. This pathway was of special interest since recent studies implicated RHOA/ROCK/SRF survival pathway as a critical target for MYC/MIZ1 complex induced apoptosis (Wiese et al. 2015). In our results a strong nuclear accumulation of SRF was noticed upon RHOA activation. Furthermore, activation of the RHOA/SRF pathway by the constitutively active forms of RHOA or SRF synergized with the previously observed MYC induction of GLS1 activity. However, we did not find evidence for a direct regulation of GLS1 by SRF, suggesting a regulation independent from a direct transcriptional targeting.

Taking in account the current results, we suggest that RHOA/SRF signaling and MYC cooperate to establish a pattern of glutaminolysis that is critical for the survival of transformed cells. With the current *in vitro* results indicating that cancer cells expressing MYC could benefit from SRF co-expression, we evaluated the levels of MYC and SRF expression in a panel of 39 clinical breast cancer samples. Interestingly, a direct correlation between high SRF protein levels and MYC was found in 67% of the analyzed samples. We therefore assume that, in breast cancers, MYC and RHOA/SRF pathways have a cooperative role in maintaining the glutamine metabolism active at a level that is required for the survival of transformed cells.

In conclusion, this study exposed a critical survival mechanism in MYC transformed cells. Targeting RHOA/SRF pathway as the potential to be an effective selective lethality therapy, aiming to therapeutically exploit the apoptotic vulnerability created by MYC in cancer cells.

STATISTICAL ANALYSIS FRAMEWORK COMBINING MMD-BASED PHENOTYPIC DISTANCES AND AVAILABLE PROTEOMIC DATA CAN BE USED TO PREDICT NOVEL BIOLOGICAL PATHWAYS. (I & III)

In the 3D shRNA screen, described in publication I, we successfully identified epithelial regulators genes that manipulate proliferation and apoptosis in a model of the mammary gland (MFC10A 3D culture). Knockdown of putative epithelial integrity regulators (hEIR), alone or in the presence of *MYC* activation, yielded 3D structures with moderate to severe altered morphology (size and/or symmetry). For the publication I, the strength of the morphological changes was evaluated by analyzing fold changes relative to the control samples in each experimental set. Although this is a perfectly correct method it limits the comparison of phenotypes obtained in the independent different experimental sets. In addition, the previous analysis method only allowed us to analyze one shape descriptor at the time, not allowing the comparison of the overall morphology of the structures (area and circularity).

In order to fully expose the data potential, we were keen to re-analyze it in collaboration with expert biostatisticians. We implemented a novel analysis framework that analysis the data at a population level, taking in account all the shape descriptors included in the initial experiments. This allows the creation of phenotype networks, were we can identify clusters of similar phenotypes and further explore the known links between the genes which upon knockdown lead to those phenotype clusters.

The morphometric analysis for all the genes included in the screen was performed in independent experimental sets in order to keep the shRNA validation and experiments manageable. The total number of 53 validated shRNA was divided into seven experimental sets, all including the proper controls, shRNA with a non-target sequence in the same vector as the genes of interest included in those sets. Dividing the experiments into manageable independent sets also allowed us to do thorough imaging of the structures shortly after staining. While this ensures minimal loss of staining intensity it also requires careful planning of the experimental and analysis schedule.

All statistical analysis should begin by a careful observation of the descriptive elements of the collected data. The calculation of means, medians, standard deviations, and frequency

histograms allows the researcher to evaluate how the data should be subsequently processed and analyzed (Ali and Bhaskar 2016). The visual inspection of the frequency histograms was enough to verify that our morphometric data had a non-gaussian distribution (not normally distributed amongst the range of the data values). Non-gaussian distribution was observed for both shape descriptors in almost all experimental conditions (III: Figure 1d-g). We further confirmed the non-normality assumption by applying the commonly used normality tests, Shapiro-Wilk and Kolmogorov-Smirnov. The non-normality of the data determines that only non-parametric statistical tests can be applied to the data.

Data pre-processing is often disregarded as it is hypothetically less crucial when the data fits the normality standards with a clear definition of outliers (Jung 2011). In our data, both shape descriptors showed a marked different in the range of values obtained in the different experimental conditions. To make the data comparable, they need to be on the same range of values, hence we log-transformed the area values and logit-transformed the circularity values. The scaling of the data does not alter the data relationships, it transforms the data populations to have similar unit variance allowing a reliable comparison between them.

Once the pre-processing of the data is completed, we proceeded to calculate the maximum mean discrepancy (MMD) between our data populations. We started by exploring the variance between the different experimental control populations and we found a noteworthy variance between them. This implies that, to make our experimental sets comparable, we had to normalized all the data populations. The experimental sets normalization was done by mean centering the control populations in a reproducing kernel Hilbert space (RKHS) (III: Figure 3c).

We could now apply the algorithm that calculates the maximum mean discrepancy (MMD) between all our data populations, including all our experimental conditions and morphometric observations. In a simplified way, the test takes into account all the morphometric parameters and transpose them into a reproducing kernel Hilbert space (RKHS) where the data populations is represented. This is a pair wise analysis and the algorithm will define a function that calculates the maximum difference between the two data populations being analyzed (III: Figure 3b). These MMD values, which inversely represent the similarity between the observed phenotypes (low MMD values = similar phenotypes), are then used to build phenotype heatmaps and networks (III: Figure 4, 5).

The MMD analysis method has been successfully used in biological analysis, being used to integrate biomarker data (Borgwardt et al. 2006), minimize measurements variations (Shaham et al. 2017) and to identify similar gene expression patterns in different experimental conditions (Vegas et al. 2016). Nonetheless, in order to validate if suitable for our data, we looked for consistency between the results obtained with MMD and with a

univariate Wilcoxon Rank-Sum statistical test. The Wilcoxon Rank-Sum only permits the independent analysis of each shape descriptor and experimental set. Validating the applicability of the MMD analysis to our data, we verified a striking overlap between the number of genes and shRNA's that were considered, by both analysis methods, to have a statistically significant impact in the observed phenotypes.

The principal aim of the study was to unravel biological pathways that could be implicated in the observed phenotypes. We hypothesized that those genes, which upon downregulation, lead to similar phenotypes, would also be related at the protein level and consequently act via similar pathways. To address this question, we integrated our phenotype networks with STRING database proteomic data (Szklarczyk et al. 2015). From our phenotype networks we selected clusters of phenotypes that more closely relate to each other (defined by the MMD lower values) and parallelly searched for solid evidence of relationships between the proteins included in the phenotype cluster in the STRING database (IV: Figure 4). The analyses of this superimposition of data proved challenging due to the relative high level of overlap, forcing us to focus on anchor genes or interesting phenotypical relationship. To amplify our analysis scope, we selected the first neighbors of our anchor or interaction of interest in the phenotype network (IV: Figure 5).

In the phenotype networks created without oncogene challenge, we focused on the known PARD6G-CDC42 interaction (III: Figure 5b-c). We noticed protein and phenotype closeness to Dishevelled Segment Polarity Protein 3/DVL3 and beta-catenin/CTNNB1. Interestingly, both DVL3 and CTNNB1 have been previously implicated as key mediators of the Wnt signaling pathway. This pathway with a critical role in development has been implicated in different types of cancer, with recent studies implicating Wnt/β-catenin signaling as a regulator of catabolic processes in cancer metabolism (Brown et al. 2017, Zhan et al. 2017). Furthermore, we also noticed that PARD6G and MOB1B knockdown resulted in similar phenotype (low MMD value) but currently there is no evidence of direct protein-protein interaction. These observations could be further studied, to either unravel a still unknown connection or examine if they operate via the same pathways.

In the phenotype networks created with oncogene challenge we focused on YAP1 (III: Figure 5e-f). This was of particular interest, since recent studies showed that MYC-induced energetic stress would ultimately inhibit YAP/TAZ transcriptional co-activators (von Eyss et al. 2015). Moreover, the YAP knockdown phenotype (reduced growth) was only exposed in the presence of MYC activation, implying a signaling crosstalk between them. Further examination of the phenotype networks revealed similar phenotypes obtained by downregulation of NF2 and YAP1. NF2 and YAP are known to indirectly interact with each other, and both are involved in the activation of the Hippo signaling pathway. Merlin protein, encoded by NF2 gene, is able to regulate Hippo signaling pathway at different

stages, and YAP/TAZ is used by Hippo pathway to control cell proliferation and tissue growth (Zhang et al. 2010, Petrilli and Fernandez-Valle 2016, Reginensi et al. 2016). Hippo pathway is a conserved kinase pathway that has been thoroughly studied with accumulated evidence of a role in tumorigenic processes (Harvey et al. 2013, Yu et al. 2015, Guerrant et al. 2016, Petrilli and Fernandez-Valle 2016, Bae et al. 2017). In addition, our data analysis suggests that downregulation of either NF2 or YAP, in the presence of MYC activity, impairs the growth of the transformed structures, which can be seen as a desired effect on MYC-driven tumors. This phenotype should be further explored and potentially exploited for therapeutic intervention.

In conclusion, this study proved that this novel analysis workflow (III: Figure 6) has the potential to find novel biochemical pathways relevant for understanding epithelial biology and cancer. Furthermore, this analytical tool can be applied to larger and more complex datasets, facilitating the creation of hypotheses. Nonetheless, all the hypotheses regarding protein-pathways relations should be further validated with appropriate laboratorial methodology.

RETINOIC ACID-INDUCED 2 (*RAI2*) WAS FOUND CRITICAL FOR THE TRANSCRIPTION OF MAMMARY EPITHELIAL INTEGRITY REGULATORS AND FOR PREVENTING EARLY HEMATOGENOUS DISSEMINATION OF TUMOR CELLS (DTC). (IV)

While tumor suppressor genes (TSG) and their modulation have been the target of several studies (Hanahan and Weinberg 2011), metastatic suppressor genes (MSG) have been scarcely characterized. Amongst the difficulties to study them, is the fact that they tend to vary more depending on cancer type and are rarely found inactivated by mutations (Smith and Theodorescu 2009, Hurst and Welch 2011). In this study, we focused on the current knowledge indicating bone marrow as a common home for breast, lung and colon carcinomas disseminated tumor cells (DTC). These DTCs, frequently resistant to the conventional therapies, can remain dormant for long periods of time, retaining the ability to exploit their new microenvironment and eventually grow into full-blown metastasis (Kang and Pantel 2013). Metastases in distant organs can be occur several years after full relapse of the primary tumor and are the most frequent cause for breast cancer patient's mortality.

Tumor cells can either travel to sentinel lymph node and subsequently invade the blood vessels, or directly intravasate into blood vessels. Since the mechanisms leading to hematogenous dissemination are still unknown, this study started by determining a genetic signature for breast cancer DTCs. This was accomplished by the analysis of whole genome

expression profiles of breast tumor samples from 32 non-treated patients, lymph-negative. Analysis was performed taking in account the presence of DTC's in the bone marrow (early metastasis indication). From the genetic profile correlated with the presence of DTC, only four genes were found upregulated. With the majority of the genes found downregulated, we assumed there is a prominent role of potential suppressors of early tumor cells dissemination. A publicly available database (GSE3494) was used to verify how the previous genes identified as potential dissemination suppressors would correlate with other breast cancer signatures. The defined DTC signature was closely related with the one observed in the most aggressive and dedifferentiated luminal sub-populations, found in basal and HER2-positive tumors (IV: Figure 1). Furthermore, the genes found in the DTC signature were validated, *in silico,* for patients' prognosis. Interestingly, from the DTC signature, the low expression of retinoic acid–inducible gene (*RAI2*) was the only one implicated in low patients' survival in all breast, lung, ovarian and colon cancer datasets (IV: Figure 2).

The specific functions of *RAI2* gene are still unclear, with some evidence indicating a role in neural development, cell growth and differentiation (Jonk et al. 1994). Furthermore, due to its chromosomal localization, *RAI2* has been also considered as a candidate for diseases mapping to the Xp22 region, like the Nance-Horan syndrome (Walpole et al. 1999, Walpole et al. 1999). A strong correlation between low *RAI2* mRNA expression and DTC-positivity, low differentiation grade, presence of mutant TP53 and tumor advanced stage strengthened the notion that low *RAI2* expression is implicated in poor prognosis. Even though tumor cells in the lymph can travel to blood vessels, lymphatic and hematopoietic dissemination routes are believed to be mainly independent (Wong and Hynes 2006, Sola et al. 2011). Since we could not correlate low *RAI2* with sentinel lymph node status, we concluded that *RAI2* effect is restrained to the hematogenous but not to the lymphatic tumor cells dissemination.

Experiments with breast cancer cell lines, demonstrated a correlation between RAI2 expression and ERα status, with the highest *RAI2* expression found in the luminal ERα-positive subtype and the lowest in HER2-positive and highly aggressive breast cancer cell lines. In fact, the non-transformed MCF10A cells presented the highest levels of *RAI2*. Moreover, by immunofluorescent staining, it was found that *RAI2* in these cells is localized in the cell nucleus, indicating a possible role in transcriptional regulation. To test if *RAI2* expression is able to sustain cells luminal differentiation we performed experiments with breast cancer cell lines. Results showed that the shRNA knockdown of *RAI2* efficiently downregulated ERα protein expression and consequently lowered the expression of GATA3, FOXA1 and GRHL2 proteins (IV: Figure 4). Since these proteins have been previously identified as regulators of the mammary epithelial differentiation, we

hypothesized that *RAI2* expression is linked to the loss of epithelial differentiation, leading to more aggressive and invasive tumors. Consistent with the previous hypothesis, we observed that the downregulation of *RAI2* in ERα-positive cell lines (MCF-7, KPL-1, and CAMA-1) results in altered cell's phenotype. Under normal contrast microscope, these cells exhibited enlarged and less refractive cell bodies, presenting microfilaments branching from the cell edges and in some sub-populations cells presented a spindle shape. These morphological changes imply the acquisition of a higher cellular plasticity upon loss of *RAI2*.

Phenotype changes derived from *RAI2* downregulation were further explored in cells grown in the presence of an extracellular matrix (Matrigel embedded). The knockdown of *RAI2* in MCF7 lead to the formation of acinar structures with normal size and number of cells, implying there was no significant alteration in the cells proliferation rate. On the other hand, *RAI2* downregulation in 2D cultures resulted in increased AKT phosphorylation, with AKT/mTOR kinase inhibitor being unable to reduce cells viability (IV: Figure 4). Curiously, the 3D structures showed signs of altered cell polarization, with cis-Golgi matrix protein (GM130; apical marker) facing either the lumen or the basal side of the structures. Moreover, loss of *RAI2* decreased the expression of E-cadherin expression at the cell junctions, which may impair the adhesion junctions and lead to loss of cell polarity. Altogether, *RAI2* seems implicated in epithelial integrity regulation (IV: Figure 4).

With the phenotype changes indicating a shift towards a higher cell plasticity, we decided to explore the possible connection between *RAI2* low expression and epithelial-to-mesenchymal transition (EMT). The induction of EMT in MCF10A, by TGFβ addition, resulted in a decreased expression of *RAI2* and E-cadherin, and in an increased expression of vimentin (IV: Figure 5). Moreover, using a Boyden chamber assay, loss of *RAI2* in the selected luminal cells was able to promote migration and invasion. Remarkably, overexpression of *RAI2* in the highly invasive cell line MDA-MB-231, was able to prevent cell invasion and migration (IV: Figure 5). Altogether, we gathered evidence that *RAI2* expression is needed to prevent invasion and migration of malignant cells, and its ectopic expression seems to be able to prevent EMT.

To understand how RAI2 is connected with all the above-mentioned processes, we searched for proteins that could interact with *RAI2*. Carboxyl-terminal binding proteins (CtBPs) were identified as suitable candidates. Immunofluorescence staining of RAI2 and CtBPs showed they co-localize in the nuclei of MCF7 cells (IV: Figure 6b). Proteins bind to CtBP1 and CtBP2 via a conserved "PXDLS" CtBP-interaction domain (Chinnadurai 2009). To identify the existence of such domains in RAI2 protein, Clustal analysis (Sievers et al. 2011) was used to perform multiple sequence alignment analysis, and two highly

conversed orthologous sequences (ALDLS) were found in the internal region of the RAI2 protein. RAI2 mutants, lacking the two previously identified potential binding sites with CtBP, showed a marked decrease in co-precipitating with CtBP, indicating a physical connection between RAI2 and CtBPs (IV: Figure 6d). Previous studies have shown that in epithelial breast cancer cells, CtBP is implicated in cell differentiation control (Di et al. 2013). We then investigated how RAI2 low expression is linked to impaired function of CtBP as a transcriptional regulator. Interestingly, the migration assays, using the RAI-2 mutants, suggested that the suppression of invasion and migration by RAI2 is partially dependent on their connection to the CtBP proteins.

In conclusion, this study thoroughly correlates the low expression of RAI2 with the presence of breast cancer DTC in bone marrow. Furthermore, low levels of RAI2 are also correlated with a low overall survival in breast, lung, ovarian and colon cancer patients. RAI2 was also correlated with the less differentiated breast tumors subtypes, which have a more invasive and aggressive phenotype. *In vitro* experiments, with breast cancer cell lines, linked low levels of RAI2 with increased activation of the AKT signaling pathway and with partial loss of epithelial integrity (loss of E-Cadherin in AJ; loss of apical-basal polarity). Moreover, RAI2 nuclear localization and *in vitro* experiments using RAI2 mutants for CtBP's binding sites, showed evidence of a direct interaction between RAI2 and CtBPs. Indicating that loss of RAI2 leads to the deregulation of CtBPs, previously suggested to control a number of key target genes in early hematological tumor cells dissemination (Birts et al. 2010, Paliwal et al. 2012).

CONCLUSIONS AND FUTURE PERSPECTIVES

This study identified and clarified the function of novel human epithelial integrity regulators in breast cancer. Some of the discoveries done during this study can be exploited for novel therapeutic approaches, in particular to target MYC-driven tumors and the discovery of biomarkers for early metastatic breast cancer. The unravelling of several genes that alter proliferation and apoptosis, either alone or in cooperation with MYC oncogene (publication I) opened several study avenues. Of note, the methods developed during the screen for epithelial integrity regulators in publication I were crucial not only for the studies included in this thesis, but also for related studies more clinically oriented using 3D cultures of patient-derived explants (related publication IV).

This study has shown that Par6 family as a critical role in the maintenance of epithelial integrity and in suppressing MYC proliferative effects. Previous studies showed that PARD6B overexpression has the ability to increase proliferation via stimulation of mitogen-activated protein kinase (MAPK) (Nolan et al. 2008). Interestingly, in our studies we observed that the downregulation of both PARD6B and PARD6G was able to sustain or increase cell proliferation via the activation of AKT pathway even in deprived cell culture conditions. In 3D cultures, higher proliferation rates were observed upon the downregulation of PAR6G alone, and the combined downregulation of PAR6B with chronic or acute MYC activation. In this study, we linked PAR6G tumor-suppressor functions to the repression of AKT pathway proliferative effects, while other studies showed that PAR6B oncogenic functions are correlated to MAPK pathway. Since in our study PAR6B downregulation enabled MYC ability to force quiescent cells into cell cycle progression, one can speculate that PARD6 genes have the ability to act via different signaling pathways depending on expression level.

Synthetic lethal interactions specific to cancer cells are tempting therapeutic approaches, due to the expected minimal effects in the normal cells. For example, the inhibition of poly (ADP-ribose) polymerase (PARP) has been observed to selectively kill BRCA1- or BRCA2-deficient cancer cells (Farmer et al. 2005). Moreover, current clinical trials using PARP inhibitors in breast cancer (BRCA) have been showing promising preliminary results (Brown et al. 2016, Bitler et al. 2017). The previous success story using a synthetic lethal interaction in clinical trials makes us believe that the observed RHOA-MYC lethal interactions (publication I and II) could be considered, in a near future, as a possible avenue to specifically target MYC-driven breast cancer.

In publication III we implemented a novel framework to perform statistical analysis of morphometric data, generated in the aforementioned reverse genetic screen (publication I). This workflow was able to combine information about known protein interactions and all

morphological changes observed in the primary 3D experiments. This is a straightforward analysis method that can be expanded to a higher number of observations and potentially assist the creation of new hypothesis to be experimentally explored. The present study included very few morphological parameters but the algorithm has the potential to process, not only a greater amount of morphological data, as other information regarding, for example, proliferation, apoptosis and polarity status. Unfortunately, we didn't perform an in-depth analysis of all the structures included in publication I, to further compare how this approach could be beneficial with a wider variety of observations. The method showed potential to identify signaling pathways involved in epithelial integrity maintenance, even with only a partial suppression of gene expression. It will be interesting to test if similar screen done with more efficient knockdown tools like CRISPR/Cas9 would reveal more signaling pathways connected to epithelial integrity maintenance.

Another critical discovery made during this study was the novel correlation between low levels of RAI2 and the presence of bone DTC in breast cancer patients. Furthermore, RAI2 loss in luminal epithelial cells lines leads to the mislocalization of polarized elements of the cell. RAI2 was also correlated with the transcription regulation of epithelial integrity regulators via their physical interaction with CtBPs. Tumor cells are able to travel through the body via lymph or blood vessels, processes called lymphatic or hematogenous tumor cells dissemination. In clinical examinations, the presence of tumor cells in the sentinel lymph node is the default way to gather information whether or not breast cancer cells have already started to disseminate into the patient's body. Cells usually travel fist to sentinel lymph node and subsequently invade the blood vessels, but some cells manage to directly intravasate into blood vessels, escaping the sentinel lymph node and consequently the clinicians' analysis. The methods to directly identify the presence of CTC in the blood vessels are still complicated for clinic routine usage and still need further development for improved sensitivity. RAI2 gene expression in the tumor biopsies has the potential to be used as a biomarker for the presence of early breast cancer metastasis disseminated into bone marrow, facilitating patient's diagnosis and follow-up treatments.

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