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Myc-dependent transcriptional programs in mammary epithelial cells

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List of abbreviations

ACK: ammonium-chloride-potassium buffer

ALDH: aldehyde dehydrogenases

Arf: alternate reading frame protein

BABB: benzylalcohol benzylbenzoate solution

Bcl-2: B-cell lymphoma 2

bHLH-LZ: basic helix-loop-helix leucine zipper

bp: base pairs

BR: basic region

BRCA1: breast cancer 1, early onset

BTB: broad complex, tramtrack, bric à brac domain

CDKI: cyclin-dependent kinase inhibitor

cDNA: complementary DNA

ChIP: chromatin immunoprecipitation

CoREST: co-repressor to the RE1 silencing transcription factor/neural restrictive silencing factor

CSCs: cancer stem cells

Ctr: control

DEGs: differentially expressed genes

DNA: deoxyribonucleic acid

Dnmt3a: DNA (cytosine-5)-methyltransferase 3A

EdU: 5-ethynyl-2'-deoxyuridine

EGF: epidermal growth factor

EMT: epithelial to mesenchymal transition

ER: estrogen receptor

eRPKM: exonic reads per kilobase per million of total reads aligned

ESCs: embryonic stem cells

FACS: fluorescence-activated cell sorting

FBS: fetal bovine serum

FC: fold change

FDR: false discovery rate

FGF: fibroblast growth factor

Fl: floxed

gDNA: genomic DNA

GEO: Gene Expression Omnibus

GO: gene ontology

GR: growth rate

GTP: guanosine-5'-triphosphate

HDAC3: histone deacetylase 3

HE: hematoxylin and eosin

HER2: epidermal growth factor receptor 2

HLH: helix-loop-helix

HMECs: human mammary epithelial cells

HSCs: hematopoietic stem cells

Ifrd2: interferon-related developmental regulator 2

IHC: immunohistochemistry

iPSC: induced pluripotent stem cell

KI: knock-in

Klf4: Kruppel-like factor 4

L-Glu: L-Glutamine

Lum-SC: luminal-stem cell

Mad: Max dimerization protein

MaSCs: mammary stem cells

Max: Myc-associated factor X

MECs: mammary epithelial cells

miR o miRNA: microRNA

Miz1: Myc-interacting zinc finger protein 1

MMECs: mouse mammary epithelial cells

MMTV: mouse mammary tumor virus promoter

Mnt: Max binding protein

mRNA: messenger RNA

Mxl1: Max-like 1

Myc VD: MycV394D mutant

Myc: myelocytomatosis oncogene

MycER: Myc estrogen receptor

MycKO: Myc knock-out

MycOE: Myc overexpression

Myo-SC: myoepithelial-stem cell

ncRNA: non-coding RNA

NLS: nuclear localization signal

NuRD: nucleosome remodelling and deacetylating complex

OHT: 4-hydroxytamoxifen

OSK: Oct4, Sox2 and Klf4

OSKM: Oct4, Sox2, Klf4 and Myc

P-TEFb: positive transcription elongation factor b

PBS: solution

PCR: polymerase chain reaction

PI: propidium iodide

POK: POZ and Krüppel

POZ: poxvirus and zinc finger

PR: progesterone receptor

Preg-SC: pregnancy-stem cell

qPCR: quantitative PCR

R26: Rosa-26 promoter

Ras: rat sarcoma virus oncogene

Rb: retinoblastoma gene

RNA: ribonucleic acid

Rplp0: ribosomal protein, large, P0

RPM: reads per million

rpm: revolutions per minute

rRNA: ribosomal RNA

RT-PCR: reverse transcriptase PCR

RT: room temperature

rtTA: reverse tetracycline transactivator

SCs: stem cells

SDS-PAGE: sodium dodecyl sulphate-polyacrylamide gel electrophoresis

SFE: sphere forming efficiency

shRNA: short hairpin RNA

SLCs: small light cells

Smpdl3b: sphingomyelin phosphodiesterase, acid-like 3B

Sox2: SRY (sex determining region Y)-box 2

TAD or TA: transactivation domain

TCGA: The Cancer Genome Atlas

TET: tetramerization domain

TGFβ: transforming growth factor β

TNBC: triple negative breast cancer

TopBP1: topoisomerase II binding protein 1

TRE: tetracycline responsive element

tRNA: transfer RNA

TSS: transcriptional start site

WT: wild type

Zbtb17: zinc finger and BTB domain containing 17

ZnF: zinc fingers

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Abstract

The transcriptional programs governing the decision of mammary epithelial stem and progenitor cells to self-renew or differentiate are still not completely outlined. Previous evidences pointed out the role of Myc, and in particular of its repressive activity with Miz1, in these processes. Since the presence of stem-like cells within the tumor, the so-called cancer stem cells (CSCs), is now considered crucial for tumor initiation and maintenance, clarifying this aspect of Myc biology could be relevant in understanding its contribution to the genesis of breast cancer. Here, we used RNA-seq technology to profile the transcriptional programs regulated by Myc in two different settings. First, we studied immortalized mammary epithelial cells, in which we induced either Myc loss or gain of function. In this setting, Myc contributed to the positive and negative regulation of different sets of genes. Activated genes are involved in proliferation, metabolism, ribosomal biogenesis, mitochondrial organization, chromatin modification, RNA processing and modification. Repressed genes, on the other hand, were mainly involved in lysosome and vesicle-mediated transport, angiogenesis, cell death, extracellular matrix interaction, cell adhesion regulation, epithelial development and morphogenesis. Second, we studied the effect of Myc activation in mammosphere cultures, which provide a measure of stem cell activity. We demonstrate that Myc, when overexpressed, is able to promote self-renewal of mammary epithelial stem cells, as assessed by increased mammosphere expansion, and confirmed by mammary gland reconstitution assays in vivo. This activity of Myc is in part dependent on the interaction with the co-repressor protein Miz1, since the Myc mutant V394D (hereby Myc VD), impaired in Miz1 binding, is defective in promoting self-renewal. Overexpression of Myc in mammospheres was associated with the de-regulated expression of about three thousand genes, with similar numbers of up- and down-regulated genes. A group of around nine hundred genes

was specifically repressed by Myc WT and not by the VD mutant. Surprisingly, the overlap between the groups of regulated genes in those mammary epithelial cells in adhesion or grown as mammospheres was limited, illustrating the context-dependency of Myc-dependent responses. Thus, transcriptional repression via Miz1 may constitute one of the mechanisms through which Myc sustains mammary epithelial cell self-renewal. We are currently setting a functional screen, among genes repressed in a Miz1-dependent manner, to identify those that are critical in this process. Our study shall shed light on the mechanisms through which Myc regulates self-renewal in mammary epithelial stem and progenitor cells. Understanding this could be crucial in order to clarify the physiopathological roles of Myc in the mammary gland.

1. Introduction

1.1 Myc: discovery and structure

The proto-oncogene *c-MYC* was discovered over three decades ago as homologous of the myelocytomatosis viral oncogene (*v-Myc*) and belongs to a family comprising also *N-MYC* (Shwab, Alitalo, & Klemphauer, 1983) and *L-MYC* (Nau et al., 1985). In normal conditions *c-MYC* expression is finely regulated in response to stimuli coming from the extracellular surrounding and mainly reflects the cellular growth state: it is induced by mitogenic stimuli and repressed by cell contact and differentiation stimuli (Oster, Ho, Soucie, & Penn, 2002). In turn, MYC, dimerizing with its partner protein MAX, regulates many biological activities (proliferation, cell growth, apoptosis, stemness/differentiation, genomic stability, adhesion/migration, angiogenesis etc.) either activating or repressing the transcription of specific sets of genes (Amati, Littlewood, Evan, & Land, 1993; Blackwell, Kretzner, Blackwood, Eisenman, & Weintraub, 1990; Blackwood & Eisenman, 1991; Herkert & Eilers, 2010; Kress, Sabò, & Amati, 2015).

In mouse, homozygous deletion of *c-myc* is embryonic lethal before 10.5 days of gestation (Davis, Wims, Spotts, Hann, & Bradley, 1993) and conditional depletion of *c-myc* demonstrated its crucial function in proliferation and cell growth (de Alboran et al., 2001; Perna et al., 2012; Trumpp et al., 2001). On the other side, ectopic *c-myc* over-expression confirmed its association with hyperproliferation and tumor development (Dang, 2012). As a matter of fact, MYC is often deregulated in human cancers by mutations, chromosomal translocation or amplification (Meyer & Penn, 2008), as well as by de-regulation of upstream signaling pathways (e.g. Ras, Wnt, Notch) (He et al., 1998; Sears, Leone, DeGregori, & Nevins, 1999; Sharma et al., 2006). Altogether, Myc over-expression

is a common feature of cancer. The MYC protein is a 439-residue transcription factor of the basic helix-loop-helix leucine zipper (bHLH-LZ) family. The N-terminus of Myc protein is composed by two homology boxes (box I, aa 44-63, and box II, aa 128-143, regions highly conserved among the Myc family members) and includes its Transactivation Domain (TAD, aa 1-143) (Figure 1A). This domain of Myc is essential for transformation and transcriptional activation/repression, ensuring the interaction with a plethora of transcription factors and co-activators (Kato, Barrett, Villa-Garcia, & Dang, 1990; Meyer & Penn, 2008; Oster et al., 2002). Moreover, the N-terminus contains two tightly controlled phosphorylation sites (Thr58 and Ser62) that regulate Myc stability and degradation (Sears et al., 2000). The Myc homology boxes IIIa (aa 180-199, conserved in c- and N- Myc), IIIb (aa 259-270) and IV (aa 304-324) are located in the middle of Myc protein, together with the Nuclear Localization Signal (NLS, aa 320-328 aa) (Figure 1A). The C-terminus of Myc includes its bHLH-LZ domain, composed of the helix-loop-helix-leucine zipper domain (HLH-LZ, aa 370-439), responsible dimerization with Max, immediately preceded by the Basic Region (BR, aa 355-369), essential for DNA binding (Blackwell et al., 1990) (Figure 1A). Myc/Max dimers preferentially associate with the DNA motif CACGTG (E-box) and variants thereof (Blackwood & Eisenman, 1991). As for other HLH and bHLH-LZ proteins, dimerization can occur in solution and is a pre-requisite for DNA binding. The crystal structure of DNA-bound bHLH-LZ domains provided a direct visualization of this phenomenon (S. K. Nair & Burley, 2003), bHLH dimers positioning the BR of both proteins for insertion into the major groove of the DNA double helix (Figure 1B).

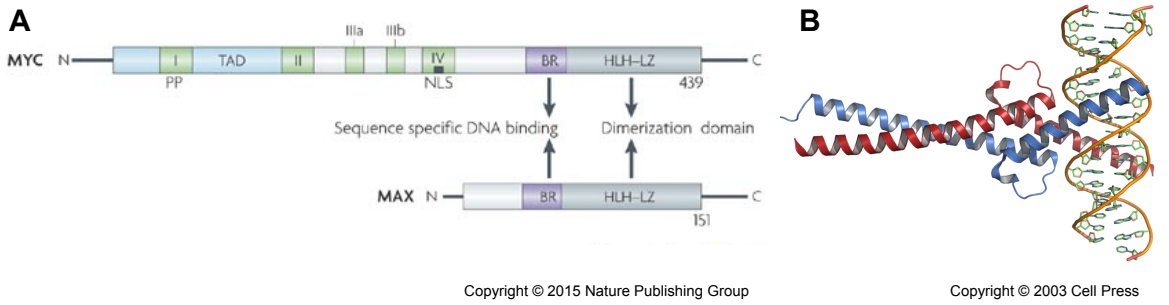

Figure 1: Myc/Max structure and interaction. (A) Schematic representation of human MYC and MAX proteins. MYC contains four homology boxes (I-IV, including a Nuclear Localization Signal (NLS)), a Transactivation Domain (TAD), a Basic Region (BR, for the binding to the chromatin) and an Helix-Loop-Helix-Leucine Zipper domain (bHLH-LZ, for the interaction with MAX) (modified from Meyer & Penn, 2008). (B) X-ray structure of Myc (red) and Max (blue) heterodimer interacting with the double helix of the DNA (modified by Wikipedia from S. K. Nair & Burley, 2003)

1.2 Myc: role in transcriptional activation

As previously mentioned, Myc is a bHLH-LZ transcription factor that, by heterodimerizing with Max, can activate or repress gene transcription. Myc/Max dimers bind preferentially the E-boxes, very common sequences in the genome acting as transcription factors binding sites (Desbarats, Gaubatz, & Eilers, 1996). The general motif, bound by bHLH and bHLH-LZ proteins, is CANNTG (where N can be any nucleotide); in particular the palindromic canonical sequence bound by Myc and Max is CACGTG (Eilers & Eisenman, 2008; Oster et al., 2002). This interaction ensures Myc functions such as cell cycle progression, apoptosis and cellular transformation (Amati et al., 1993; Blackwell et al., 1990; Blackwood & Eisenman, 1991).

Max or 'Myc-associated factor X' is a bHLH-LZ transcription factor able to heterodimerize with Myc to allow its transcriptional activity (Amati et al., 1993; 1992). In addiction, Max is able to homodimerize or heterodimerize with other proteins belonging to the same class of transcription factors, such as Mad or Mxl1, that therefore act as Myc antagonists (Grandori, Cowley, James, L. P., & Eisenman, 2000).

In vivo, the E-box may not be the sole, or even the principal, determinant of DNA binding by Myc (Jiannan Guo et al., 2014; Sabò & Amati, 2014). Chromatin binding analyses have shown a preferential association of Myc to CpG islands, active chromatin features (H3K4me3, H3K27ac), presence of the basal transcription machinery and sites hypersensitive to digestion by DNaseI (Deaton & Bird, 2011; Fernandez, 2003; Guccione et al., 2006; C. Y. Lin et al., 2012; Martinato, Cesaroni, Amati, & Guccione, 2008; Nie et al., 2012; Sabò et al., 2014; Zeller et al., 2006). In particular, genome-wide binding profiles have pointed out that Myc occupies both active promoters and distal sites (enhancers), depending on its expression levels. In particular, at physiological level (low expression), Myc preferentially binds high affinity sites, containing E-box sequences; while at supra-physiological level (high expression), it can “invade” also other low affinity sites such as promoter and enhancers in an active chromatin state (C. Y. Lin et al., 2012; Nie et al., 2012; Sabò et al., 2014; Walz et al., 2014).

Starting from the widespread genomic distribution of Myc, two recent studies have questioned the presence of a Myc-specific transcriptional program, proposing instead that Myc acts as ‘amplifier’ of those programs already active in the cell. According to this model, Myc interacts with all active loci, invading also active enhancers, and that leads to an increase recruitment of the P-TEFb complex to promoters and consequently of the RNA polymerase processivity. Therefore, Myc would act always as a transcriptional activator, and that would be the cause of the increment in the RNA content per cell (the so-called “RNA amplification”) (C. Y. Lin et al., 2012; Nie et al., 2012).

The alternative scenario proposes that Myc is as transcriptional activator/repressor of distinct gene sets, arguing that the RNA amplification, when present, occurs only as a secondary consequence of the activity of Myc-specific targets (Sabò et al., 2014). Indeed, among Myc-regulated genes, the main enriched ontology

categories are related to protein synthesis, cell proliferation/growth, energetic metabolism, RNA processing etc. Thus, global increase in RNA levels is associated to DNA synthesis and increment in cell size, and occurs only concurrently with Myc selective transcriptional regulation (Kress et al., 2015). This model reconciles the phenomenon of RNA amplification with the Myc-dependent biological activities individuated so far. In this regard, the main transcriptional activations of Myc are listed below (Figure 2):

- **Cell cycle/proliferation:** Myc is an early growth response gene; in response to mitogens, it induces cell cycle transition from G0 to S phase or progression in G1 (Amati, Alevizopoulos, & Vlach, 1998; Oster et al., 2002).
- **Protein synthesis/Ribosome biogenesis:** Myc induces the synthesis of ribosomal RNA (rRNA), ribosomal proteins and transfer RNA (tRNA) promoting the activity of the RNA Polymerase I, II and III respectively (Arabi et al., 2005; Gomez-Roman, Grandori, Eisenman, & White, 2003; Ji et al., 2011).
- **Metabolism:** Myc regulates several enzymes involved in glucose metabolism (e.g. enolase A, hexokinase, lactate dehydrogenase, phosphor-fructokinase, glucose transporter) or lipogenesis (e.g. carnitine-palmitoyl transferase 1A) enhancing glucose uptake, glycolysis and fat acid oxidation; it has also a pivotal role in mitochondrial biogenesis and function, iron metabolism, purine and pyrimidine biosynthetic pathways (Dang et al., 2006; Stine, Walton, Altman, Hsieh, & Dang, 2015).
- **Cell growth**: Myc roles both in cell metabolism and in protein synthesis regulation cause accumulation of cell mass/size, thus promoting cell growth (Iritani & Eisenman, 1999; Schuhmacher et al., 1999)**.**
- **RNA processing/stabilization:** Myc induces expression of alternative splicing factors (e.g. serine/arginine-rich splicing factor 1, protein-tyrosine phosphatase-β) (Hirsch et al., 2015; Koh et al., 2015) or regulates the activity of other RNA

binding proteins able to influence transcripts stability or quality control (e.g. tristetraprolin, Smad nuclear-interacting protein 1) (Cleveland et al., 2012; Fujii et al., 2006).

- **Apoptosis:** Myc up-regulates key pro-apoptotic molecules such as Bax, Bak, BH3-only family (e.g Bim, Noxa) (Meyer & Penn, 2008; Oster et al., 2002).

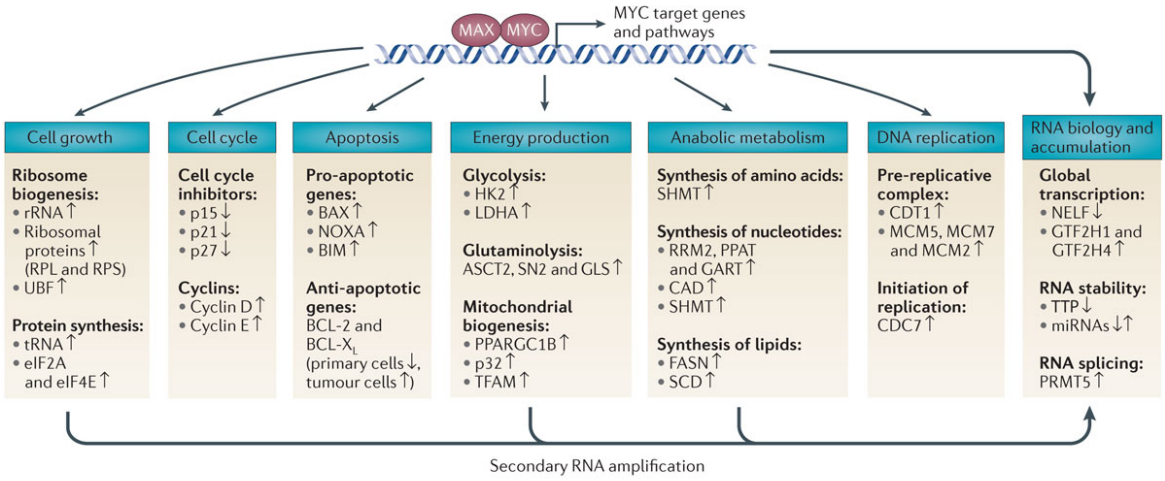

Figure 2: Myc transcriptional activation functions. The main pathways, and their components, that are regulated by Myc/Max transcriptional activation. This transcriptional network results in a global RNA production. The genes listed are examples (from Kress et al., 2015).

1.3 Myc: role in transcriptional repression

Beyond its function as an activator, Myc also acts as transcriptional repressor, in particular when over-expressed (Walz et al., 2014). First, Myc is able to down-regulate its own expression; this repression is proportional to Myc cellular concentration thus it acts as a negative feedback mechanism for maintaining cell homeostasis (L. J. Penn, Brooks, Laufer, & Land, 1990). Gene repression by Myc has been proposed to occur through interaction with several other transcription factors among which Miz1 (Staller et al., 2001), Sp1/Sp3 (Gartel et al., 2001), NF-Y (Izumi et al., 2001), Smad2/Smad3 (Feng, Liang, Liang, Zhai, & Lin, 2002) and YY1 (Shrivastava et al., 1993), the best characterized and possibly the most general partner in Myc-mediated repression being Miz1 (Peukert et al., 1997),

which will be introduced in further detail below. The main functional categories of genes repressed by Myc are briefly listed here:

- **Negative regulators of cell cycle/proliferation**: c-Myc represses the expression of anti-mitogenic molecules such as Gadd45, Gadd153, c/EBPα, Cyclin-Dependent Kinase Inhibitors (CDKIs) as $p15^{INK4B}$ (Cdkn2b), $p18^{INK4C}$ (Cdkn2c), $p21^{CIP1}$ (Cdkn1a), $p27^{KIP1}$ (Cdkn1b), $p57^{KIP2}$ (Cdkn1c) (Amati et al., 1998; Gartel et al., 2001; Steffi Herold et al., 2002; Knoepfler, Cheng, & Eisenman, 2002; Oster et al., 2002; Seoane, Le, & Massagué, 2002; Staller et al., 2001; Warner, Blain, Seoane, & Massagué, 1999). In this context, it is worth mentioning the antagonistic role between Myc and TGFβ signaling: in epithelial cells TGFβ is a major anti-mitogenic signal. Through its effectors, the Smad proteins, it is able to induce cell cycle arrest in G_1 phase, by up-regulating the Cdk4-inhibitor $p15^{INK4B}$ and down-regulating *c-myc* expression. Forced expression of Myc prevents TGFβ-dependent cytostatic response, since it induces Cyclin D expression and blocks the Smad-dependent transcription of $p15^{INK4B}$ (Seoane et al., 2001; Warner et al., 1999).
- **Survival:** Myc down-regulates anti-apoptotic proteins such as Bcl-2 and Bcl-X_L (Patel & McMahon, 2006). Moreover, Myc represses the Serum Response Factor (SRF)-responsive genes, leading to a pro-apoptotic effect (Wiese et al., 2015).
- **Cell adhesion/cytoskeleton:** Myc down-regulates the expression of genes encoding for collagen, adhesion (β1-integrin, N-cadherin) and cytoskeletal proteins (Actin, Cdc42) (Coller et al., 2000; Gebhardt, 2006; Herkert et al., 2010; Shiio et al., 2002). This may affect cell morphology, enhance anchorage-independent growth and cause mobilization of stem cells from their niche, all functions that are tightly linked to the transforming potential of Myc (discussed below).

1.3.1 Myc and Miz1: functions and role in tumorigenesis

The Myc-interacting zinc finger protein1 (Miz1), encoded by the *Zbtb17* gene, is a transcription factor of the POK (Poxvirus and Zinc finger (POZ) and *Krüppel)* family (Peukert et al., 1997). The *Zbtb17* gene encodes for a protein of 297 residues and molecular mass of 32.5 kDa. The N-terminus of Miz1 protein contains a POZ/BTB (POZ/Broad complex, Tramtrack, Bric à brac) domain (Costoya, 2007; Stogios, Downs, Jauhal, Nandra, & Privé, 2005) (Figure 3). This region is essential for homo- and hetero- oligomerization, enabling the formation of dimers and tetramers in solution (Stead et al., 2007); moreover, it allows the recruitment of non-POZ proteins, the association to the chromatin and the fulfilment of the transcriptional functions (Kelly & Daniel, 2006). Indeed, deletion of the POZ domain dampens Miz1 binding to DNA (Gebhardt et al., 2007; Kosan et al., 2010). The central region of Miz1 protein consists in a Transactivation (TA) Domain and 13 consensus Cys_2Hys_2 *Krüppel*-type zinc finger domains (Peukert et al., 1997; 1997). This region is also involved in mediating Miz1 binding to DNA, which happens preferentially to a long non-palindromic sequence placed close to the transcriptional start site (TSS) of the regulated genes (Barrilleaux et al., 2014).

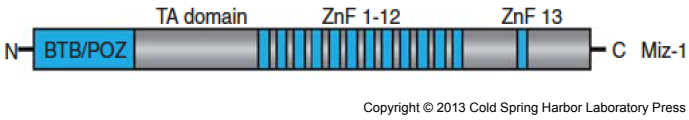

Figure 3: Miz1 structure. Schematic representation of Miz1 protein, it contains: a BTP/POZ domain (essential for oligomerization, association to the DNA and fulfilment of the transcriptional functions), a transactivation (TA) domain and 13 Zinc Fingers (ZnF) domains (functional to DNA interaction) (modified from Wiese et al. 2013).

Miz1 can interact with different partners and, depending on that, the transcriptional effect can vary. In particular: Miz1 activates transcription when associated with the histone acetyltransferase p300 and nucleophosmin (Staller et al., 2001; Wanzel et

al., 2008); whereas it plays a repressive function when in conjunction with Myc (Peukert et al., 1997), Bcl-6 (Phan, Saito, Basso, Niu, & Dalla-Favera, 2005), Gfi1 (Q. Liu, Basu, Qiu, Tang, & Dong, 2010) or Zbtb4 (Weber et al., 2008). In particular, the Myc/Max dimer displaces Miz1 co-activators and/or recruits co-repressors (DNA methyltransferases, Histone Deacetylases) thus blocking the positive effect of Miz1 on the transcription of its target genes (Brenner et al., 2005; Varlakhanova, Cotterman, Bradnam, Korf, & Knoepfler, 2011) (Figure 4).

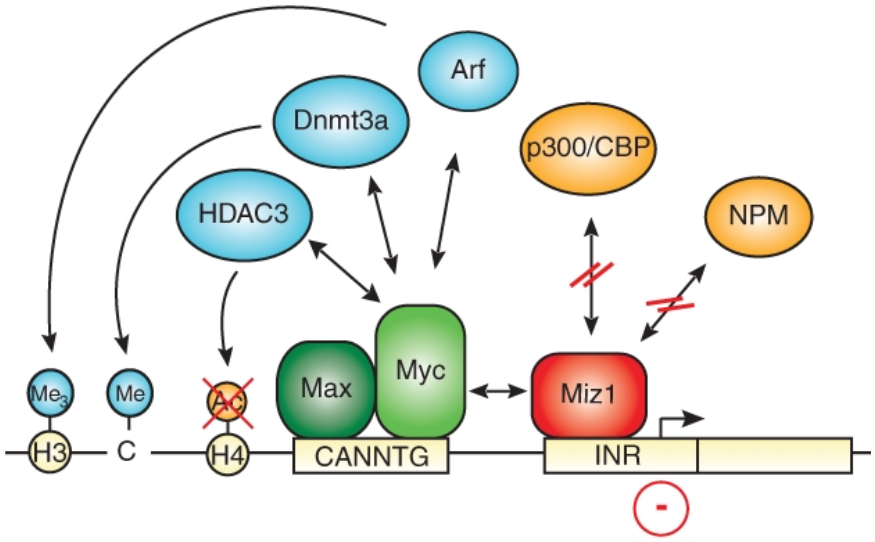

Figure 4: Myc/Max/Miz1 and transcriptional repression. Miz1 alone activates transcription by interaction with the histone acetyltransferase p300/CBP (CREB-binding protein) and nucleophosmin (NPM). Associating with Myc/Max, Miz1 plays a repressive function. Indeed, Myc/Max dimer displaces Miz1 co-activators and/or recruits co-repressors (e.g. Histone Deacetylase 3 (HDAC3), DNA (cytosine-5)-methyltransferase 3a (Dnmt3a), Alternate reading frame protein (Arf)) to repress the transcription (from Herkert et al., 2010).

The Myc/Miz1/Max trimer down-regulates the transcription of two groups of genes: proliferation/survival genes and cell adhesion-related genes. The first group includes $p15^{INK4B}$ (Staller et al., 2001), $p21^{CIP1}$ (Steffi Herold et al., 2002), $p27^{KIP1}$ (Yang et al., 2001), $p57^{KIP2}$ (Hönnemann, Sanz-Moreno, Wolf, Eilers, & Elsässer, 2012), proteins involved in cell cycle arrest and reduction of cell proliferation. For example, TGFβ induces $p15^{INK4B}$ expression by promoting binding of its effectors Smad3/4, Miz1 and Sp1 upstream of the promoter while Myc/Max binding to Miz1

block it (Seoane et al., 2001). A similar mechanism happens on the $p21^{CIP1}$ promoter, where upon UV irradiation and after release of the interacting topoisomerase II binding protein 1 (TopBP1), Miz1 activates the gene transcription ensuring DNA damage-induced cell cycle arrest. Myc binding to Miz1 blocks $p21^{CIP1}$ expression, switching the cell cycle arrest response to apoptosis (Steffi Herold et al., 2002). In fact, Miz1 plays a central role also in the regulation of Myc-mediated apoptosis, as shown by the dampening of the apoptotic response in cells that overexpress a form of Myc unable to bind Miz1 (the MycV349D mutant, hereafter MycVD) (Steffi Herold et al., 2002) compared with cell overexpressing wild type Myc (Patel & McMahon, 2006) (Figure 5). Indeed, Miz1 alone up-regulates the transcription of the pro-survival gene *Bcl2*, but together with Myc represses *Blc2*, thus favouring apoptosis (Patel & McMahon, 2007).

The Myc/Miz1/Max complex represses also a second class of genes coding for molecules involved in cell-cell adhesion, actin cytoskeleton and interaction with the extracellular matrix (Gebhardt, 2006). For example, in either keratinocytes or hematopoietic stem cells, the repression of integrin-coding genes by Myc is Miz1-dependent and influences the interaction of the stem cells with the stem niche (Gebhardt, 2006; Wilson, 2004) (discussed below).

Recent genomic studies pointed out the complexity of Myc and Miz1 interaction in transcriptional regulation. In particular, in primary cells Miz1 has been shown to bind a small set of target genes, mainly involved in vesicular transport, endocytosis, lysosomal biogenesis, cell-cell contact, cell differentiation and growth (Gebhardt et al., 2007; Kosan et al., 2010; E. Wolf et al., 2013a; Ziegelbauer, Wei, & Tjian, 2004). Almost all the promoters of these genes contain the Miz1-binding motif (Barrilleaux et al., 2014) and comparison between different models (murine neural progenitor cells and human mammary epithelial cells) showed that the binding sites of Miz1 are conserved between different species and cell type (E.

Wolf et al., 2013a).On the other hand, in tumor cells, in which Myc levels are high, Miz1 is targeted by Myc also to new binding sites (containing E-box but not only) (Walz et al., 2014). The transcriptional output of the concerted binding of the two proteins to promoters seems to be governed by their relative amount: for Myc-activated genes Myc quantity is higher than Miz1, for Myc-repressed genes it is similar.

Depletion of Miz1, mediated by shRNA, has been described to promote de-repression of a large part (25-40%) of Myc down-regulated genes, while having no effect on Myc activated genes (Walz et al., 2014). A similar result can be observed when the mutant Myc VD (unable to bind Miz1) is overexpressed instead of wild type Myc: impairment in gene repression and no effect on gene activation (Walz et al., 2014).

Multiple data *in vivo* suggested that Miz1 could be crucial in Myc-dependent tumorigenesis (Wiese et al., 2013). Indeed, Myc VD overexpression in murine transgenic models strongly delays lymphomagenesis compared to Myc WT up-regulation, with a reduced cell growth and enhanced TGFβ–induced senescence (Möröy, Saba, & Kosan, 2011; Müller et al., 2014; van Riggelen et al., 2010). In addiction, Myc/Miz1 repression of $p15^{INK4B}$ and $p21^{CIP1}$ expression, in response to stimuli that enhance proliferation, have been shown to support skin papilloma formation (Hönnemann et al., 2012; Inoue et al., 2013), as well colon carcinogenesis (Wiese et al., 2013).

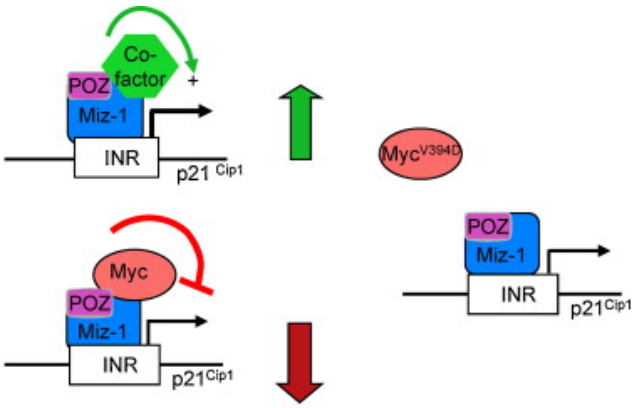

Figure 5: Myc/Miz1 functions. Miz1 induces transcription of its target genes (e.g. $p21^{CIP1}$), while Myc antagonizes the process binding Miz1 and inducing repression of these targets. Myc VD (Herold et al., 2002), a Myc mutant impaired in Miz1 binding, loses Miz1-dependent transcriptional repression (from Moroy et al, 2011).

1.4 Mammary Epithelial Cells (MECs)

The mammary gland is an organ developed as an appendage of the skin, a large and complex glandular system capable to produce nutrient-rich secretions. It has been thought to derive from an apocrine-like skin gland (Oftedal, 2002) that, by secreting milk, has developed during evolution to allow nutrition and microbial protection to the offspring (Peaker, 2002).

The mammary gland is organized in two compartments: the epithelium and the stroma. The epithelium is formed by ducts (that transport the milk) and alveoli (spherical structures). Two types of cells surrounding a lumen constitute these structures: the luminal cells, that in the alveoli are specialized to produce milk, and the basal cells, which constitute the myoepithelial contractile component. The stroma, surrounding and supporting the epithelial structures, is constituted mostly by adipocytes, but also fibroblasts, haematopoietic cells and blood vessels (Hennighausen & Robinson, 2005) (Figure 6).

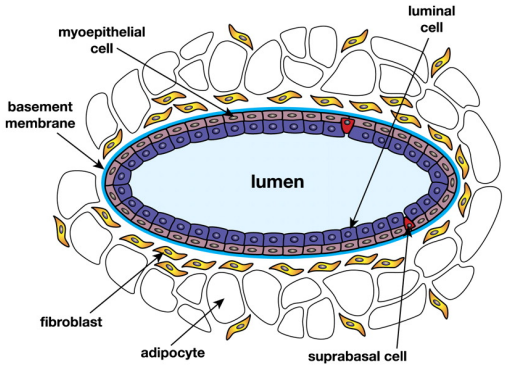

Figure 6: Schematic structure of the mammary gland ducts. Two types of cells surrounding a lumen make the epithelium of the mammary ducts: the luminal cells and the myoepithelial cells. The supporting stroma is constituted mostly by adipocytes, fibroblasts, haematopoietic cells and blood vessels (from Visvader, 2009).

The mammary gland is a highly dynamic tissue, which undergoes extensive cellular remodelling and morphological changes throughout life, in particular during puberty and pregnancy (Hennighausen & Robinson, 2005). Mammary stem cells (MaSCs) are fundamental both in the organogenesis and in the homeostatic maintenance of the mammary gland. The MaSCs are a small pool of cells (<5%) in the mammary epithelium, displaying stem cells features such as capacity of multi-lineage differentiation and self-renewal. The MaSCs generate the mature mammary epithelium, giving rise to either the luminal or the myoepithelial lineage through different lineage-restricted intermediates (Visvader, 2009).

Two main models have been proposed to explain the differentiation hierarchy within the mammary gland epithelium (P. A. Joshi & Khokha, 2012; Visvader & Smith, 2011). The first scenario suggests the existence of a multipotent stem cell able to generate both luminal (ductal or alveolar) and myoepithelial differentiated cells (Stingl et al., 2006). A strong evidence in support of this model is the demonstration that a single basal cell, can generate an entire and functional mammary gland contributing to both luminal and myoepithelial lineage (Shackleton et al., 2006). The second scenario distinguishes between basal and luminal

unipotent stem cells, which differentiate in a lineage-restricted way. Indeed, lineage-tracing experiments demonstrated the existence of unipotent stem cells in the postnatal and adult epithelium. These cells would be able to maintain either the luminal or the basal epithelial populations but not both. According to this model, multipotent stem cells, with higher self-renewal ability, would be present only in puberty, pregnancy or in case of tissue regeneration and transplantation assay, not in normal tissue maintenance (Van Keymeulen et al., 2011) (Figure 7).

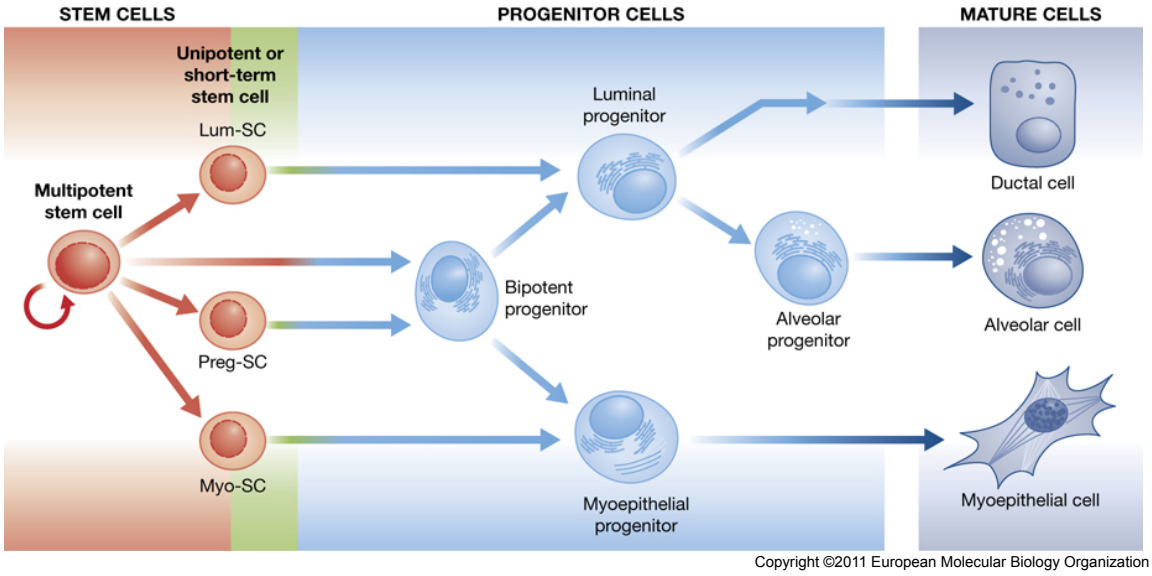

Figure 7: Schematic representation of epithelial cell hierarchy in the mouse mammary gland. Two different models explain the hierarchy within the mammary gland epithelium. The first one proposes the existence of one true multipotent stem cell, able to generate both luminal and myoepithelial differentiated cells through descendent progenitor cells, that could be both long- and short- lived lineage restricted cells. The alternative scenario suggests the presence of unipotent stem cells: luminal-stem cell (Lum-SC) and myoepithelial-stem cell (Myo-SC), in addiction to the pregnancy-stem cells (Preg-SC, emerging only during pregnancy). In this case, real multipotent stem cells would be present only in puberty or recruited in case of wound healing/tissue regeneration, not in mammary gland homeostasis (from Lindeman & Visvader, 2011).

The relationship between multipotent and unipotent stem cells could be better clarified by unifying the experimental methods used for identification and isolation of MaSCs. One of the first methods used was based on morphological analysis by electron microscopy. It evidenced the presence of Small Light Cells (SLCs) in the basal layer, constituting the 3% of the epithelial population and having properties that resemble those of the stem cells (Chepko & Smith, 1997). Since then, new

methods have been developed to study and functionally characterize putative stem cells. *In vitro* one of the most used clonogenic assays is the growth of cells in non-adherent conditions in which only stem cells can grow and give rise to a new colony, called mammosphere (Debnath & Brugge, 2005; Dontu & Wicha, 2005; Dontu et al., 2003). *In vivo* instead, to evaluate self-renewal capacity, the most used technique is the serial dilution transplantation. In this case, the putative stem cells are transplanted in serial dilutions into the cleared fat pad of syngeneic or immunodeficient host mice and the frequency of mammary stem cells is calculated on the basis of positive reconstitution (evaluated by the presence of branched ductal trees with lobules and/or terminal end buds) (Deome et al., 1959; Illa-Bochaca et al., 2010; Makarem et al., 2013). Currently, the most commonly used methods to fractionate the epithelial compartments and isolate potential stem cells are based on staining of surface markers, clonogenic assays, specific enzymatic activity and dye-retention (Cicalese et al., 2009; Dontu et al., 2003; Ginestier et al., 2007; Stingl, Eaves, Zandieh, & Emerman, 2001; Visvader, 2009).

Great efforts have also been made to understand which mechanisms and pathways are involved in the maintenance and function of MaSCs. In particular, several genomic studies focused on the characterization of the specific transcriptional programs active in the different epithelial subpopulations (Kendrick et al., 2008; Lim et al., 2009; 2010; Sheridan et al., 2015). Multiple pathways have been discovered to play an essential role in governing stem cells status, including steroid hormones, Notch, BMP/TGFβ, Wnt/β-catenin, and JAK-STAT signaling (Fridriksdottir, Petersen, & Rnnov-Jessen, 2011; Fuchs, Tumbar, & Guasch, 2004). In general, the microenvironment surrounding the stem cells, commonly named “niche”, is pivotal in providing the adequate stimuli to either keep the ability to self-renew or to direct the fate through a complex repertoire of differentiation programs (Manavathi, Samanthapudi, & Gajulapalli, 2014; Visvader, 2009;

Visvader & Smith, 2011). Indeed, in the niche, specialized differentiated cells secrete specific factors and interact with the extracellular matrix (through cadherins, adherent junctions and integrins) such as to determine the retention or the release, and subsequent differentiation, of the stem cells (Fuchs et al., 2004).

1.4.1 Mammary Cancer Stem Cells (CSCs)

The stem cell concept has been applied also to tumors. Through the dissociation and transplantation of human cancer cells into immune-deficient mice, a subpopulation of cells capable of tumor reconstitution has been identified (Reya, Morrison, Clarke, & Weissman, 2001), which led to the so-called cancer stem cells hypothesis. This posits the existence of a minor population of self-renewing cancer stem cells (CSCs) able of reconstitute tumors upon transplantation (Kreso & Dick, 2014). These cells, which have been extensively characterized, comprise the 1-10% of the total population and are identified in human tumors by specific patterns of surface markers ($CD44^{+}$, $CD24^{-/low}$), as well as by high ALDH enzymatic activity (Groner, Vafaizadeh, Brill, & Klemmt, 2009). CSCs may generate through mutational events in normal stem cells or progenitors, which allows unlimited propagation of these cells and tumor maintenance (Cobaleda, Cruz, González-Sarmiento, Sánchez-García, & Pérez-Losada, 2008). Indeed, the cancer stem cells acquire the ability to symmetrically divide in two cells able to self-renew; while, usually the stem cells divide asymmetrically generating a stem cell and a cell undergoing to differentiation (Morrison & Kimble, 2006).

Experimental observations showed that acquisition of a more migratory phenotype due to epithelial to mesenchymal transition (EMT) is also associated with the appearance of stem cells properties, increasing the number of self-renewing cells able to seed mammospheres and, when implanted into cleared fat pads, to give rise to the entire epithelial tree (Mani et al., 2008). In fact, human mammary epithelial cells (HMECs) can generate SCs upon activation of pathways driving

EMT such as the Ras-MAPK cascade, Twist, Snail, Slug (Wenjun Guo et al., 2012; Mani et al., 2008; Morel et al., 2008). Several stimuli such as hypoxia, growth factors, cytokines and all the elements of the microenvironment of the stem niche can shape the mammary epithelial cells and let them acquiring stem cells features. However, this theory has recently been reformulated in the light of observations suggesting that distinct EMT programs govern normal and cancer stem cells (Ye et al., 2015).

Two main hypotheses have been formulated to explain the contribution of CSCs to the tumor formation and characteristics (Cobaleda et al., 2008). The first model considers the grade of differentiation of the initiating cells as the main factor that shapes the final tumor subtype (Ince et al., 2007). The second model proposes that all the tumor subtypes derive from the same cancer stem cell, which could then partially undergo differentiation and, through a random combination of oncogenic events, determines the tumor phenotype (Tsai et al., 1996).

According to transcriptional profiling experiments of mammary subpopulations and tumors, a transcriptional similarity between the different tumors subtypes and specific epithelial subpopulations has been observed (Figure 8). In particular, Claudin^{low} and 'Normal-like' tumors, having mesenchymal features, show a signature similar to the MaSCs subset. Surprisingly, the poorly differentiated basal-like tumors share more similarity with the luminal progenitors than the MaSCs. As expected, the signature of the more differentiated tumor subtypes Luminal A and B is similar to the differentiated luminal cells. Finally, the HER2-overexpressing tumors don't show a clear association with a normal counterpart of epithelial cells, but probably derive from cells with a luminal predisposition (Granit, Slyper, & Ben-Porath, 2013; Visvader, 2009). Hence elucidating the structure of the normal mammary epithelial hierarchy shall be functional to understand the

cellular origins of the different tumor subtypes and to shed light on the causes of breast cancer heterogeneity (Skibinski & Kuperwasser, 2015).

The presence of the CSCs may also explain the differential sensitivity to conventional therapies (chemo- and radio- therapy) and the relapse events of some breast tumors. Indeed, these cells are dormant or slow-cycling and mutagenic events alter their expression of multi-drug transporters, growth factors and anti-apoptotic signals. All these features allow the CSCs persistence after the cancer therapies. Actually these treatments, targeting the rest of the tumor, cause the selection of the CSCs and their increase in proportion in the total cancer mass. This results in the regeneration of the tumor, once the therapies are suspended (Kakarala & Wicha, 2008; Mimeault, Hauke, Mehta, & Batra, 2007).

Altogether, the above observations indicate that eradication of the cancer stem cells, by induction of their differentiation or apoptosis, would be essential to definitively cure breast cancer. Given the recent demonstration that distinct programs govern the normal and the cancer stem cells (Ye et al., 2015), a better knowledge of the pathways that specifically control cancer stem cells is needed to design new treatment strategies able to specifically target CSCs without causing toxicity to normal stem cells (Mimeault et al., 2007).

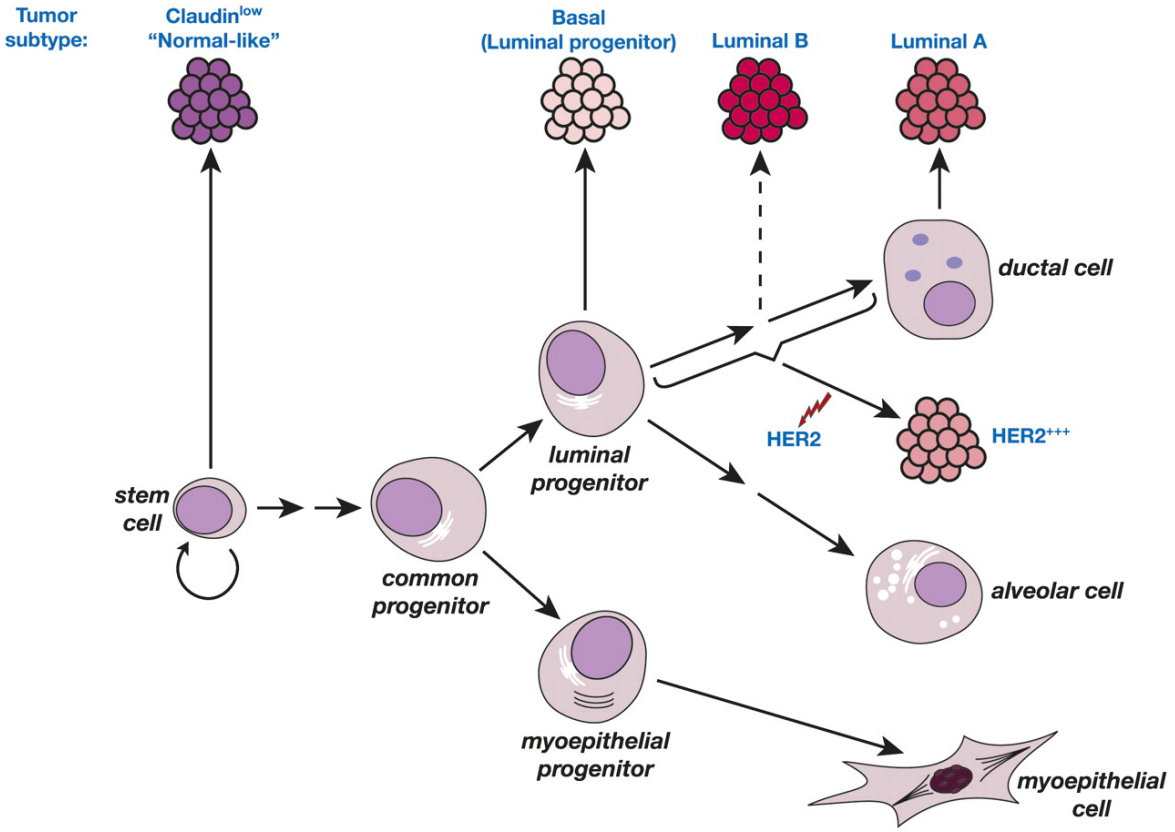

Figure 8: Model of human mammary epithelium hierarchy and potential derivation of breast tumor subtypes. According to data obtained with transcriptional profiling, six mammary tumor types (Claudin^{low}, Normal-like, Basal, Lumina A, Luminal B, HER2-overexpressed) have been associated to the normal epithelial cell counterpart (MaSCs, luminal progenitors or mature luminal cells) (from Visvader, 2009).

1.5 Myc: role in balancing differentiation and self-renewal

The inhibition of differentiation was one of the first biological activities attributed to *c-myc* oncogene (Coppola & Cole, 1986; Prochownik & Kukowska, 1986). This function has been confirmed by Myc gain/loss of function studies in cell lines and primary tissues of heterogeneous origin (Leon, Ferrandiz, Acosta, & Delgado, 2014), leading to the concept that inhibition of differentiation is a critical mechanism in Myc-mediated tumorigenesis.

Myc dampens differentiation mainly by preventing the exit from the cell cycle, (Buttitta & Edgar, 2007; Oster et al., 2002). Furthermore, Myc antagonizes the transcriptional function of Mxd or Mad proteins (Mad 1-4, Mnt), that dimerizing with Max causes the inhibition of proliferation and apoptosis, favouring cell

differentiation (Zhou & Hurlin, 2001). Lastly, MYC inhibits the expression of transcription factors that are in charge of driving differentiation in different tissues. For example it blocks c-JUN, GATA1, C/EBPα, ERG1, 'master regulators' of neuronal, erythroid, adipocytic and macrophagic differentiation, respectively (Leon et al., 2014).

Beyond its role in inhibition of cell differentiation, Myc plays an active role in promoting stemness. Expression of *c*- and *N*- *myc* is pivotal in embryonic stem cells (ESCs). Gene expression signatures have highlighted that the Myc transcriptional program is highly expressed in these cells (Ben-Porath et al., 2008) and accounts for the higher similarity between ESCs and cancer cells (Kim et al., 2010).

The network by which Myc can contribute to the maintenance of pluripotency and ESCs identity is complex. Myc induces the expression of Polycomb repressive complexes components or activates microRNA (e.g. *miR*-17-92), able to repress genes involved in cell differentiation (C.-H. Lin, Jackson, Guo, Linsley, & Eisenman, 2009; Neri et al., 2012). Finally, it represses developmental genes such as Gata6 or the Hox cluster interacting with the NuRD and the CoREST complexes (Chappell & Dalton, 2013).

Yamanaka and colleagues established pluripotency *in vitro* using Myc, together with Oct4, Sox2 and Klf4 (OSKM) and reprogrammed a differentiated somatic cell to an induced pluripotent stem cell (iPSC) (Takahashi & Yamanaka, 2006). Many variations to the initial approach have been described. Some of them showed that Myc overexpression is not essential in establishing pluripotency (Nakagawa et al., 2007), however it has been demonstrated that its lack reduces the efficiency and delays the reprogramming. It seems that OSK have a pioneer activity in binding closed chromatin, whereas Myc enhances OSK binding to the DNA (Soufi, Donahue, & Zaret, 2012).

Despite all these evidences corroborating the role of Myc in self-renewal and against differentiation, there are as many models where Myc plays a pro-differentiation role. Hematopoietic stem cells (HSCs) are one of the best-characterized systems in which Myc plays a pivotal role in homeostasis. Indeed, Myc deletion produces expansion of HSCs, whereas Myc overexpression causes depletion of the stem cell pool. This is due to Myc activity in regulating adhesion properties within the stem cell niche. As a matter of fact, Myc down-regulates integrins and N-cadherin expression, allowing the release of the stem cells from their protective microenvironment and anticipating the HSCs differentiation (Wilson, 2004).

The skin epidermis is another model in which Myc over-expression promotes differentiation (Gandarillas & Watt, 1997; Watt, Frye, & Benitah, 2008). Myc is expressed in the proliferative basal layer and in the bulge, its up-regulation modifies the adhesion properties (decreased α6β4 integrin expression), causes the formation of hemidesmosomes and the assembly of actinomyosin cytoskeleton (Frye, 2003). These changes of the local microenvironment stimulate keratinocytes stem cells progression to transient-amplifying cells, promoting differentiation along the epidermal and sebaceous lineages (Arnold & Watt, 2001).

The mechanisms of action of Myc in mammary epithelial stem cells and their microenvironment are still incompletely characterized. Both basal cells and luminal progenitors express high levels of Myc (Kendrick et al., 2008). Myc deletion in progenitor alveolar cells delays their differentiation and proliferation in pregnancy, decreasing also the expression of proteins involved in milk synthesis, during lactation (Stoelzle, Schwarb, Trumpp, & Hynes, 2009). Instead, Myc deletion from the basal cells leads to gland hypoplasia, reduces the proliferation rate both in luminal and basal epithelial compartments (suggesting a control of the luminal compartment by the basal cell layer), affects the self-renewal ability of the stem

and progenitor cells present in the basal layer impairing both their clonogenic potential *in vitro* and their reconstitution efficiency *in vivo* (Moumen et al., 2012). On the other side, Myc up-regulation in mammary epithelial cells causes mammospheres amplification and increased frequency of cleared fat pad repopulation (Pasi et al., 2011). All these observations highlight the role of Myc in expanding the mammary stem and progenitor compartments, even if remains still unclear where Myc exerts its function (luminal or basal compartment) and if it changes during the developmental stages of the mammary gland and/or in pathological conditions.

To understand the function of Myc in the mammary gland, and in particular in the self-renewing population, could be functional also to the comprehension of its role in breast cancers. In human, MYC oncogenic activity is associated with an aggressive tumor phenotype (BRCA1 alterations, ER-negativity, basal-like subtype) and poor prognosis (Blancato, Singh, Liu, Liao, & Dickson, 2004; Y. Chen & Olopade, 2008). In murine models, Myc up-regulation in the mammary glands raises the incidence of cancer, even if it needs the cooperation with other oncogenes (H-Ras being the most common) (Cowling, D'Cruz, Chodosh, & Cole, 2007; Schoenenberger et al., 1988). Several processes could underline the Myc-enhanced tumorigenesis, for example it has been observed that abnormal activation of Myc can lead to breast oncogenesis reinforcing β-catenin-dependent amplification of stem cells (Moumen et al., 2013) or inducing anchorage-independent growth and a general morphological transformation of mammary epithelial cells (Cowling et al., 2007). Anyhow, Myc deregulation, not only leads to the acquisition of a malignant stem cell-like phenotype (R. Nair et al., 2014), but it impacts also on processes related to survival, proliferation, apoptosis and genome instability in mammary epithelial cells (Hynes & Stoelzle, 2009; Pasi et al., 2011; Wang et al., 2011).

Myc has been shown to either promote or be required for epithelial to mesenchymal transition (EMT) both *in vitro* and *in vivo* (Bin Cho, Cho, Lee, & Kang, 2010; Jackstadt et al., 2013; A. P. Smith et al., 2008; Trimboli et al., 2008), supporting a migratory cellular phenotype in breast. Nevertheless, the effects of Myc on migration are not univocal and depend on cell-type or context (Cappellen, Schlange, Bauer, Maurer, & Hynes, 2007; H. Liu et al., 2012). Gene expression profiles in mammary tumors showed that Myc, like Ras, is able to induce features associated to EMT and cancer stem cell (M. Liu et al., 2009). The repression of the extracellular matrix proteins (fibronectin and collagen), reduced adhesiveness, cytoskeletal dysregulation are all hallmarks of Myc cell transformation (Akeson & Bernards, 1990; Coller et al., 2000; Cowling & Cole, 2007; Elkon et al., 2015; Shiio et al., 2002).

In general, Myc is a key transcription factor in the genesis of tumor. Also in other cellular systems, its down-regulation can cause senescence, loss of tumor-initiation capability and a general impairment of the neoplastic features (Akita et al., 2014; Civenni et al., 2013; Shachaf et al., 2004). In tumorigenesis, the regulation of self-renewal can be one crucial activity of Myc. Indeed, if physiological level of Myc are fundamental in stem cell maintenance, its up-regulation can support the formation of cancer stem cells (Laurenti, Wilson, & Trumpp, 2009) (Figure 9). Since inactivation of Myc is often associated to cell differentiation and regression in Myc-addicted tumors, the future challenge will be to identify a way to make its complex pathway druggable also in breast cancer.

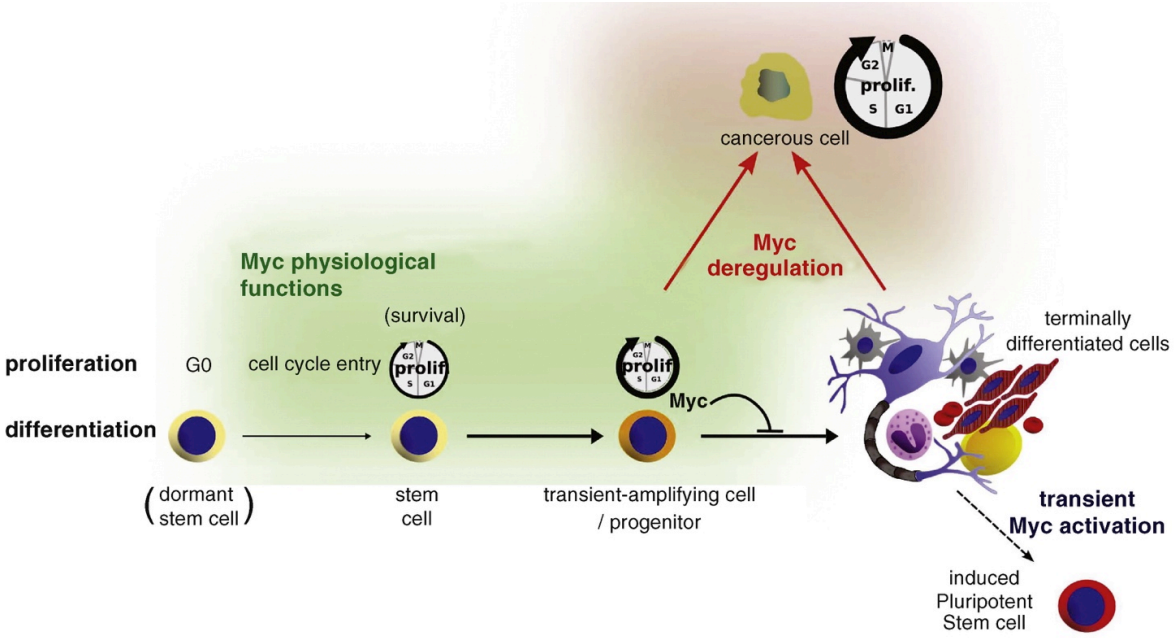

Figure 9: Role of Myc in differentiation of stem cells. The quiescent stem cells keep the tissue homeostasis in standard conditions. When activated, Myc (physiological levels) induces stem cells self-renewal, this process ensure both stem cells pool maintenance and transient-amplifying/progenitors cells production. The latter cells extensively proliferate before terminal differentiation into mature cells. During these phases, Myc inhibits terminal differentiation and has an essential role in proliferation. However, in some tissues at this stage, Myc can also regulate the release from the stem cell niche, thus differentiation. Transient reintroduction of Myc, in cooperation with Oct4, Sox2 and Klf4, induces reprogramming of somatic cells into pluripotent stem cells. Finally, deregulated Myc activity can, in association with other oncogenic events, induce the formation of Cancer Stem Cells (CSCs) (from Laurenti et al., 2009).

1.6 Aim of the project

The role of Myc in transcriptional repression is still one of the controversial aspects of Myc biology. Contrasting data and interpretations have led to debate even the existence of a Myc-dependent repressive function (Nie et al., 2012). To clarify this aspect of Myc transcriptional regulation is a crucial point, given the previous observations about its involvement in cell adhesion, self-renewal, survival and other functions central in tumorigenesis.

Unquestionably, Myc plays a pivotal role in balancing self-renewal and differentiation, but the fact that in different tissues the outcomes of Myc deregulation are different is a confounding factor. Although Myc is one of the key players in self-renewal function, its role in mammary epithelial stem cells is still

unclear. The goal of this study was to understand the role of Myc in transcriptional regulation of mouse mammary epithelial cells (MMECs) by genome-wide analysis. Taking advantage of a Myc mutant (Myc VD), unable to bind Miz1 (Steffi Herold et al., 2002), we specifically investigated the role of the Myc/Miz1 complex in controlling stem cells properties and the balance between self-renewal and differentiation. This could be clinically relevant for breast cancer patients, in which deregulation or overexpression of MYC are common features (about 20% of the cases) (Liao & Dickson, 2000), especially if we consider the relevance that Myc/Miz1 complex showed in other contexts of tumorigenesis (Wiese et al., 2013). In breast tumors, MYC can promote invasiveness and metastasis formation (Wolfer et al., 2010), its oncogenic activity is associated with more aggressive tumors and a poorer prognosis (Blancato et al., 2004; Y. Chen & Olopade, 2008). We thus decided to understand if Miz1 could play a role in these processes.

2. Material and Methods

2.1 Mouse models and genotyping

The following mouse strains were used for this study: *c-myc*$^{fl/fl}$ mice (Trumpp et al., 2001), R26-MycER^{T2} mice (Murphy et al., 2008), TET-MYC WT and TET-MYC VD mice (van Riggelen et al., 2010), R26rtTA mice (Jansson & Larsson, 2012), MMTVrtTA mice (Jackson lab, Stock #010576) (Whisenhunt et al., 2006), AthymicNude-Foxn1nu (Harlan). *c-myc*$^{fl/fl}$, R26-MycER^{T2}, R26rtTA and MMTVrtTA mice were maintained on a C57/Bl6 background. TET-MYC WT and TET-MYC VD mice were on a FVB background. Mice obtained from combination of strains in different background were used only at the first generation, in order to keep the genetic composition constant.

Primers used for genotyping are listed in Table 1.

Strain	Primers
c-myc$^{fl/fl}$	CACCGCCTACATCCTGTCCATTC TACAGTCCCAAAGCCCCAGCCAAG
R26-MycER^{T2}	AAAGTCGCTCTGAGTTGTTAT (comm.) GCGAAGAGTTTGTCCTCAACC (WT) GGAGCGGGAATGGATATG (KI)
TET-MYC WT	GGCGCTGCGTAGTTGTGCTGATGT AGCCCCGAGCCCCTGGTG
TET-MYC VD	CTGCTGCCAAGAGGCTCAAG GTATGCTGTGGCTTTTTTAAGGATAT
R26rtTA	AAAGTCGTCCTGAGTTGTTAT (comm.) GCGAAGAGTTTGTCCTCAACC (WT) GGAGCGGGAGAAATGGATATG (KI)
MMTVrtTA	GGCGAGTTTACGGGTTGTTA CTGGTCATCATCCTGCCTTT

Table 1: Primers used for mouse genotyping. A common primer (comm.) and a specific one were used to discriminate between wild type (WT) and knock-in (KI) mice.

Experiments involving animals were performed in accordance with the Italian laws (D.L. 116/92 and following additions), which enforce EU 86/609 directive (Council

Directive 86/609/EEC of 24 November 1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes).

2.2 Mammary gland dissection

The thoracic (5th and 6th gland pair) and inguinal (7th and 8th gland pair) mammary glands of 6- to 12-week-old virgin mice were dissected to obtain primary mouse mammary epithelial cells (MMECs). Inguinal mammary glands were collected also for whole-mount staining and histology. Figure 10 is a schematic representation of the murine mammary glands localization.

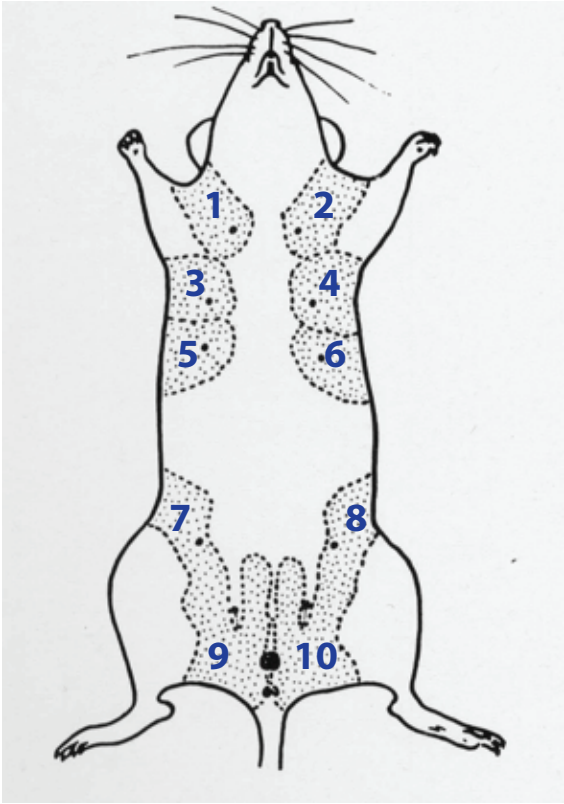

Figure 10: Schematic representation of the anatomy of murine mammary glands. Mouse mammary glands, in total five pairs, are divided in two subgroups: thoracic (1st to 6th glands) and inguinal (7th to 10th glands). The 5th and 6th thoracic gland pair and the 7th and 8th inguinal gland pair are usually dissected to isolate murine mammary epithelial cells (modified from Murphy E.D., 1966).

2.3 Mouse Mammary Epithelial Cells (MMECs)

2.3.1 Isolation and FACS analysis

Primary MMECs were isolated from thoracic and inguinal mammary glands. Tissues were mechanically minced, then digested with 200 U/ml collagenase I (Sigma), 10 mg/ml hyaluronidase IV (Sigma) in DMEM/F12 medium (Lonza) in rotation at 37°C for 3 hr. The samples were washed with PBS and centrifuged at 600 rpm for 5 min to enrich for mammary epithelial cells and organoids and eliminate stromal contamination. The organoids were further digested with 0.05% trypsin (Lonza) for 5 min and 5 mg/ml dispase (Stem Cell Technology) plus 100 µg/ml DNase (Roche) for 5 min. The cells were then filtered through 40 µm cell strainers to obtain a suspension of single cells (Wenjun Guo et al., 2012). The cell suspension was then centrifuged at 1200 rpm, 5 min at 4°C. The pellet was gently resuspended in a mix 1:1 of PBS and ACK lysing buffer (Lonza) for 1 min at room temperature, to lyse the red blood cells. The lysis was then stopped by dilution of the ACK buffer with extra PBS, then the samples were centrifuged 1200 rpm, 5 min, 4°C and resuspended in the culture medium.

For adhesion cell cultures, cells were plated in DMEM/F12 medium (Lonza) supplemented with 5% North American FBS (Hyclone), 10 ng/ml EGF (Peprotech), 4 mg/ml heparin (Hospira), 50 ng/ml cholera toxin (Sigma), 500 ng/ml hydrocortisone (Sigma), 5 µg/ml insulin (Roche), 50-100 I.U./ml penicillin, 50 to 100 µg/ml streptomycin, 0.5 mM L-Glu, 25mM Hepes (Sigma). For suspension cells culture, cells were plated in low attachment plates (BD Falcon) previously coated twice with polyhema (Sigma) in MEBM medium (Lonza), 2% B27 supplement (Life Technology), 10 ng/ml EGF (Peprotech), 10 ng/ml FGF (Peprotech), 4 mg/ml heparin (Hospira), 500 ng/ml hydrocortisone (Sigma), 5 µg/ml insulin (Roche), 50-100 I.U./ml penicillin, 50 to 100 µg/ml streptomycin.

The purity of the epithelial cells culture was assessed three days after seeding by

FACS analysis (MACSQuant® Analyzer 10, Miltenyi Biotec). Cells were stained on ice for 30 min and were all positive for the epithelial markers CD24 (eBioscience) and negative for not-epithelial markers such as CD31, Tert119 and CD45 (eBioscience) (Table 3).

2.3.2 Immortalization of primary MMECs

Primary MMECs have been isolated from *c-myc*$^{fl/fl}$ and R26-MycER^{T2} mice in order to obtain epithelial cells in which we could modulate the levels of Myc.

To get a more stable and homogenous culture, we immortalized the cells through p53 and Rb knock-down (Elenbaas et al., 2001; Karantza Wadsworth & White, 2008) by infecting them with the MLS-shp53 (Hemann et al., 2003) and LentiLox-shRb (Rubinson et al., 2003) viral vectors, respectively. Unlike the human counterpart, murine cells do not need the expression of the telomerase catalytic subunit to be immortalized, given the higher length of murine telomeres. Over a period of 4-6 weeks, both not/single-infected epithelial cells and contaminating cells died, whereas immortalized MMECs started growing. MMECs colonies showed typical cuboidal and packaged epithelial shape. About ten independent single cell clones were picked and characterized by real-time RT-PCR analysis with a selected panel of epithelial (*E-cadherin*, *ZO-3*) and mesenchymal (*Vimentin*, *N-cadherin*) markers (primers sequences in Table 2). Two clones have been selected from each preparation as biological replicates on the basis of their epithelial features both at morphological and gene expression levels. In regular tissue culture MMECs clones were kept at 50-70% confluence at 37°C, 5% CO_2, 5% O_2 and split every 3-5 days, at 1:5 dilution.

2.3.3 Modulation of Myc expression

To achieve the knock-out of the *c-myc* gene, *c-myc*$^{fl/fl}$ immortalized MMECs were treated with 100 µg/ml TAT-Cre recombinant protein (in-house prepared) and 10 µM chloroquine (Sigma) for two hours in Optimem medium (Invitrogen) and

harvested after 24 hours. The Cre recombinase is an enzyme of the bacteriophage P1 able to catalyse the site-specific recombination between two regions of DNA called LoxP sites. In this case the LoxP sites were present into the 3' untranslated region and the Intron 1 of *c-myc* gene sequence, thus after the cut the genes is irreversibly excided from the genome and switched-off. The TAT peptide derived from HIV-TAT protein, containing a nuclear localization sequence, renders the TAT-Cre recombinant fusion protein cell membrane-permeant.

The immortalized MMECs from R26-MycER^{T2} mice, suitable for Myc activation, express the *MycER* transgene from the ubiquitous Rosa26 locus. *MycER* encodes for a fusion protein between the ligand-binding domain of a mutant estrogen receptor (ER) and the Myc protein. The ER domain, without its intrinsic transactivation activity, responds to the synthetic steroid 4-hydroxytamoxifen (OHT). Hence, it maintains the chimeric MycER protein (that is constitutively expressed) inactive in the cytoplasm, until OHT is added to the culture and allows its translocation to the nucleus.

R26-MycER^{T2} MMECs have been stimulated with 200 nM OHT (Sigma) or EtOH (control) for 24 hours, and then harvested.

c-myc knock-out and activation were evaluated at DNA and/or RNA level by real-time RT-PCR and at protein level by western-blot analysis.

2.4 DNA extraction and analysis

Total genomic DNA was purified by DNeasy Blood & Tissue Kit (Qiagen) following manufacturer's instructions. 10 ng of DNA were then used for qPCR reactions with FAST SYBR Green master Mix (Applied Biosystems), the sequences of the primers used are listed in Table 2.

2.5 RNA extraction and analysis

Total RNA was purified by RNeasy columns (Qiagen) and treated on-column with DNaseI (Qiagen). RNA quality was checked with the Agilent 2100 Bioanalyser (Agilent Technologies). Complementary DNA (cDNA) was produced using the reverse transcriptase ImPromII (Promega). 10 ng of cDNA were used for real-time PCR reactions with FAST SYBR Green Master Mix (Applied Biosystems), the sequences of the primers used are listed in Table 2.

2.6 Protein extraction and analysis

Cell extracts were lysed by RIPA buffer (20 mM Hepes at 7.5 pH, 100 mM NaCl, 5 mM EDTA, 10% Glycerol, 1% Triton X-100, 0.1% SDS supplemented with protease inhibitors (Mini, Roche) and phosphatase inhibitors (0.4 mM Sodium Ortovanadate, 10 mM Sodium Fluoride), then sonicated. 30 µg of total cell extracts, quantified with the Protein Assay kit (Bio-Rad), were separated by SDS-PAGE using 7.5% or 12% polyacrylamide gels (Bio-Rad). The proteins were then transferred to a nitrocellulose membrane by Trans-Blot Turbo Transfer System (BioRad) for 30 min (25V- 1.0 A). Membranes were washed in TBS-T (10 mM TrisHCl, 100 mM NaCl, 0.1% Tween at pH 7.4) and blocked with 5% milk in TBS-T for 1 hour, then immunoblotted over-night with the indicated primary antibodies (Table 3): c-Myc (Y69, Abcam) and Vinculin (V9264, Sigma). After incubation of the membranes with appropriate secondary antibodies (Table 3) for 1 hour at room temperature, chemiluminescent imaging was performed by ChemiDoc MP System (BioRad) using Western ECL reagent (Pierce).

2.7 MMECs self-renewal and growth assays

Wild type (Ctr), TET-MYC WT and TET-MYC VD mice were crossed with R26rtTA

mice and the resulting offspring used to isolate primary MMECs for 3D culture mammosphere assay. This is an inducible system in which the TetO sequence (containing a tetracycline responsive element, TRE) is placed upstream of the promoter regulating the transcription of the transgene (in this case human *c-MYC* WT or VD). The reverse tetracycline transactivator (rtTA) is able to bind the TetO operator only in the presence of the antibiotic Tetracycline or its analogues Doxycycline (Tet-On system). In this way, the expression of the transgene is reversibly activated.

c-MYC up-regulation was evaluated at RNA level by real-time RT-PCR and at protein level by western-blot analysis.

A clonal sphere-forming assay was used to evaluate the self-renewal capacity of MMECs, that consists in growing them in non-adherent conditions as mammospheres (Dontu et al., 2003). Primary MMECs, isolated as previously described, were grown in ultra-low attachment plates at concentration of 20000 cell/ml in a serum-free mammary epithelial growth medium. In these conditions only stem cells can grow and give rise to a new colony, called mammosphere. After one week in culture mammospheres were collected and centrifuged at 300 rpm for 5 min at room temperature (the low centrifuge speed allow the sedimentation only of the spheres and not of single cells). The spheres were then resuspended in a small volume of fresh medium and manually disgregated by pipetting and using a syringe (25G needle), then filtered through 40 μm cell strainers to obtain single-cells suspension. The cells were counted and seeded at the initial concentration, in the presence or in the absence of 2 μg/ml Doxycycline (Sigma) to induce *c-MYC* overexpression, as indicated in each experiment.

The Growth Rate (GR) was calculated as the number of cells counted after mammospheres disgregation per number of cells seeded.

To establish the Sphere Forming Efficiency (SFE), the cells were seeded in

MethoCult™ (Stem Cell Technology) diluted 1:1 in cell culture medium (supplemented with the relative growth factors). Cells were then counted by 4x magnification on EVOS® Digital Microscopes (Electron Microscopy Sciences) after one week. SFE was calculated by dividing the number of newly formed mammospheres (considering only the spheres with diameter bigger than 80 μm) for the number of cells seeded, and reported as percentage.

To calculate the sphere size, for each sample ten microscopic fields were acquired and the area of the spheres was measured using ImageJ 1.43u software (NIH, USA) analysis, considering only the spheres with diameter bigger than 80 μm and > 0.2 circularity (to exclude the formation of mere aggregates).

2.8 EdU cell proliferation assay

To measure the percentage of MMECs in S phase, small mammospheres formed 48 hours after seeding of single-cells (between the M2 and M3 passage) have been incubated with EdU (5-ethynyl-2'-deoxyuridine, a nucleoside analogue of thymidine) 10 nM for 1.5 hours and then fixed with 2% formaldehyde for 5 minutes at room temperature. Samples were stained using anti-EdU antibody according to the manufacturer's instructions (Click-iT® kit for cell proliferation, Life Technology) and then incubated with propidium iodide over-night. The percentage of proliferating cells was assessed by FACS analysis (MACSQuant® Analyzer 10, Miltenyi Biotec).

2.9 Annexin V apoptosis assay

The presence of apoptotic cells was measured using Annexin V staining following the protocol of the Annexin V/Dead Cell Apoptosis Kit (Life Technology). Before the staining, small mammospheres formed 48 hours after seeding of cells between

M2 and M3 passages were mechanically isolated into single-cells. Annexin V labels apoptotic cells, while living cells remain unstained. Propidium Iodide (PI) staining (50 µg/ml in PBS for 5 min) marked the necrotic cells. The number of labeled dead cells was measured by FACS analysis (MACSQuant® Analyzer 10, Miltenyi Biotec).

2.10 Cleared fat pad reconstitution assay

To functionally measure the stem cell frequency of MYC WT and VD over-expressing mammospheres we performed mammary reconstitution assays transplanting single cells obtained from Ctr, TET-MYC-WT and TET-MYC-VD R26rtTA mammospheres, harvested at 48 hours after seeding at M2 passage. These cells were then transplanted in serial dilutions (1000, 500, 100 cells) into the cleared fat pads of host mice. We decided to use AthymicNude-Foxn1nu mice (homozygous, Harlan) as recipients since the donor cells were obtained from mixed background mice, in order to avoid any immune reject response. Cell aliquots suspended in PBS containing 25% Matrigel (BD-Bioscience) were injected into the inguinal mammary fat pads of AthymicNude-Foxn1nu mice at 3 weeks of age, previously cleared of the endogenous mammary epithelium. Transplanted mammary fat pads were examined for gland reconstitution 12 weeks post-injection. The presence of branched ductal trees with lobules and/or terminal end buds was considered as positive reconstitution. The frequency of Mammary Stem Cells (MaSCs) in the transplanted cell population was calculated through the serial dilution transplantation using the Extreme Limiting Dilution Analysis Program (http://bioinf.wehi.edu.au/ software/elda/index.html) (Wenjun Guo et al., 2012; Hu & Smyth, 2009).

2.11 Whole-mount carmine alum staining

In order to visualize the ductal development of whole mouse mammary glands we performed carmine alum staining. Inguinal mammary glands (7th and 8th gland pair in Figure 10) were spread on a glass slide and fixed either in 4% formaldehyde (in case of subsequent IHC analysis) or ethanol/acetic acid (Sigma, in proportion 1:3) at room temperature over-night. They were washed in 70% EtOH for 30 min, rinsed in distilled water for 10 min and stained in carmine alum (1 g carmine (Sigma), 2.5 g aluminum potassium sulfate (Sigma), 500 ml distilled water) at room temperature, until the white of the adipose tissue was not visible anymore. Then, the mammary glands were washed in 70%, 95% and 100% ethanol for 30 minutes each. Next, they were soaked in BABB solution (Benzylalcohol (Fluka): Benzylbenzoate (Sigma), 1:2), until fat pad clarifies. Mammary glands were photographed using a stereomicroscope (Olympus, SZX16) equipped with an Infinity 1 camera (Lumenera).

2.12 Immunohistochemistry

Formalin-fixed paraffin-embedded sections were deparaffinized in histolemon two times for 10 min each. The tissue sections were hydrated through graded alcohol series (100%, 95%, 70%, H_2O), 5 min each, at room temperature. The antigen unmasking was done by citrate buffer (Biogenex), at pH 6 for 30 min at 99°C. After cooling, the slides were washed in water, then the quenching of endogenous peroxidases was performed in 3% H_2O_2 for 5 min at room temperature. After a wash in TBS (10 mM TrisHCl, 100 mM NaCl, 0.1%), the slides were pre-incubated with a blocking solution (Dako diluent solution) for 20 min at room temperature. The slides were incubated with primary antibody for 2 hours at room temperature (c-Myc (Y69, Abcam) or Ki67 (M7249, Dako), see Table 3, in Dako diluent

solution). After a wash in TBS, they were incubated with secondary antibodies (Table 3) for 30 minutes at room temperature. Then, they were washed in TBS and incubated in peroxidase substrate solution (DAB, Dako) for 10 min. The reaction was blocked in water, the tissue sections were counterstained with hematoxylin for 1 min. After rinsing with running tap water, the tissue sections were dehydrated through graded alcohol series and mounted with Eukitt.

2.13 Murine tumor models

To test the tumorigenic potential of MYC WT and VD overexpression in mice, we crossed MMTVrtTA mice and TET-MYC WT or VD mice. In this case, the rtTA protein is specifically expressed in the breast epithelia of the mammary ductal system under the control of the mouse mammary tumor virus (MMTV) promoter. In this way, we obtained an *in vivo* breast-specific conditional up-regulation of Myc WT and VD. Immediately after weaning (4-weeks), 6 mice for each experimental group were induced by Doxy diet (Mucedola Srl), a nutritionally complete diet containing Doxycycline (625 mg/kg). The induction of MYC expression (both WT and VD) and proliferation was monitored in immunohistochemistry by c-Myc and Ki67 (Table 3) staining respectively after one and three moths of induction by Doxy in two mice for each experimental group. At the same time points we also performed whole mount staining to verify any structural change in the mammary gland. TET-MYC mice without MMTVrtTA element induced as well by Doxycycline or TET-MYC/MMTVrtTA mice with standard diet were used as controls. Since the described latency for the tumor onset in TET-Myc MMTVrtTA mice is around 22 weeks (D'Cruz et al., 2001), after one year the mice, that did not show any sign of tumor development, were sacrificed and the Lin- cells were analysed by FACS for CD49f (Biolegend) and Epcam (Biolegend) surface markers to monitor possible

changes in the cell type distribution of the epithelial compartment.

2.14 RNA-seq analysis

2.14.1 Library preparation

Libraries for mRNA-seq were prepared from 2 µg of total RNA for the immortalized MMECs and 300 ng of total RNA for the primary mammospheres, using TruSeq RNA Sample Prep Kits v2 (Illumina) following manufacturer's instruction.

2.14.2 Data filtering and analysis

RNA-seq libraries were sequenced in a 50 bp pair-end mode with 70 million reads depth. NGS reads were filtered using FASTX-Toolkit suite (fastq_quality_trimmer and fastq_masker tools, http://hannonlab.cshl.edu/fastx_toolkit/). Their quality was confirmed by FastQC application (www.bioinformatics.babraham.ac.uk/projects/fastqc/). RNA-seq NGS reads were aligned to the mm9 mouse reference genome, the corresponding gtf and bed files were downloaded from the UCSC Table Browser. The TopHat aligner (version 2.0.6) with default parameter was used for the alignment. In case of duplicated reads, only one read was kept. Read counts were associated to each exon using the HTSeq software (http://www.htseq.org). Absolute gene expression was defined determining the number of exonic reads per kilobase per million of total reads aligned (eRPKM). Two-tailed Student's *t*-test was used to calculate p-value; the p-value adjusted (q-value) for multiple comparisons was obtained by Benjamini correction. Differentially expressed genes (DEGs) were identified using the Bioconductor package DESeq2 as genes whose q-value relative to the control sample is lower than 0.01.

RNA-seq of *c-myc*$^{fl/fl}$ and R26-MycER^{T2} immortalized MMECs was performed on two biological replicates (independent clones, derived from a bulk population that

was a mix of cells isolated from five mice) and two technical replicates. RNA-seq of primary mammospheres was performed on three independent samples for each experimental group (each sample was a mix of cells isolated from five mice).

2.15 Gene Ontology biological process analysis

Functional annotation analysis to determine enriched Gene Ontology biological processes was performed using DAVID (Huang, Sherman, & Lempicki, 2008; 2009).

2.16 microRNA-seq analysis

2.16.1 RNA extraction and library preparation

Total RNA, including small RNA species was extracted using miRNeasy mini kit (Qiagen). 300 ng of total RNA, for each condition in biological triplicate, were used to prepare small RNA libraries using Illumina TrueSeq™ Small RNA kit following manufacturer's instructions. The libraries were sequenced in 50 bp single-read mode at 20 million read depth on an Illumina Hiseq 2000 Platform.

2.16.2 Data filtering and analysis

After de-multiplexing by CASAVA software (that generates reads in FastQ format) and removing the adapters by the Flexible Adapter Remover software (FAR version 2.15), the reads of 22 bases (which correspond to miRNAs) were filtered. They were aligned to a custom genome that includes sequences of all canonical mature miRNAs (1975 mouse miRNAs according to the version 20 of miRBASE, http://www.mirbase.org) and the relative sequence variants or isoforms (named isomiRs). The isomiRs considered included the 5'- or 3'- end modified miRNA or the trimmed 3'- form. Reads shorter than 18 bp were discarded because considered unreliable. Alignment was performed with Bowtie ultrafast short-read

aligner (-v 0 alignment mode, no mismatches allowed). The number of aligned reads was counted by IsomiRage JAVA tool (Nicassio, 2014) (http://cru.genomics.iit.it/Isomirage/). The reads number was normalized for sequencing depth (standard reads per million normalization, RPM). The average expression in each experimental group was calculated between three biological replicates. Species with RPM >10 in at least one sample were selected for further analysis. miRNAs differentially regulated ($|Log_2$ fold change$|>0.5$) were identified, with a p-value ≤ 0.05. Statistic analysis (Student's *t* test) and heatmaps were realized by JMP 10 (SAS) software.

2.17 Human tumors analysis

In order to understand the relevance in human breast cancer of the genes identified differentially repressed between MYC WT and VD, we analysed their expression in a cohort of 1070 breast tumors present in the TCGA database (for which the MYC status is known). The tumors were divided in subcategories (Luminal A, Lumina B, Luminal unknown, Triple Negative Breast Cancer (TNBC), HER2+) on the basis of the available information about estrogen receptor (ER), progesterone receptor (PR), Epidermal Growth Factor receptor 2 (HER2) and Ki67 expression (Voduc et al., 2010). The expression data were defined as Log_2 fold change respect to the median of Normal breast tissue (112 sample). Out of 888 genes identified in our signature, 754 had a clear human counterpart and were used for the analysis. A *t*-test analysis was used to define genes regulated in statistically significant way.

2.18 Analysis of published datasets

Data from other published datasets (Kendrick et al., 2008; Lim et al., 2009; 2010; Sheridan et al., 2015; Stingl et al., 2006; Wiese et al., 2015) will been considered to select genes biologically significant from our Myc/Miz1 repressed genes list. All the expression data have been downloaded, from the supplementary tables of the paper or the Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) database, as GSM matrix. The data will be normalized on the median of the control, if it is present a clear control, otherwise on the median of all the samples and expressed as Log_2 fold change. Only those genes, of the Myc/Miz1 repressed signature, coherently regulated in each dataset (down-regulated in MaSCs vs. other subpopulations, down-regulated in CSCs vs. other tumors cells) will be taken into account.

	Amplicon	Forward primer	Reverse primer
RNA	*c-myc (mouse)*	TTTTTGTCTATTTGGGGACAGTG	CATCGTCGTGGCTGTCTG
	c-MYC (human)	CTGCGACGAGGAGGAGAACT	GGCAGCAGCTCGAATTTCTT
	Ifrd2	CACTTTGTTGAGGGTGGTGA	AGAGCACTTCCAGTCCGAAG
	Smpdl3b	GGATGGGGAGATGGTGTATG	GAAGCTGTCGGTATGGTGGT
	Rplp0	TTCATTGTGGGAGCAGAC	CAGCAGTTTCTCCAGAGC
	E-cadherin	CACCTGGAGAGAGGCCATGT	TGGGAAACATGAGCAGCTCT
	ZO-3	ACCCTATGGCCTGGGCTTC	CCCGGGTACAACGTGTCC
	N-cadherin	ATGTGCCGGATAGCGGGAGC	TACACCGTGCCGTCCTCGTC
	Vimentin	CTTGAACGGAAAGTGGAATCCT	GTCAGGCTTGGAAACGTCC
DNA	*c-myc* $^{fl/fl}$	TCTAGACTTGCTTCCCTTGCTGT	TTCCTGTTGGTGAAGTTCACGT
	c-myc $^{-/-}$	AAATAGTGATCGTAGTAAAATTTAG CCTG	ACCGTTCTCCTTAGCTCTCACG
	Nucleolin	GGCGTGGTGACTCCACGT	CGAAATCACCTCTTAAAGCAGCA

Table 2: Oligonucleotide sequences used in real-time PCR analysis for mRNA and genomic DNA evaluation.

Primary antibodies			
Antigen	**Technique**	**Info**	**Dilution**
CD24-PE	FACS	-	1:200
Lin+ (CD45-, CD31-, Ter119- biotin)	FACS	-	1:300 (each)
CD49f-APC	FACS	-	1:20
Epcam-PeCy7	FACS	-	1:600
Myc (Y69)	Western Blot	Rabbit monoclonal	1:10000
Vinculin (V9264)	Western Blot	Mouse monoclonal	1:10000
Myc (Y69)	IHC	Rabbit monoclonal	1:100
Ki67 (M7249)	IHC	Rat monoclonal	1:500

Secondary antibodies		
Antigen	**Technique**	**Dilution**
Streptavidin-Alexa488	FACS	1:100
Anti-Rabbit IgG	Western Blot	1:5000
Anti-Mouse IgG	Western Blot	1:10000
HRP-rabbit	IHC	1:100
HRP-rat	IHC	1:100

Table 3: List of primary and secondary antibodies used.

3. Results

3.1 Immortalized mammary epithelial cell lines from c-*myc*$^{fl/fl}$ and R26-MycER^{T2} mice

In order to study the transcriptional role of Myc in mammary epithelial cells, we isolated primary epithelial cells from the mammary gland of *c-myc*$^{fl/fl}$ (Trumpp et al., 2001) and R26-MycER^{T2} (Murphy et al., 2008) mice, allowing conditional deletion and activation of Myc, respectively. In particular, *c-myc*$^{fl/fl}$ cells have LoxP sites into the 3' untranslated region and Intron 1 of the *c-myc* gene, leading to deletion of the gene upon exposure to Cre recombinase. Here, we used a membrane permeable TAT-Cre fusion protein (S. K. Joshi, Hashimoto, & Koni, 2002) to introduce Cre in the cells. Instead the R26-MycER^{T2} cells express the *MycER* transgene under the ubiquitous Rosa26 locus; they are suitable for Myc activation, upon OHT treatment, since the constitutively expressed MycER chimeric protein is inactive in the cytoplasm, until OHT is added to the culture and allows its translocation to the nucleus.

Epithelial cells were isolated from both thoracic and inguinal mouse mammary glands by mechanical and chemical digestion. The epithelial nature of the resulting cell cultures was assessed three days after isolation by FACS analysis: cells were positive for the epithelial surface marker CD24 (Figure 11) and negative for markers of endothelial, hematopoietic cells or fibroblasts (CD45, CD31, Ter119) (Figure 12).

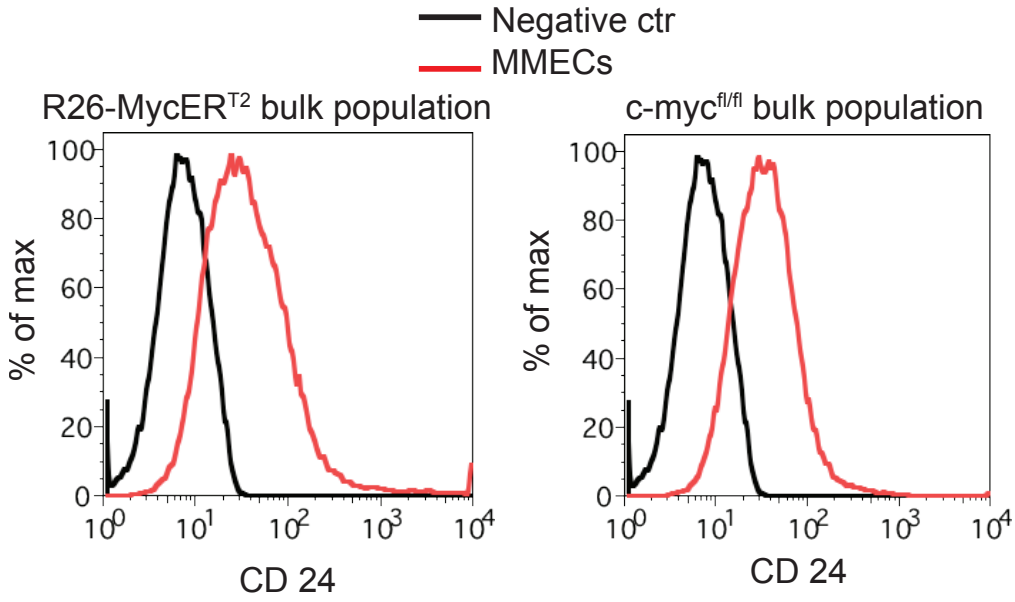

Figure 11: CD24 expression (epithelial marker) by FACS analysis. Histogram representing the percentage of cells positive for CD24 for R26MycER^{T2} (on the left) and *c-myc*$^{fl/fl}$ (on the right) cells respectively, after three days of culture. The primary mammary epithelial cells isolated (red) are compared to fibroblasts as negative control (black).

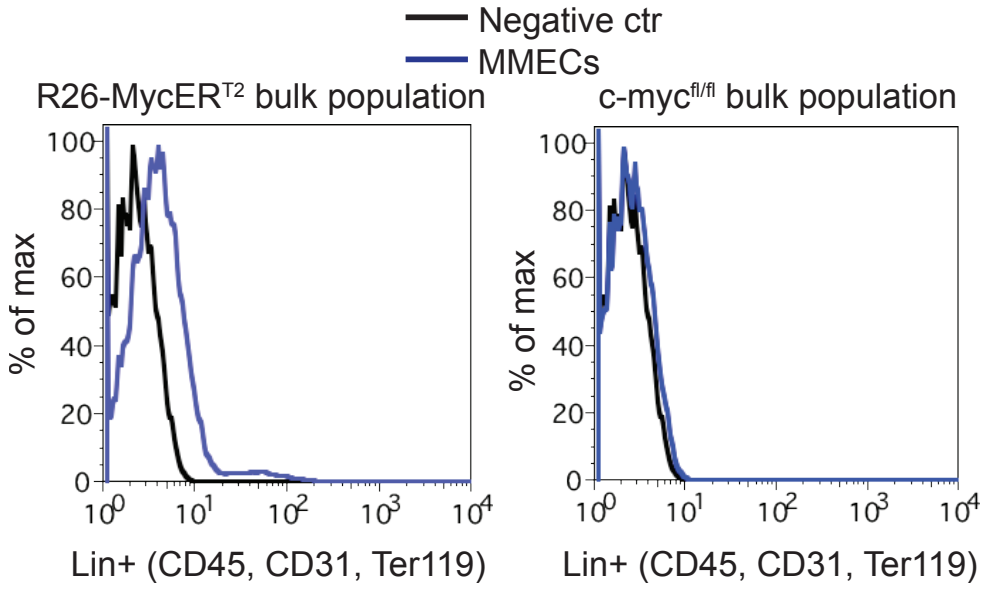

Figure 12: Lineage markers expression (CD45, CD31, Ter119) by FACS analysis. Histogram representing the percentage of cells positive for Lineage markers for R26-MycER^{T2} (on the left) and *c-myc*$^{fl/fl}$ (on the right) cells respectively, after three days of culture. The primary mammary epithelial cells isolated (blue) are compared to not stained cells as negative control (black).

The primary cells cultures, confirmed epithelial by FACS analysis, spontaneously underwent an apparent Epithelial to Mesenchymal Transition (EMT) after one

passage in conditions of adhesion, with a marked fibroblastoid appearance (Figure 13A). Hence, we thought to obtain a more stable system through cell immortalization by dual p53 and Rb knock-down, as previously described (Elenbaas et al., 2001; Karantza Wadsworth & White, 2008). We thus infected freshly isolated MMECs with viral vectors expressing shRNAs against the two proteins. Over a period of 4-6 weeks, both non-infected and single-infected epithelial cells died, whereas double-infected MMECs started growing and became immortalized. As expected, the immortalized clones grew in clusters and showed a cuboidal shape that was maintained over passages (Figure 13B).

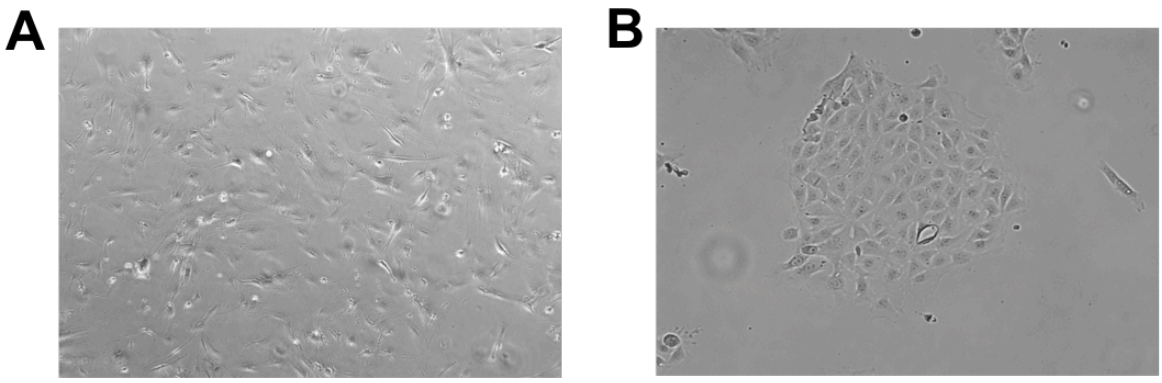

Figure 13: Morphology of the primary and immortalized mammary epithelial cells. (A) A representative image of the MMECs after one passage in culture in adhesion conditions. They showed flat, mesenchymal-like shape. (B) The same cells after immortalization by p53 and Rb knockdown showed an epithelial shape.

Several single cell clones from each immortalized bulk population were picked and expanded. The mRNA expression of epithelial (*E-cadherin, ZO3*) and mesenchymal markers (*Vimentin, N-cadherin*) was then analysed by RT-qPCR, both in the bulk population and in each clone at the same passage (Figure 14). On the basis of their morphological aspect and molecular profile, the two clones with the strongest epithelial phenotype of each population were selected for further analysis (clones C3 and E4 for R26-MycER^{T2} cells, clones A2 and A4 for *c-myc*$^{fl/fl}$ derived cells).

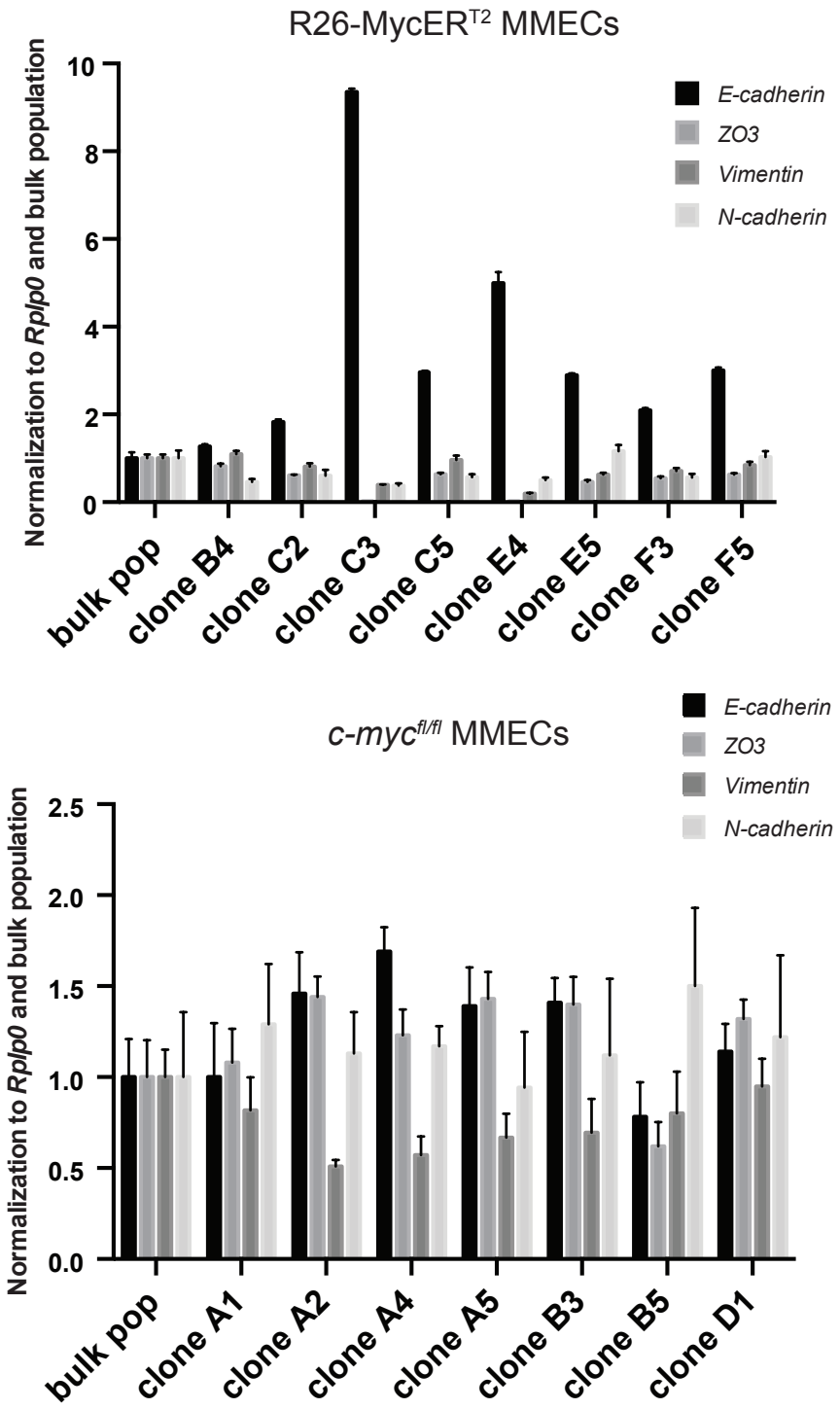

Figure 14: Gene expression analysis of epithelial clones by RT-qPCR analysis. mRNAs level of epithelial (*E-cadherin*, *ZO3*) and mesechymal (*Vimentin*, *N-cadherin*) genes in different clones picked from the immortalized bulk populations of mammary epithelial cells. In the upper panel are showed data from R26-MycER^{T2} MMECs, in the lower panel from *c-myc*$^{fl/fl}$ MMECs, respectively. All data were normalized respect to the *Rplp0* housekeeper gene and the reference bulk population. The bar plots represent the average values, standard deviations are calculated between three independent measurements.

3.2 Regulation of Myc levels in immortalized MMECs

Once we obtained stable epithelial cell clones of the desired genetic background, we set up the best conditions for Myc super-activation or deletion.

3.2.1 R26-MycER^{T2} MMECs

Epithelial clones originated from R26-MycER^{T2} mice were stimulated by standard concentration of OHT (200 nM) known to induce translocation of the MycER chimera in the nucleus in other cellular systems (Littlewood, Hancock, Danielian, Parker, & Evan, 1995). We confirmed the activation of MycER transcriptional activity by RT-qPCR analysis of known Myc target genes (*Smpdl3b* and *Ifdr2*) (Marinkovic et al., 2004) in a 48 hours time course experiment (Figure 15). The best time point (24 hours) of transcriptional response to MycER was selected for the following experiments.

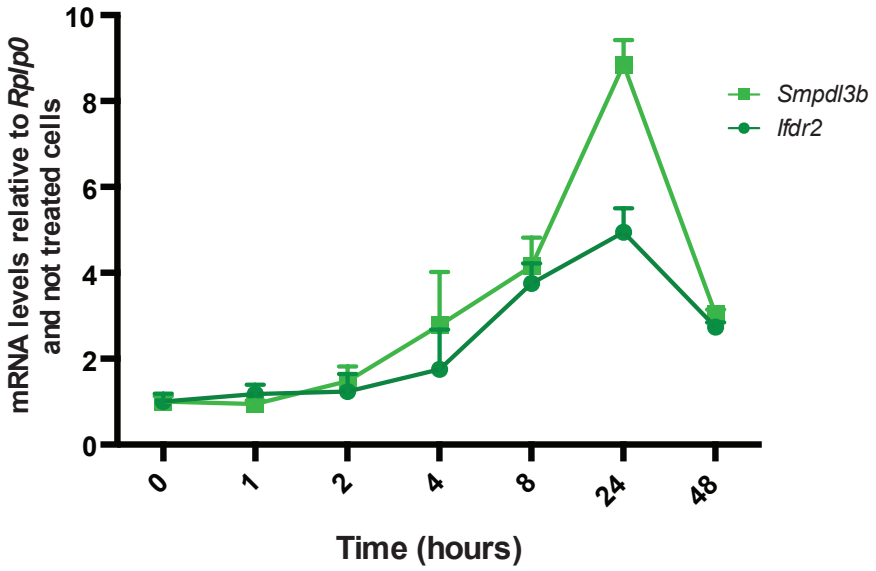

Figure 15: Time course of MycER transcriptional activation by OHT stimulation of R26-MycER^{T2} MMECs. The mRNAs level of known Myc targets genes (*Smpdl3b* and *Ifdr2*) was monitored in MycER MMECs stimulated by 200 nM OHT for 1, 2, 4, 8, 24 and 48 hours. The data are normalized respect to the *Rplp0* housekeeper gene and not treated samples. The line charts displays the average values and standard deviation between two independent clones (C3 and E4), each one done in technical duplicate, at different time points.

We compared the expression of the same genes in MMECs without MycER relative to MMECs R26-MycER (not treated by OHT), to confirm the absence of leakiness in the system (Figure 16).

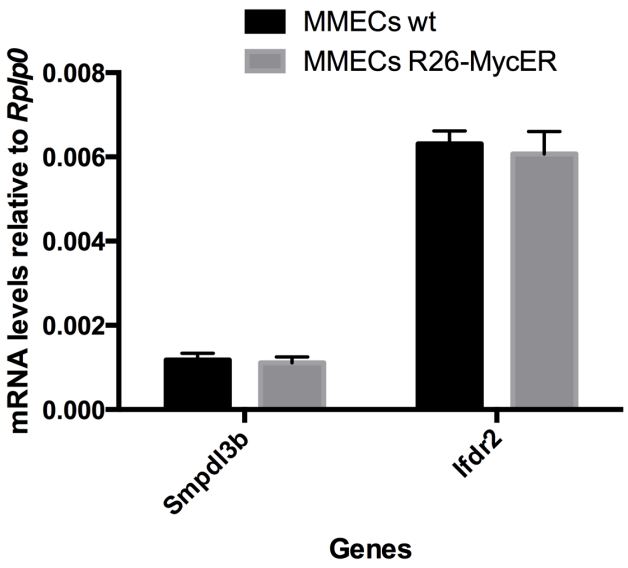

Figure 16: Control of the absence of leakiness of R26-MycER^{T2} MMECs. The mRNA levels of known Myc targets genes (*Smpdl3b* and *Ifdr2*) was monitored in MMECs without R26-MycER compared to MMECs with R26MycER, not treated by OHT. The data are normalized respect to the *Rplp0* housekeeper gene. The bars plot displays the average values and standard deviation between two measurements on the bulk population.

We also verified the expression of the exogenous human MycER protein by western blot (Figure 17): 24h of OHT treatment increased the level of the chimeric protein (possibly by increasing its stability) and led to a reduction in the levels of endogenous Myc (possibly by the transcriptional repressive activity exerted by the exogenous protein (Facchini, Chen, Marhin, Lear, & Penn, 1997; L. J. Penn et al., 1990).

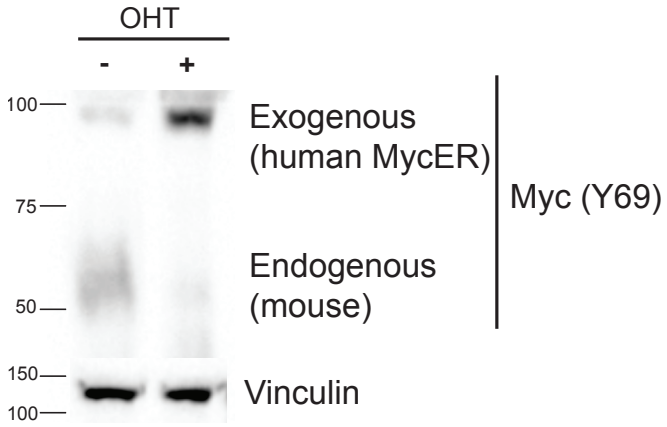

Figure 17: Myc super-activation in R26-MycER^{T2} mammary epithelial clones stimulated by OHT for 24 hours. Myc protein detected by western blot analysis using Myc Y69 antibody (Abcam) in not treated (+EtOH, vehicle) and treated samples (+ 200 nM OHT) for 24 hours. The upper band (about 97 kDa) represents the exogenous human MycER protein; the lower band (52 kDa), instead, is the endogenous murine Myc protein. Vinculin protein (130 kDa) is used as reference for protein level normalization**.**

3.2.2 c-myc$^{fl/fl}$ MMECs

In *c-myc$^{fl/fl}$* MMECs, deletion of the *c-myc* allele was achieved treating the cells with the recombinant TAT-Cre recombinase (100 µg/ml) for 2 hours. As judged by either genomic DNA (gDNA) or mRNA analysis 24 hours after treatment, an effective *c-myc* knockout (above 80-90%) was reached in these conditions (Figure 18).

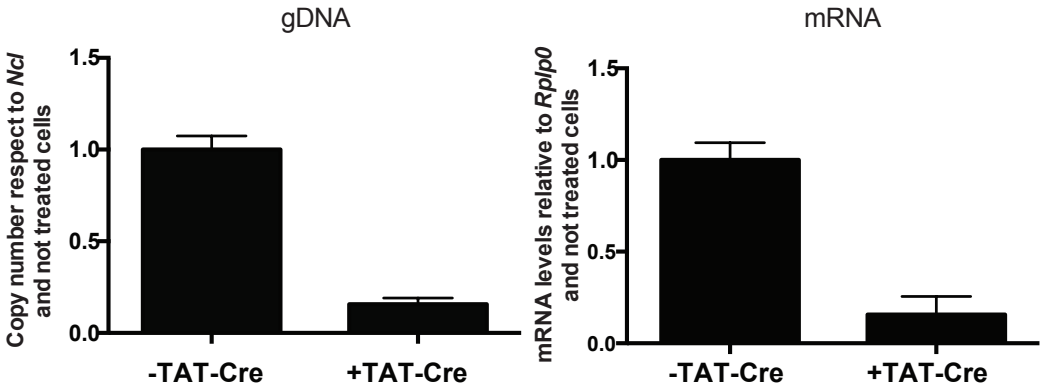

Figure 18: gDNA and mRNA levels of *c-myc* in *c-myc$^{fl/fl}$* MMECs treated with TAT-Cre recombinase. Myc levels in *c-myc*$^{fl/fl}$ epithelial clones treated or not with TAT-Cre (100 µg/ml, 2 hours) and analysed after 24 hours. The bars plot on the left shows genomic DNA copy number respect to the *Nucleolin* amplicon and not treated cells. Instead, the bars plot on the right shows the mRNA levels relative to *Rplp0* housekeeper gene and not treated cells. The average and standard deviation between two independent clones (A2 and A4), each one done in technical duplicate is reported.

We confirmed the complete ablation of the endogenous c-Myc in these conditions also at protein level (Figure 19).

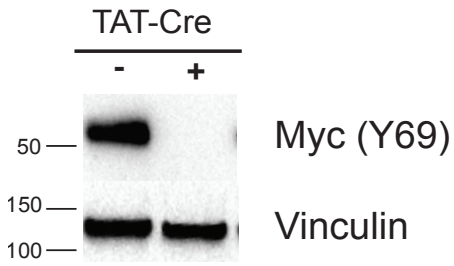

Figure 19: Myc protein ablation in *c-myc*$^{fl/fl}$ mammary epithelial clones treated with TAT-Cre. Myc protein detected by western blot analysis using Myc Y69 antibody (Abcam) in not treated (-TAT-Cre) and treated samples (+ 100 µg/ml TAT-Cre, 2 hours). As expected, there is the complete disappearance of Myc protein (52kDa) 24 hours after the TAT-Cre treatment. Vinculin protein (130kDa) is used as reference for protein level normalization.

3.3 Myc negatively controls the expression of genes involved in extracellular matrix interaction, cell adhesion and epithelial morphogenesis regulation

c-myc$^{fl/fl}$ and R26-MycER^{T2} MMECs treated with TAT-Cre or OHT to induce Myc deletion or activation were subjected to mRNA profiling by RNA-seq: for each condition, two different cell clones (biological replicates) were used and for each clone two technical replicates were prepared, producing a total of 4 replicates per sample. Differentially Expressed Genes (DEGs) between treated samples (+TAT-Cre or +OHT) and control (-TAT-Cre or -OHT) were identified using the Bioconductor package DESeq2 considering genes whose q-value relative to the control sample was lower than 0.01 (FDR ≤ 1%) and whose eRPKM expression value was ≥ 1 in at least one condition (see Material and Methods). We thus identified 4787 DEGs (2340 up- and 2447 down- regulated) upon MycER activation (akin to Myc Overexpression, henceforth MycOE) and 4687 DEGs (2262 up- and 2425 down- regulated) upon Myc deletion (Myc Knock-Out, henceforth MycKO) relative to the corresponding control sample.

In order to identify the core Myc transcriptional program in MMECs, we searched for genes regulated in opposite way by Myc up-regulation and deletion. For this purpose, we first identified the genes expressed in both systems (eRPKM ≥ 1 in both systems). Starting from 12568 genes expressed in MycOE and 12514 genes expressed in MycKO, we found 11930 genes commonly expressed. We plotted their changes in expression relative to control (as Log_2 fold change) upon Myc modulation in the two conditions (Figure 20).

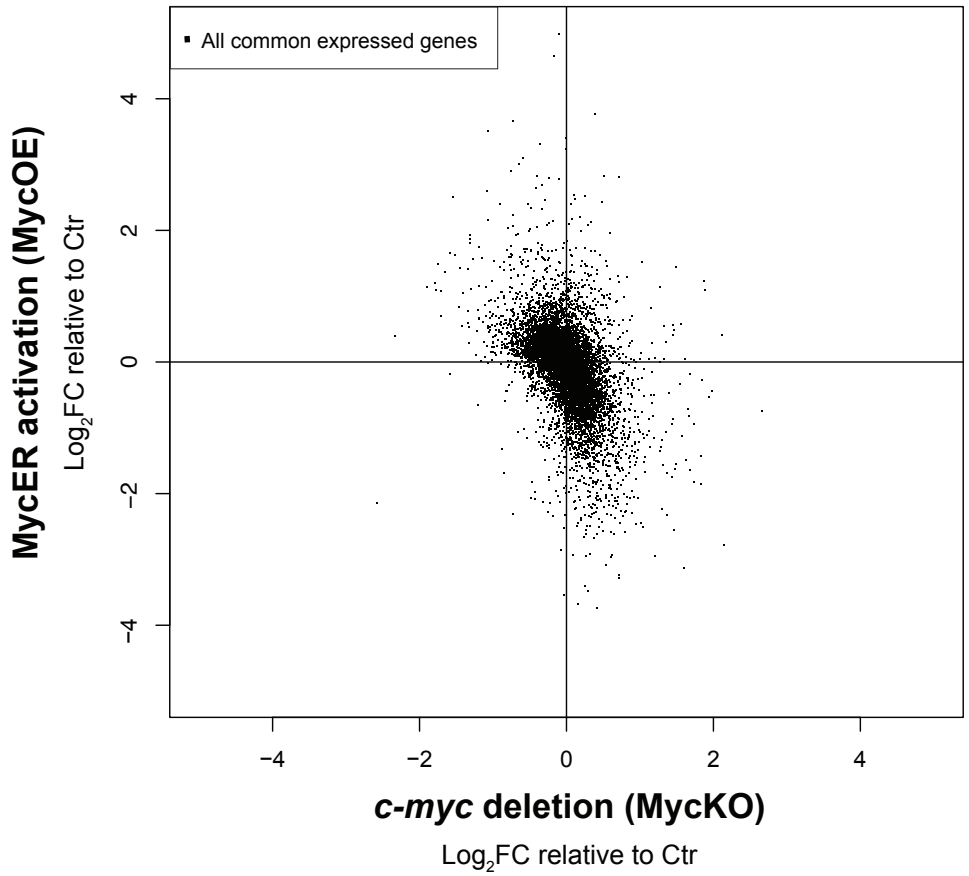

Figure 20: Transcriptional changes of common expressed genes in Myc overexpressing and *c-myc* deleted cells. The scatter plot shows all the 11930 common expressed genes between Myc overexpressing (MycOE) and *c-myc* deleted (MycKO) cells. Each dot is a gene and it is positioned on the basis of its changes in expression in MycOE (y axes) and MycKO (x axes) samples relative to the Control (expressed as Log_2 fold change). The two datasets show moderate anti-correlation, calculated by Pearson coefficient ρ_{XY}= -0.48, p-value < 2.2e-16.

As expected, we observed a moderate anti-correlation in the transcriptional responses to Myc activation and deletion (Pearson coefficient = -0.48). We then selected those genes that were regulated in a statistically significant manner in

both systems (q-value ≤ 0.01; FDR ≤ 1%, 2409 genes), thus the anti-correlation between the two systems improved (Pearson coefficient = -0.67). Among the significant genes, we selected those that were regulated in an opposite manner in the two systems. In this way we identified 1180 Myc-induced genes (up-regulated in MycOE and down-regulated in MycKO) and 1065 Myc-repressed genes (down-regulated in MycOE and up-regulated in MycKO) (Figure 21).

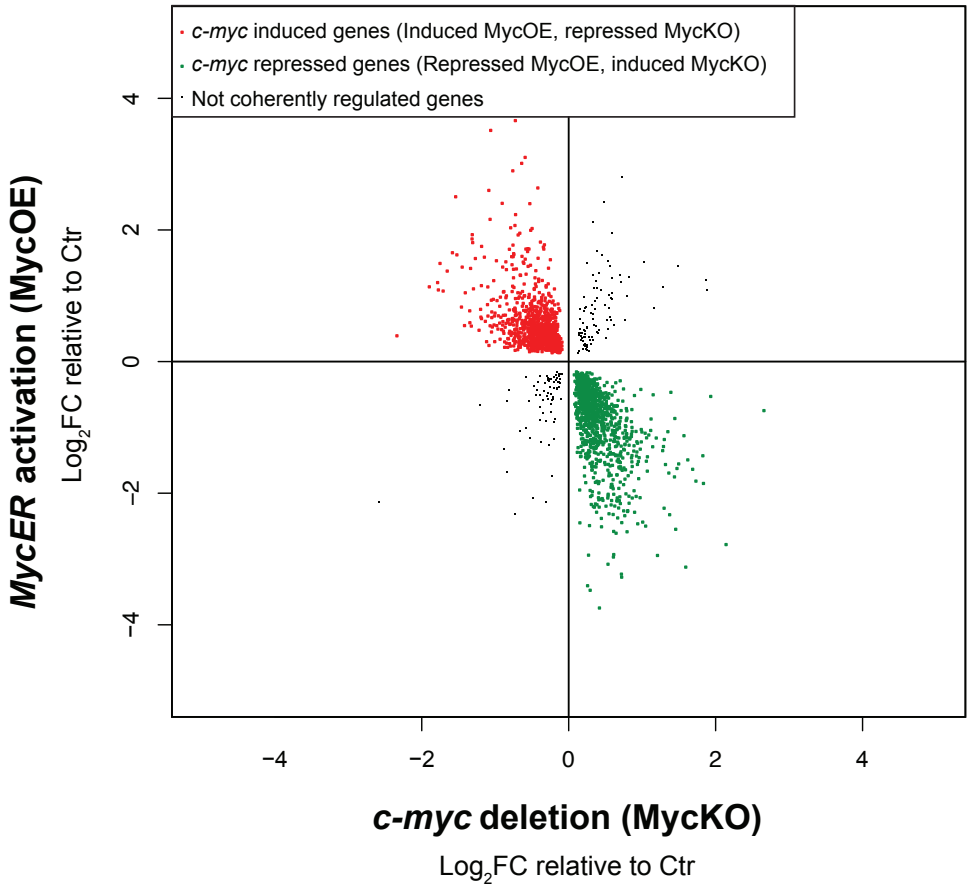

Figure 21: **The core of Myc transcriptional program in MMECs.** The scatter plot shows all the 2409 genes regulated in a statistically significant manner (q-value ≤ 0.01; FDR ≤ 1%) both in MycER activation (MycOE) and *c-myc* deleted (MycKO) cells. Each dot represents a gene and it is positioned on the basis of its changes in expression in MycKO (x axes) and MycOE (y axes) samples relative to the Control (expressed as Log_2 fold change). Genes regulated oppositely by Myc modulation in the two systems represented the core of Myc transcriptional program. In particular in green are highlighted Myc-repressed genes, (down-regulated upon MycER activation and up-regulated upon *c-myc* deletion), instead in red are highlighted the Myc-induced genes (up-regulated upon MycER activation and down-regulated upon *c-myc* deletion). The black dots are those genes that were not coherently regulated in the two cellular models, which as expected were very few genes. Considering only the significant genes, the anti-correlation between the two datasets, calculated by Pearson coefficient, increases ρ_{XY}= -0.67, p-value < 2.2e-16.

Gene Ontology analysis of the 1180 Myc-induced genes, identified the main biological function promoted by Myc in MMECs as proliferation, metabolism, ribosomal biogenesis, RNA processing and modification (mRNA, rRNA, ncRNA), mitochondrial organization, chromatin modification, all categories that were as also previously found in several other cell types (Dang, 2014; Eilers & Eisenman, 2008; Kress et al., 2015) (Table 4).

GO ID	Term	p-value
GO:0034660	ncRNA metabolic process	1.96E-54
GO:0042254	ribosome biogenesis	7.04E-47
GO:0044429	mitochondrial part	1.55E-14
GO:0000166	nucleotide binding	3.55E-14
GO:0006259	DNA metabolic process	1.84E-13
GO:0004386	helicase activity	6.99E-12
GO:0007049	cell cycle	1.17E-11
GO:0006397	mRNA processing	4.60E-11
GO:0009451	RNA modification	3.39E-10
GO:0016568	chromatin modification	3.59E-07

Table 4: The top ten Gene Ontology categories of Myc–induced genes in MMECs in adhesion. The individuated 1180 Myc-induced genes in MMECs in adhesion have been submitted to Gene Ontology analysis (http://david.abcc.ncifcrf.gov). The top ten scoring GO categories are listed with the corresponding p-value.

The same analysis has been performed for the 1065 Myc-repressed genes. This list of genes was enriched for categories such as lysosome and vesicle-mediated transport, angiogenesis, cell death, extracellular matrix and cell adhesion regulation, epithelial development and morphogenesis (Table 5).

GO ID	Term	p-value
GO:0005764	lysosome	9.05E-10
GO:0043169	cation binding	2.91E-05
GO:0001568	blood vessel development	4.76E-05
GO:0030695	GTPase regulator activity	6.47E-05
GO:0031012	**extracellular matrix**	**1.67E-04**
GO:0007155	**cell adhesion**	**1.00E-03**
GO:0016192	vesicle-mediated transport	1.00E-03
GO:0008219	cell death	2.00E-03
GO:006042	**epithelium development**	**1.00E-02**
GO:0002009	**morphogenesis of an epithelium**	**1.00E-02**

Table 5: The top ten Gene Ontology categories of Myc–repressed genes in MMECs in adhesion. The individuated 1065 Myc-repressed genes in MMECs in adhesion have been submitted to Gene Ontology analysis (http://david.abcc.ncifcrf.gov). The top ten scoring GO categories are listed with the corresponding p-value.

Among the biological functions repressed by Myc in epithelial cells, we focused our attention on extracellular matrix interactions, cell adhesion and differentiation. Previous observations highlighted the pivotal role of Myc in mammary epithelial cells in controlling stem cells and progenitors function (Moumen et al., 2012; Pasi et al., 2011) in part by promoting the acquisition of more mesenchymal and aggressive features associated to EMT and cancer stem cell generation (Bin Cho et al., 2010; Jackstadt et al., 2013; M. Liu et al., 2009; A. P. Smith et al., 2008; Trimboli et al., 2008). We hypothesized that these functions of Myc could be mediated, at least partially, by its repressive activity (Gebhardt, 2006; Kerosuo et al., 2008).

3.4 A role for Miz1 binding in Myc-induced self-renewal of mammary epithelial stem cells

Among the co-factors reported to cooperate with Myc in transcriptional repression, Miz1 appears to be the most relevant (Peukert et al., 1997) and may be required

for a large fraction (25-40%) of Myc-down-regulated genes (Walz et al., 2014). Normally, Miz1 acts as transcriptional activator in association with the histone acetyltransferase p300 and nucleophosmin. The co-operation with Myc or other transcription factors, turns Miz1 in a repressive factor (Steffi Herold et al., 2002; Staller et al., 2001; Wanzel et al., 2008). The repressive role of Myc/Max/Miz1 trimers is well documented on survival/apoptotic genes and on cell adhesion and differentiation/self-renewal related genes (Gebhardt, 2006; Kerosuo et al., 2008). To further study the transcriptional repressive role of Myc, and in particular its interaction with Miz1 in self-renewal of mammary epithelial stem cells, we took advantage of a Myc mutant unable to bind Miz1 (MycV394D, henceforth Myc VD) (Steffi Herold et al., 2002). This mutation in the Myc protein does not affect its association with Max, allowing the identification of the Miz1-dependent functions of Myc (Walz et al., 2014).

3.4.1 Evaluation of self-renewal and growth rate

Primary mammary epithelial cells were isolated from Tet-On mouse models able to ubiquitously overexpress MYC WT or VD upon Doxycycline treatment (TET-MYC-WT/R26rtTA and TET-MYC-VD/R26rtTA mice). The cells were grown in non-adherent conditions in a mammosphere assay (Dontu et al., 2003), to measure *in vitro* their self-renewal ability. Indeed, the mammospheres are enriched in early progenitor/stem cells able to propagate in culture and to differentiate along all three mammary epithelial lineages. On the contrary the late progenitor/mature cells, also present within the mammospheres, when seeded in the following passages stop growing or undergo to cell death for anoikis, because of their inability to regenerate spheres in non-adherent conditions (Figure 22).

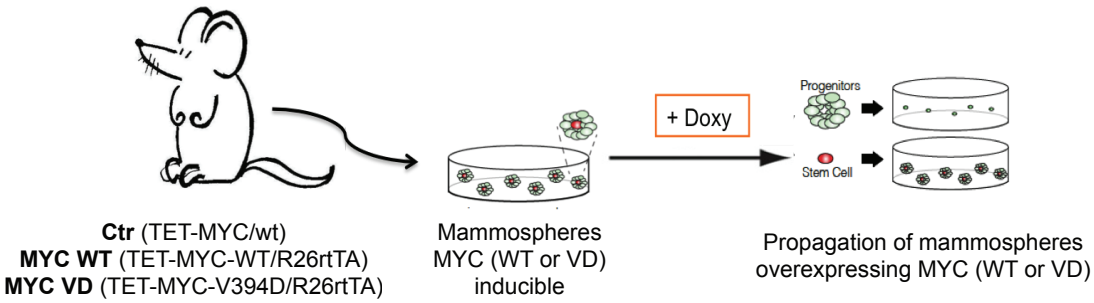

Figure 22: Schematic representation of mammospheres assay. Mammary epithelial cells derived from Ctr (TET-MYC/wt), MYC WT (TET-MYC-WT/R26rtTA) and MYC VD (TET-MYC-VD/R26rtTA) mice cultured in suspension in undifferentiated conditions are able to form mammospheres. This assay is a surrogate of stem cells propagation *in vitro*, since only early progenitors/stem cells are able to propagate and regenerate spheres in serial passages. On the contrary late progenitors/mature cells differentiate and stop growing or undergo to cell death for anoikis (modified from (Dontu et al., 2003).

Freshly isolated mammary epithelial cells of the selected genotypes were plated in non-adherent conditions for two weeks (M0-M1) in order to remove all the contaminant cells (above all fibroblast, endothelial and hematopoietic cells). After that, a single-cell suspension was seeded and the resulting mammospheres were serially passaged (M2, M3, M4, M5) once a week in the presence of Doxycycline (2 µg/ml, administered twice a week) (Figure 23). At each passage, we evaluated the Sphere Forming Efficiency (SFE), Growth Rate (GR) and the area of the newly formed mammospheres in order to characterize the stem potential of cells over-expressing both forms of MYC, relative to control wild type cells.

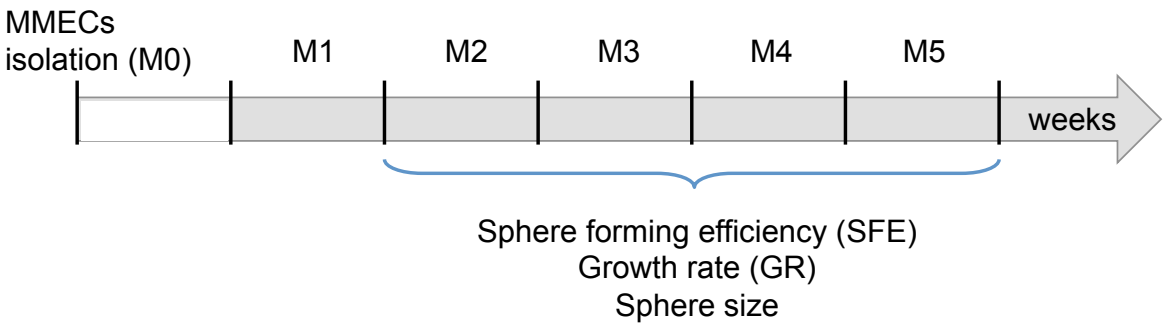

Figure 23: Schematic representation of the experimental design. Murine mammary epithelial cells were selected in culture for two weeks (M0-M1). Hence, they were serially passaged (M2-M5) in the presence of Doxycycline (grey arrow), each passage lasted one week. During these passages Sphere Forming Efficiency (SFE), Growth Rate (GR) and spheres size were evaluated.

First of all we verified the absence of leakiness of the Tet-On system. We compared the expression of two Myc-targets (*Smpdl3b* and *Ifdr2*) in primary MMECs both TET-MYC/R26rtTA without Doxycycline and TET-MYC/wt upon induction with Doxycycline (for 48h) (Figure 24). As expected there are no effect due to Doxycycline, neither any leakiness of the Tet-On system.

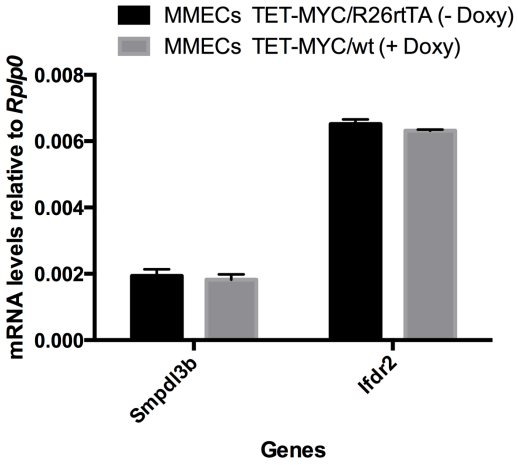

Figure 24: Control of the absence of leakiness of the TET-MYC MMECs. The mRNA levels of known Myc targets genes (*Smpdl3b* and *Ifdr2*) were monitored in TET-MYC/R26rtTA MMECs (-Doxy) compared to TET-MYC/wt MMECs (+Doxy for 48 hours). The data are normalized respect to the *Rplp0* housekeeper gene. The bars plot displays the average values plus standard deviation between two independent measurements.

Then, we checked the over-expression of *c-MYC* WT and VD transgenes both at mRNA and protein levels. In particular the mRNA was analysed immediately after one week of culture of the mammary epithelial cells (M0, before starting the treatment with Doxycycline) and after one (M1), three (M3) and five (M5) weeks of *c-MYC* induction by Doxycycline. Starting from the same levels of endogenous c-myc (mouse), the induction of the exogenous c-MYC (human) is comparable between MYC WT and VD in the first passage (M1) and slightly higher in MYC VD samples respect to MYC WT at M3 and M5. As expected, the induction of c-MYC was also able to repress the endogenous c-myc and to induce Myc targets such as Ifdr2 and Smpdl3b (Figure 25).

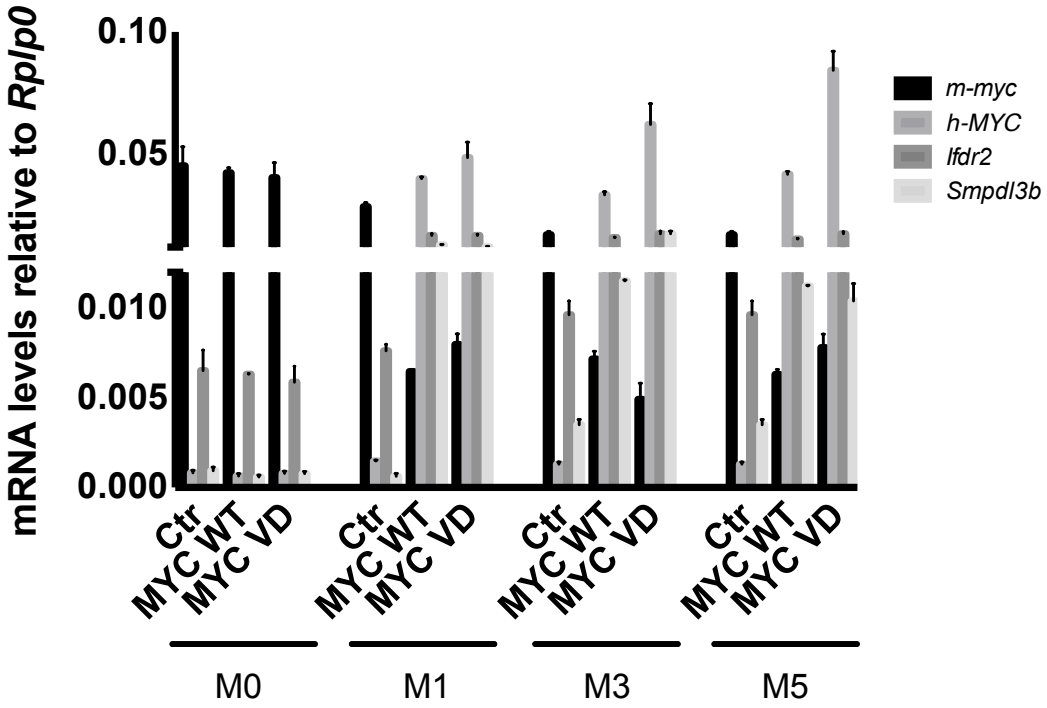

Figure 25: Gene expression analysis in mammospheres overexpressing MYC WT and VD. mRNAs extracted from primary mammary epithelial cells (Ctr, MYC WT and MYC VD) grown as mammospheres before (M0) and after Doxycycline induction for the entire duration of the experiment (M1, M3, M5). The bars plots show the mRNA levels of the endogenous *c-myc* (mouse), exogenous Doxycycline-induced *c-MYC* (human) and Myc targets (*Ifdr2* and *Smpdl3b*) relative to *Rplp0* housekeeper gene. Standard deviations are measured considering three replicates of the experiment.

In the end, we verified the expression of the *c-MYC* WT and VD transgenes at the protein level after one (M1), three (M3) and five (M5) weeks of c-MYC induction by Doxycycline and found that Myc quantity was comparable between the WT and the mutant VD protein (Figure 26).

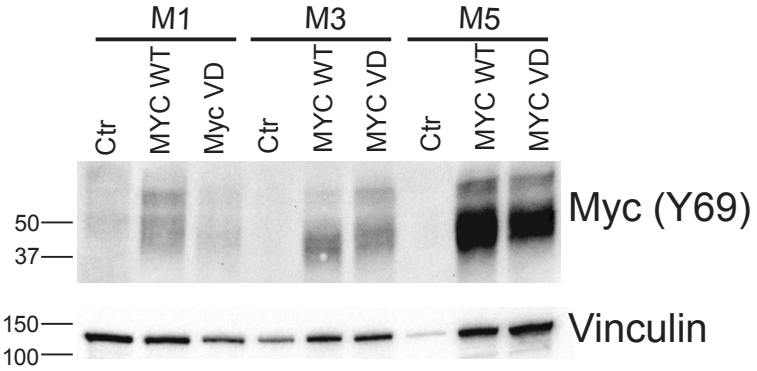

Figure 26: Myc protein overexpression in mammospheres. Myc protein detected by western blot analysis using Myc Y69 antibody (Abcam) in primary mammospheres (Ctr, MYC WT and MYC VD) treated by 2 µg/ml Doxycycline for one (M1), three (M3) and five (M5) weeks. c-Myc protein is represented by two bands (52 kDa, 40 kDa). Vinculin protein (130kDa) is used as reference for protein level normalization.

Since in non-adherent conditions only the stem cells were able to survive and give rise to small aggregates of proliferating cells, called mammospheres, the number of growing spheres was an indirect measurement of the stem cells contained in the population. We measured the Sphere Forming Efficiency (SFE) as percentage of sphere-forming units for a certain number of cell seeded. In general, mammospheres originated from MYC-overexpressing cells were different relative to the control, showing increases in both number and size (Figure 27). Compared with MYC WT, this effect was reproducibly lower in MYC VD overexpressing cells.

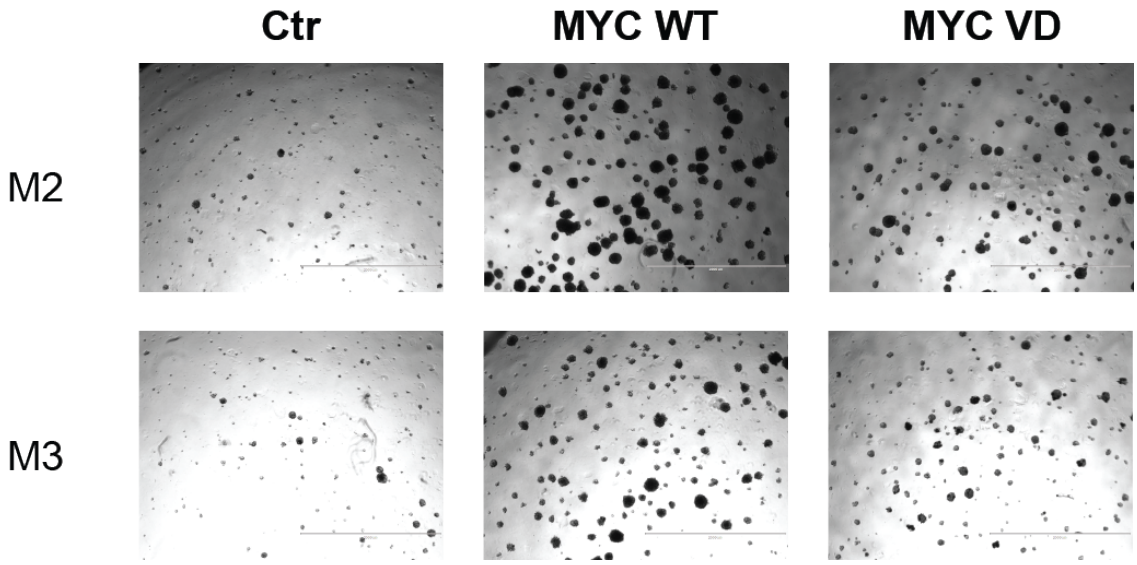

Figure 27: Primary mammospheres originated from MYC overexpressing (WT and VD) and control cells. Representative images of mammospheres originated from control (Ctr) and MYC (WT and VD) overexpressing cells at M2 and M3 passages of the assay. The scale bar of reference is 2000 µm.

In particular, MYC-overexpressing cells showed increases in SFE of four to ten folds relative to control cells (Figure 28). This difference was higher in the first part of the experiment (M2-M3), whereas it decreased in the second part (M4-M5). MYC VD showed the same behaviour of MYC WT, but with a milder increase of the SFE (five to two folds).

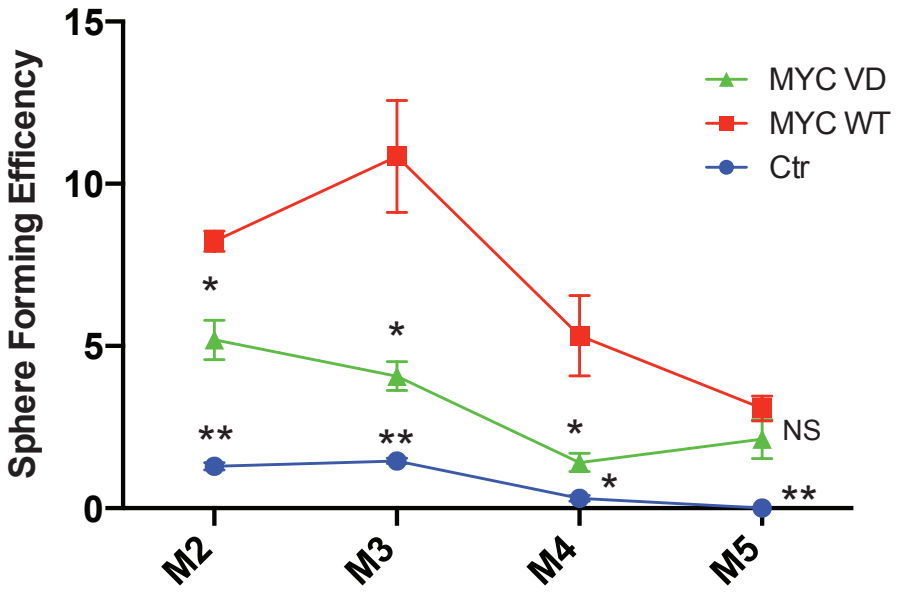

Figure 28: Sphere forming efficiency (SFE) of MYC overexpressing (WT and VD) and control mammospheres. The Sphere Forming Efficiency (SFE) for Ctr (blue), MYC WT (red) and MYC VD (green) overexpressing cells over the passages (M2-M5). The lines chart display the average values calculated between three independent experiments (two technical replicates for each) ± standard deviation. *t*-test statistical analysis between Ctr or MYC VD samples respect to MYC WT showed respectively: NS= Not Significant, ∗ = p-value ≤ 0.05, ∗∗ = p-value ≤ 0.01 significant difference, at each passage.

In addition, we measured the Growth Rate (GR) as number of cells counted after mammospheres disgregation for number of cells seeded to assess the proliferative potential of the mammospheres. We observed that MYC WT over-expression significantly enhanced the growth rate (three to five fold) of the mammospheres (Figure 29). These differences were observed at all passages, but in particular at M2 and M3. As above, MYC VD had a milder phenotype but in this case the differences between MYC WT and VD were not statistically different.

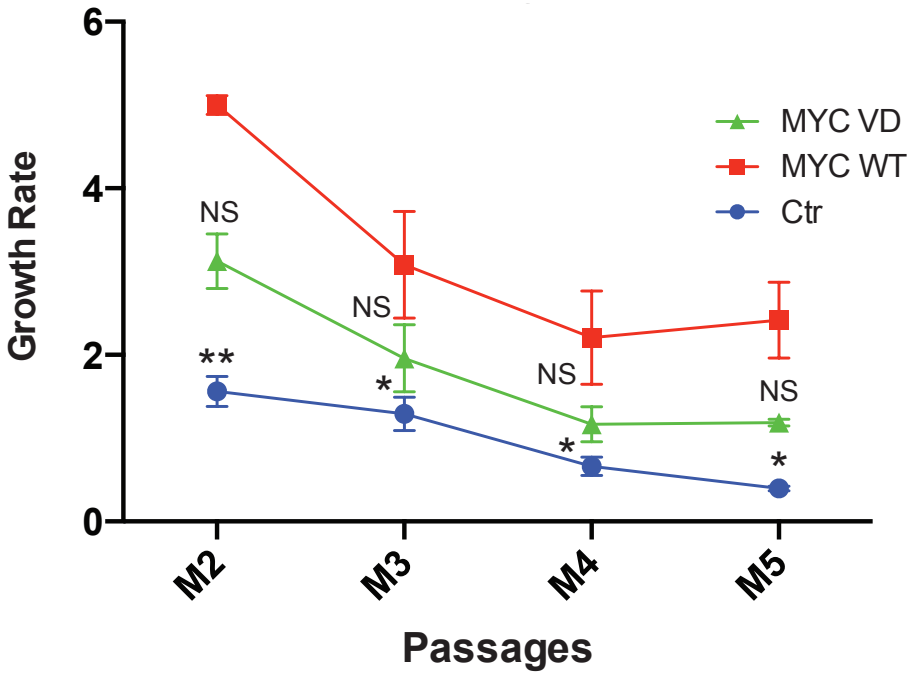

Figure 29: Growth rate (GR) of MYC overexpressing (WT and VD) and control mammospheres. The Growth Rate (GR) has been measured for Ctr (blue), MYC WT (red) and MYC VD (green) overexpressing cells as the number of cells originate from disgregation of newly formed mammospheres divided by the number of seeded single-cells over the passages (M2-M5). The lines chart display the average values calculated between three independent experiments (two technical replicates for each) ± standard deviation. *t*-test statistical analysis between Ctr or MYC VD samples respect to MYC WT showed respectively: NS= Not Significant, ∗ = p-value ≤ 0.05, ∗∗ = p-value ≤ 0.01 significant difference, at each passage.

Lastly, we compared the sphere size in the three experimental groups and we found that MYC WT over-expression increased the area of the mammospheres significantly respect to the control cells. These differences were observed at all passages, but in particular at M2. Again MYC VD had a milder phenotype that was significantly different from the WT at M2 and M3 passages (Figure 30).

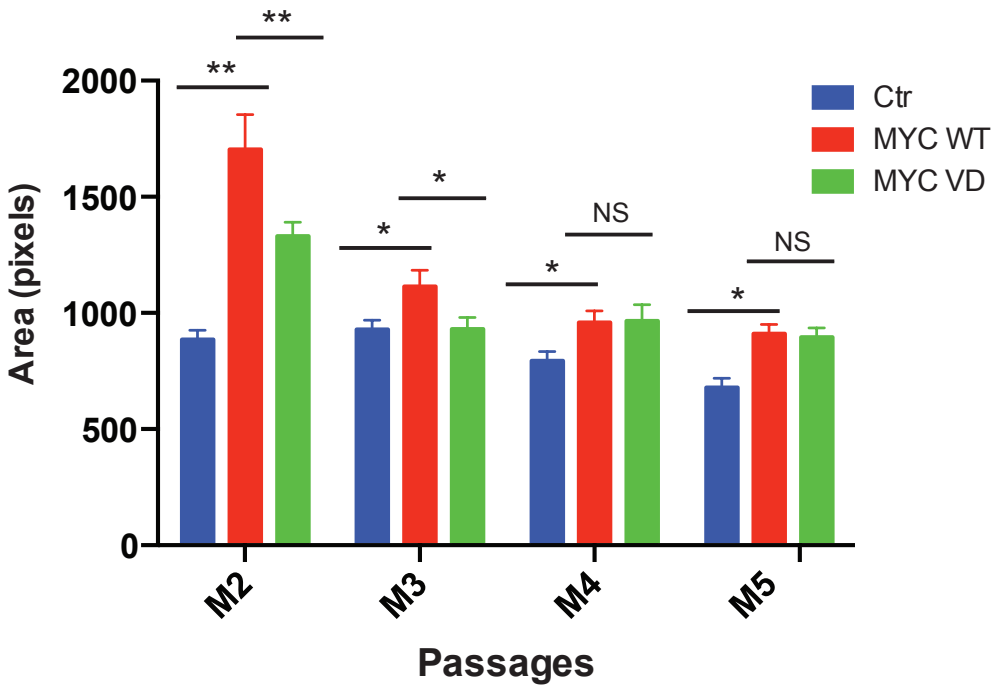

Figure 30: Size (Area) of MYC overexpressing (WT and VD) and control mammospheres. The sphere size has been measured for Ctr (blue), MYC WT (red) and MYC VD (green) overexpressing cells. The area of the spheres is calculated in pixels considering spheres with diameter more than 80 μm (≥600 pixels) and circularity >0.2 (this parameter considers the roundness of the spheres to exclude mere cell aggregates), using ImageJ software. The bars plot show the average values calculated between three independent experiments (two technical replicates for each) ± standard deviation. *t*-test statistical analysis between Ctr or MYC VD samples respect to MYC WT showed respectively: NS= Not Significant, ∗ = p-value ≤ 0.05, ∗∗ = p-value ≤ 0.01 significant difference, at each passage.

In conclusion MYC overexpressing mammary epithelial cells showed an increased sphere forming efficiency *in vitro* in mammosphere assays, suggesting enhanced self-renewal ability. The MYC VD mutant was less effective suggesting that the interaction between Myc and Miz1 could be partially responsible for the observed phenotype.

3.4.2 Evaluation of proliferation and apoptosis

Since Myc is a well-known regulator of cell cycle progression and proliferation (Amati et al., 1998; Oster et al., 2002), and given the prominent role of Myc/Miz1 interaction in the regulation of apoptosis (Patel & McMahon, 2006; Seoane et al., 2002; Staller et al., 2001), we verified if the observed differences between MYC WT and VD were attributable to an alteration in these functions rather than in self-

renewal ability. To distinguish between these two hypotheses, we evaluated cell cycle distribution and cell death in Ctr or MYC (WT or VD) over-expressing mammospheres 48 and 96 hours after seeding between M2 and M3 passages.

As expected, Myc overexpressing cells had a higher proliferation rate compared to control, with more cells in S-phase, as shown by increases EdU staining, both at 48 and 96h (Figure 31). MYC VD overexpression was slightly less effective than MYC WT at 48h, while it had a comparable effect in proliferation at 96h, while it was significant different, as MYC WT, relative to the control (at bot time points).

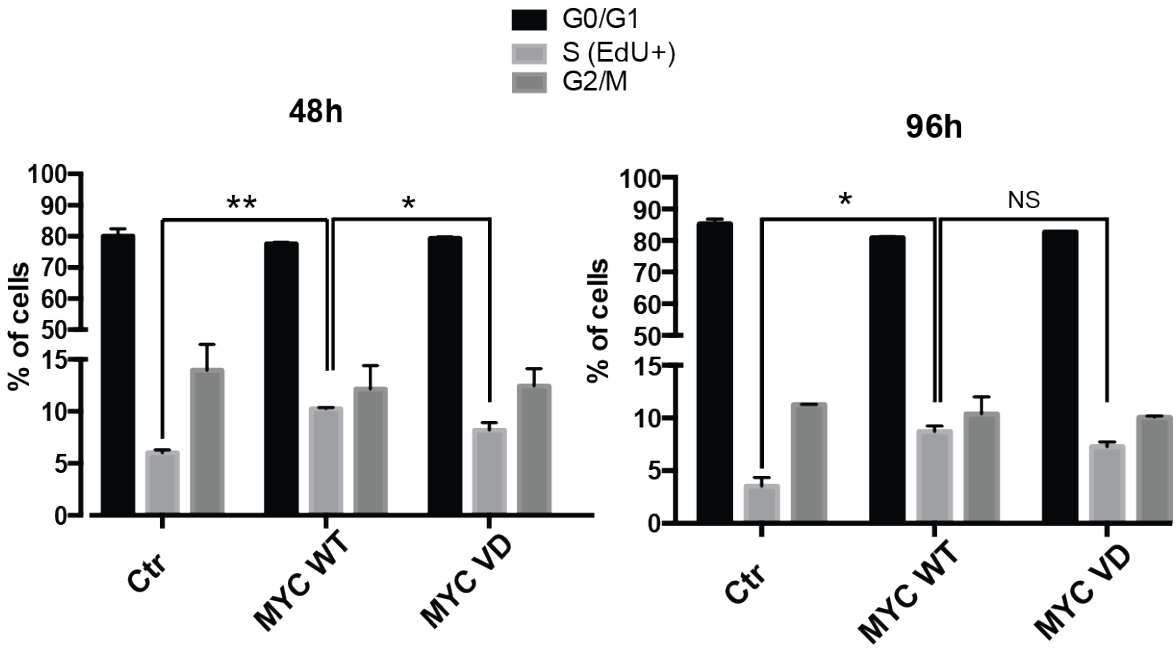

Figure 31: Cell cycle distribution of MYC overexpressing (WT and VD) and control cells. The cell cycle distribution has been evaluated by EdU/PI staining in Control and Myc overexpressing (WT and VD) mammospheres, at 48 and 96 hours after seeding (passage M2-M3). Bar plots representing the average values of two independent experiments ± standard deviation. *t*-test statistical analysis between Ctr or MYC VD samples respect to MYC WT showed respectively: NS= Not Significant, ∗ = p-value ≤ 0.05, ∗∗ = p-value ≤ 0.01 significant difference.

We also evaluated the apoptotic rate in the same conditions (Figure 32). Although Myc is a known inducer of apoptosis (Patel & McMahon, 2006), it has been demonstrated that this occurs only when its levels raise above a certain threshold (Murphy et al., 2008). In our conditions, the level of MYC overexpression obtained seemed to be rather protective versus apoptosis, since control cells showed the

highest cell death. This paradoxical observation may be explained by the fact that in non-adherent conditions cells undergo massive cell death due to anoikis and Myc overexpression could increase the number of cells resistant to anoikis (Paoli, Giannoni, & Chiarugi, 2013). There were minor, not statistically significant, differences between MYC WT and MYC VD.

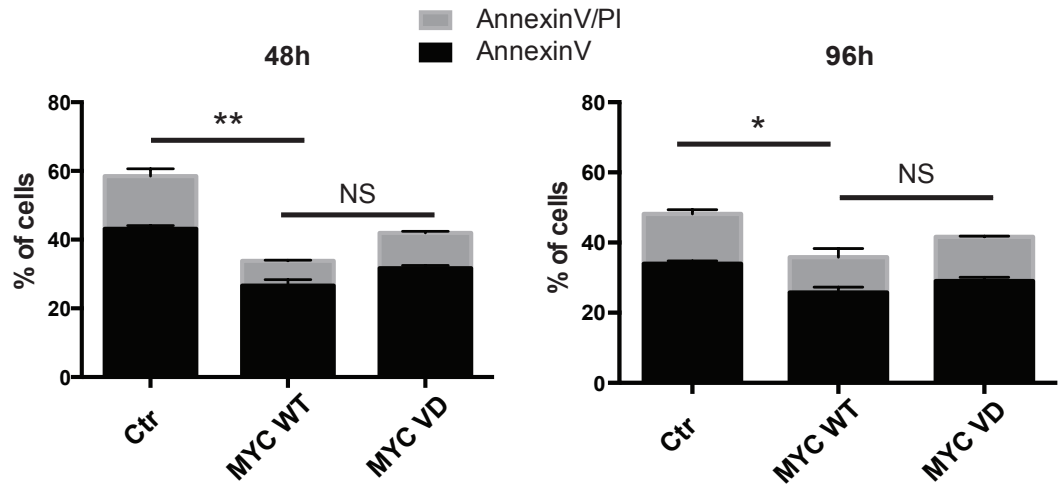

Figure 32: Cell death of MYC overexpressing (WT and VD) and control cells. Cell death has been evaluated by AnnexinV/PI staining in Control and MYC overexpressing (WT and VD) samples, at 48 and 96 hours after seeding (passage M2-M3). AnnexinV marks the cells in early apoptosis; instead the double staining AnnexinV/PI marks the cells in late apoptosis/necrosis. Myc overexpressing samples displayed significantly less cell death respect to the Control, at both time points. There was no significant difference between MYC WT and VD cells. Bar plots representing the average values of two independent experiments ± standard deviation. *t*-test statistical analysis between Ctr or MYC VD samples respect to MYC WT showed respectively: NS= Not Significant, ∗ = p-value ≤ 0.05, ∗∗ = p-value ≤ 0.01 significant difference.

In conclusion Myc overexpression increases the proliferation rate and decreases apoptosis of mammary epithelial cells grown as mammospheres. This appears to be largely independent from Miz1, since MYC WT and VD have very similar effects. Thus, proliferation and apoptosis alone could not explain the differences in SFE between MYC WT and VD, suggesting that the defect of MYC VD relates to its lower ability to promote self-renewal, relative to MYC WT.

3.4.3 In vivo mammary reconstitution assays

Since we wanted to prove that Myc has a Miz1-dependent function in promoting self-renewal, we decided to functionally measure stem cell frequencies in MYC

WT and VD overexpressing populations *in vivo*. Thus, we performed mammary reconstitution assays (Illa-Bochaca et al., 2010).

We obtained mammospheres from Ctr, TET-MYC-WT and TET-MYC-VD R26rtTA mice, and collected the cells 48 hours after seeding as single-cell suspensions, between the M2 and M3 passages. We then transplanted them in serial dilutions (1000, 500, 100 cells) into the cleared fat pads of 3 weeks old AthymicNude-Foxn1nu host mice. Transplanted mammary fat pads were examined for gland reconstitution 12 weeks post-injection by whole mount staining. The presence of branched ductal trees with lobules and/or terminal end buds was considered as positive reconstitution. A representative image of mammary gland reconstitution with MYC WT cells is shown in Figure 33.

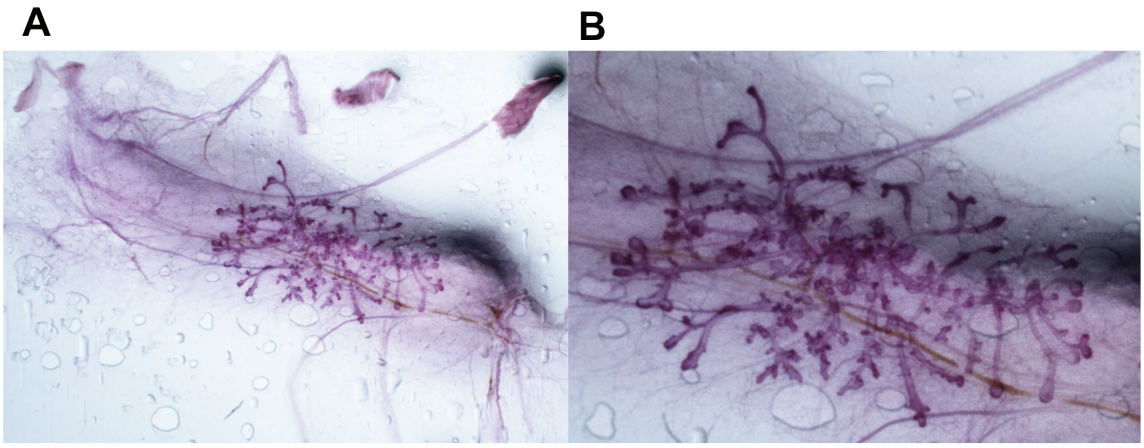

Figure 33: Whole-mount analysis of the transplanted mammary glands. (A) A representative image of the transplanted mammary glands in which is occurred reconstitution. The exogenous reconstitution is distinguishable from the endogenous one because of its typical central growth in the gland. (B) A magnification (2X) of the image clearly shows the morphology of the branched ductal trees.

The frequency of Mammary Stem Cells (MaSCs) in the transplanted cell population was calculated through the serial dilution transplantation using the Extreme Limiting Dilution Analysis Program (http://bioinf.wehi.edu.au/software/elda/index.html) (Wenjun Guo et al., 2012; Hu & Smyth, 2009) and

reported in Table 6. The calculated SCs frequencies were significantly higher in MYC WT than in Ctr and MYC VD cells, while there are no significant differences between Ctr and MYC VD cells.

Group	n. injected cells	Mammary glands outgrowths/ injection	Estimate stem cells frequency (upper and lower limits)	p-value
Ctr	1000	0/4	1:8348 (1:1161-1:59998)	0.0215
	500	1/8		
	100	0/6		
MYC WT	1000	1/6	1:1193 (1:557-1:2553)	-
	500	2/8		
	100	5/9		
MYC VD	1000	0/2	1:4816 (1:1550-1:14963)	0.0274
	500	1/4		
	100	2/10		

Table 6: Frequency of stem cells in different mammary cells population. Cell suspensions from mammospheres were injected in the mammary glands of 3 weeks old Athymic Nude-Foxn1nu host mice. Results are shown as number of outgrowths per number of injections, per each concentration of cells injected. SCs frequencies (estimates and upper/lower limits) were calculated by limiting dilution analysis. The statistical significance of the difference in SCs frequencies (p-value) between Ctr or MYC VD and MYC WT cells is calculated by *t*-test.

3.5 MYC WT and VD overexpression and tumorigenesis

To test the tumorigenic potential of MYC WT and VD overexpression in mice, we crossed MMTVrtTA mice and TET-MYC WT or VD mice. In this way we obtained an *in vivo* breast-specific conditional up-regulation of either MYC WT or VD. The induction of MYC, both WT and VD, and proliferation were monitored in immunohistochemistry by staining with anti Myc (Y69) and Ki67 (M7249) antibodies respectively after one and three months of induction by Doxycycline diet in two mice for each experimental group. As shown in the panels in Figure 34, after one month of induction we scored neither a real activation of the MYC transgenes, nor an induction of proliferation.

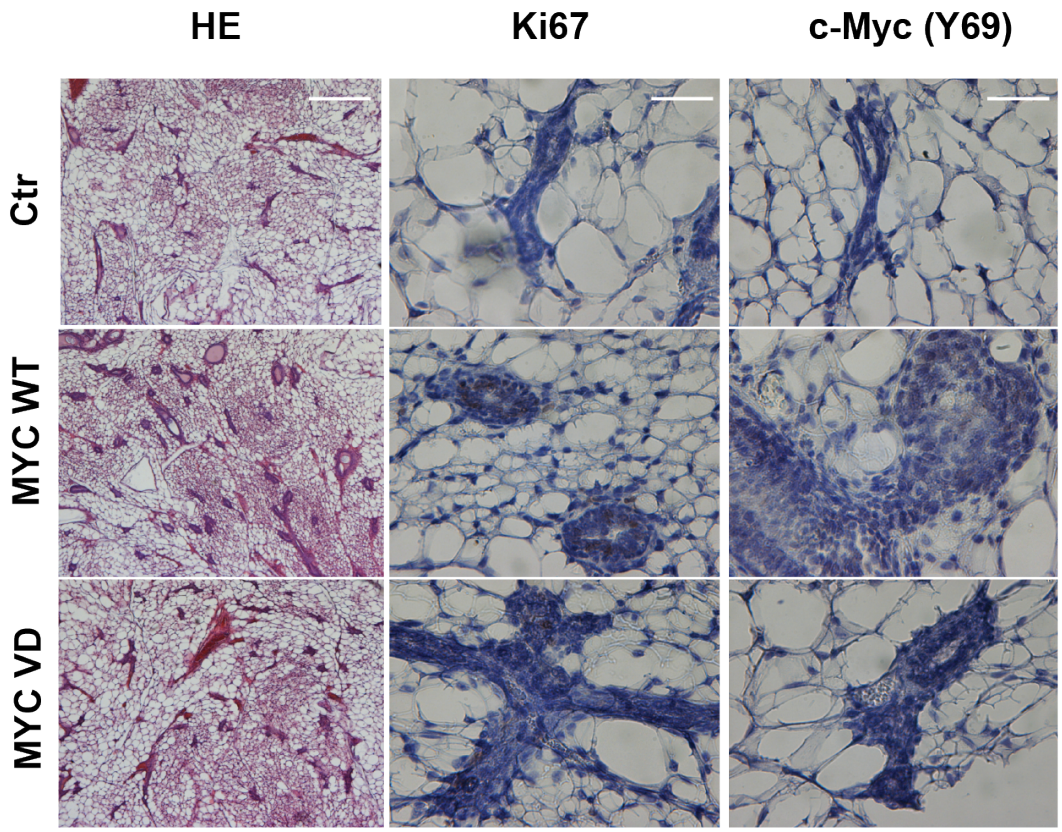

Figure 34: Immunohistochemical analysis of TET-MYC (WT and VD) MMTVrtTA and control mice. Hematoxylin and Eosin (HE), Ki67, c-Myc (Y69) immunohistochemistry were performed on Ctr and TET-MYC (WT and VD) MMTVrtTA mice induced *in vivo* by Doxycycline food for 30 days. Both from a structural and a proliferative point of view, the TET-MYC mice did not show any significant difference respect the control mice. The over-expression of the transgenes was too low at this time point. (Scale bar: HE= 200 μm, Ki67 and c-Myc =50 μm).

We verified also MYC overexpression *in vivo* after three months of induction by Doxycycline in two mice for each experimental group. As shown in the panels in Figure 35, at this stage the activation of MYC transgenes was much improved. We were able to notice a more homogenous induction of MYC (WT and VD) in all the cells of the same ductal structure and in all the ducts of the mammary gland. Moreover the transgenes seemed to be expressed prevalently in the luminal, more than in the basal compartment of the ducts. Also the proliferation, evaluated by Ki67 staining, was higher respect to the mammary gland of the control.

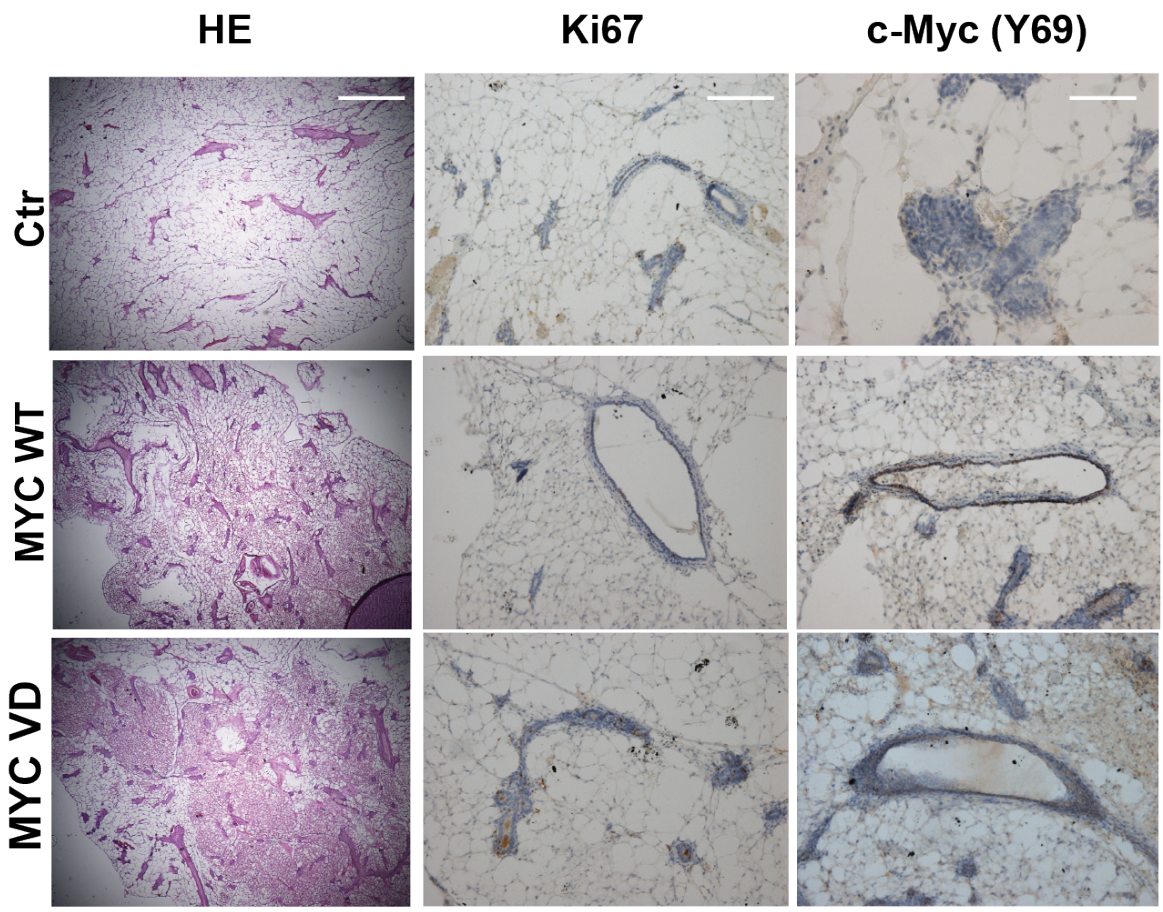

Figure 35: Immunohistochemical analysis of TET-MYC (WT and VD) MMTVrtTA and control mice. Hematoxylin and Eosin (HE), Ki67, c-Myc (Y69) immunohistochemistry were performed on Ctr and TET-MYC (WT and VD) MMTVrtTA mice induced *in vivo* by Doxycycline food for 90 days. Both Myc and Ki67 staining were significantly increased in TET-MYC mice respect the control mice. (Scale bar: HE= 400 µm, Ki67 and c-Myc =100 µm).

In order to verify if there was any structural change due to MYC up-regulation we verified the structural development of the mammary glands by whole mount staining. We did not score any structural difference between Myc WT and VD, neither between control and MYC-overexpressing cells (Figure 36).

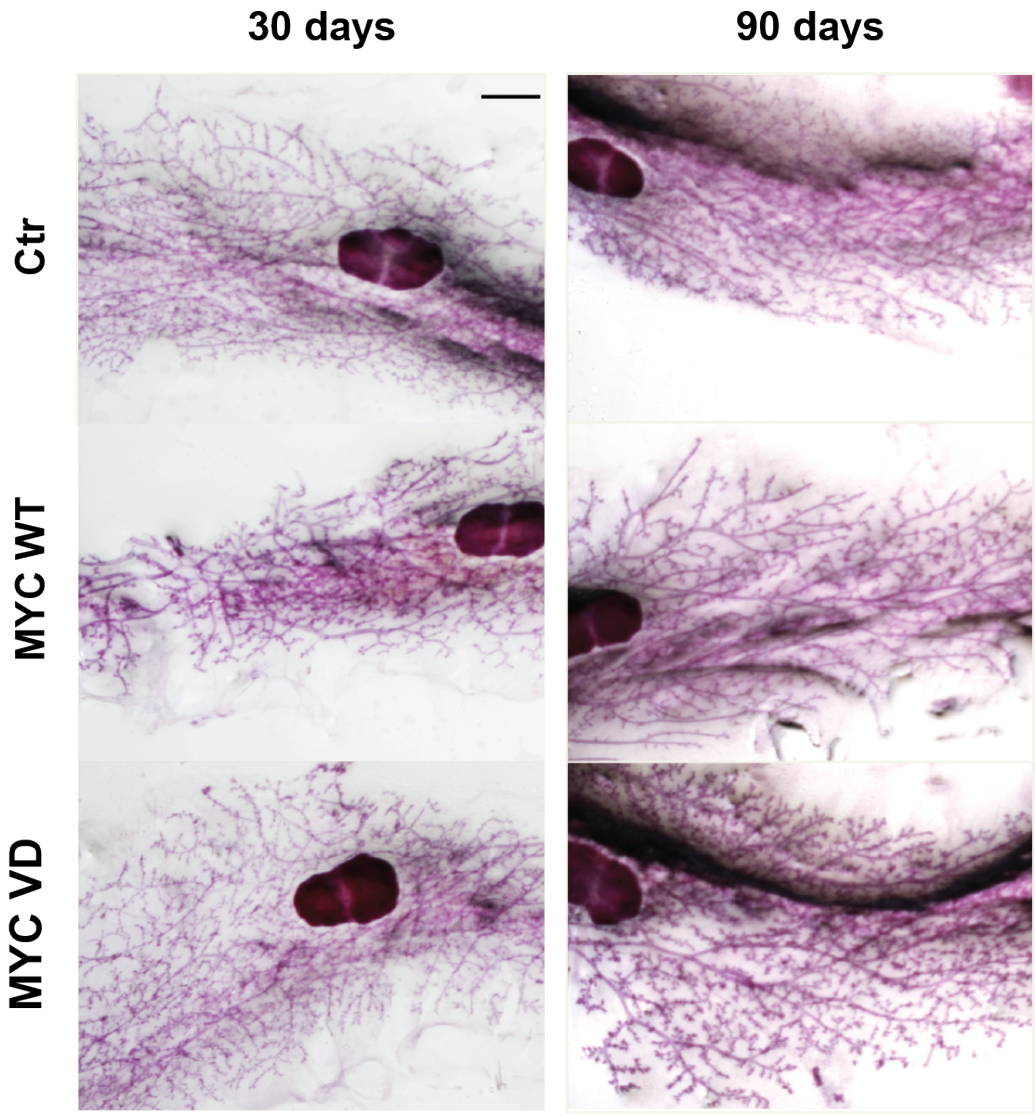

Figure 36: Whole mounts of mammary glands of TET-MYC (WT and VD) MMTVrtTA and control mice. The whole structure of the mammary gland was stained, in order to verify any structural change in Ctr and TET-MYC (WT and VD) MMTVrtTA mice induced *in vivo* by Doxycycline food for 30 or 90 days. (Scale bar 2mm).

Since the expected latency for the onset of these tumors is about 22 weeks, after one year without tumor development, we decided to sacrifice the animals and analyse by FACS whether there was any alteration in the distribution of subpopulations (basal and luminal) of the epithelial compartments upon MYC overexpression. As shown in Figure 37, we were not able to individuate significant differences in the expression of basal vs. luminal surface markers in MYC overexpressing cells compared to control.

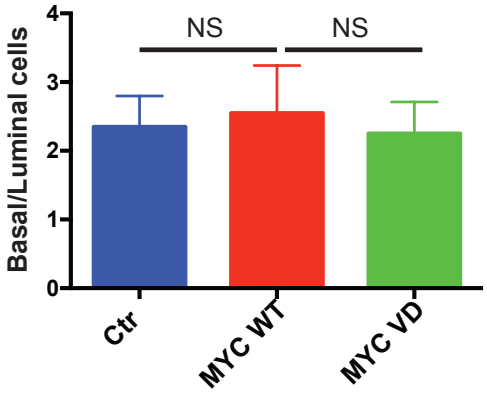

Figure 37: Basal/Luminal cells in TET-MYC (WT and VD) MMTVrtTA and control mice. The ratio between Basal ($CD49f^{high}/Epcam^{high}$) and Luminal ($CD49f^{low}/Epcam^{high}$) cells subpopulation in the epithelial compartment (Lin-) was evaluated in Ctr and TET-MYC (WT and VD) MMTVrtTA mice induced *in vivo* by Doxycycline food for one year. Bar plots representing the average values of two independent experiments ± standard deviation. *t*-test statistical analysis between Ctr or MYC VD samples respect to MYC WT showed not significant (NS) differences.

The lack of any phenotype of the MMTV-Myc mice, not even the ones reported in literature (D'Cruz et al., 2001), and also of a clear indication of transgene overexpression and activity, made us to conclude that we need to address the tumorigenic potential of MYC WT and VD in mammary epithelial cells with a different experimental approach. One possibility that we are currently evaluating would be to transplant MYC-overexpressing (WT and VD) and control epithelial cells in 6 weeks old AthymicNude-Foxn1nu host mice, after having infected them with a viral vector expressing other oncogenes (i.e. Ras) to shorten the latency of tumorigenesis.

3.6 Myc/Miz1 interaction is necessary for the regulation of genes involved in extracellular matrix interaction, cell adhesion and epithelial morphogenesis

3.6.1 RNA-seq analysis

In order to find the transcriptional program underlying the observed phenotypes, MYC WT and VD overexpressing mammospheres together with control samples

(collected at 48 hours after seeding as single-cell suspensions between the M2 and M3 passages) were subjected to mRNA profiling. Differentially expressed genes (DEGs) were identified by RNA-seq analysis using the Bioconductor package DESeq2 considering genes whose q-value relative to the control sample is lower than 0.01 (FDR ≤ 1%) and whose expression is higher than eRPKM of 1 (see Material and Methods). We found 3902 up- and 3799 down-regulated genes in MYC WT overexpressing cells relative to the control and 2960 up- and 3005 down- regulated genes in MYC VD respect to the control.

To map the genes responsible for the differences in SFE in MYC WT relative to MYC VD, we first selected the genes expressed at least in one condition (Ctr, MYC WT or VD) and plotted those 13919 genes in a scatter plot in witch each gene is a dot and it is positioned on the basis of its changes in expression (Log_2 fold change) in MYC WT and MYC VD overexpressing mammospheres relative to the control (Figure 38). The majority of the dots were positioned on the diagonal, suggesting a good correlation in the gene regulation between the two systems (Pearson coefficient ρ_{XY}= 0.85).

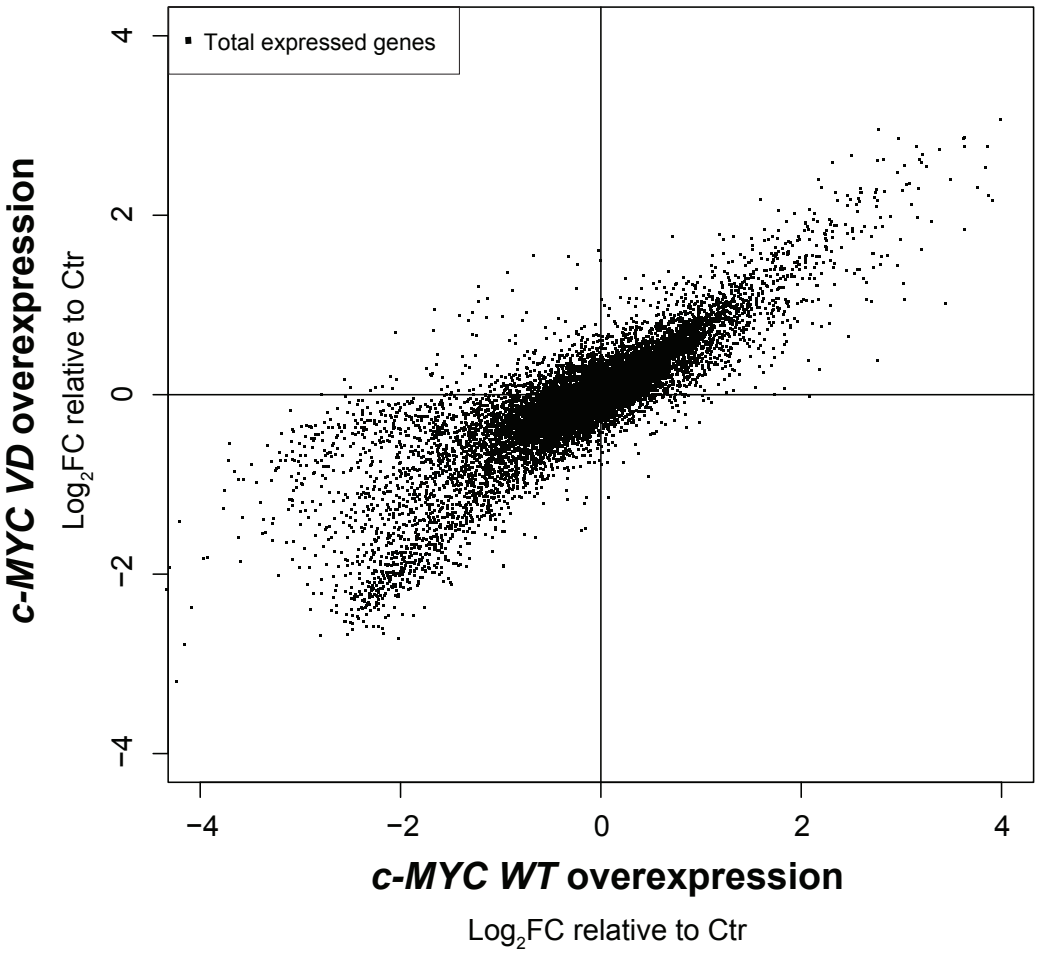

Figure 38: Transcriptional changes in *c-MYC WT* and *c-MYC VD* overexpressing primary mammospheres. The scatter plot shows all the 13919 expressed genes in *c-MYC WT* and *c-MYC VD* overexpressing cells. Each dot is a gene and it is positioned on the basis of its changes in expression in *c-MYC WT* (x axes) and *c-MYC VD* (y axes) overexpressing samples relative to the Control (expressed as Log_2 fold change). The two datasets show good correlation, calculated by Pearson coefficient ρ_{XY}= 0.85, p-value < 2.2e-16.

After this, we selected those genes regulated in a statistically significant manner in at least one system (q-value ≤ 0.01; FDR ≤ 1%, 8445 genes) and among them only the genes differentially regulated in the two systems, having a difference of at least 1.5 fold change (|Log_2FC WT - Log_2FC VD |>0.585). We identified 305 genes induced in MYC WT overexpressing samples and less induced/repressed by MYC VD overexpression (red/green dots in Figure 39). As expected, the differences between MYC WT and VD were even more consistent for the repressed genes, since we found 888 genes repressed by MYC WT and less repressed by MYC VD (green dots in the scatter plot Figure 39). Indeed, it has been already shown that

dampening Myc/Miz1 interaction (by shRNA against Miz1 or tacking advantage of the MYC VD mutant) has no effect on Myc activated genes, while it causes de-repression on part of Myc down-regulated genes (Walz et al., 2014; Wiese et al., 2015).

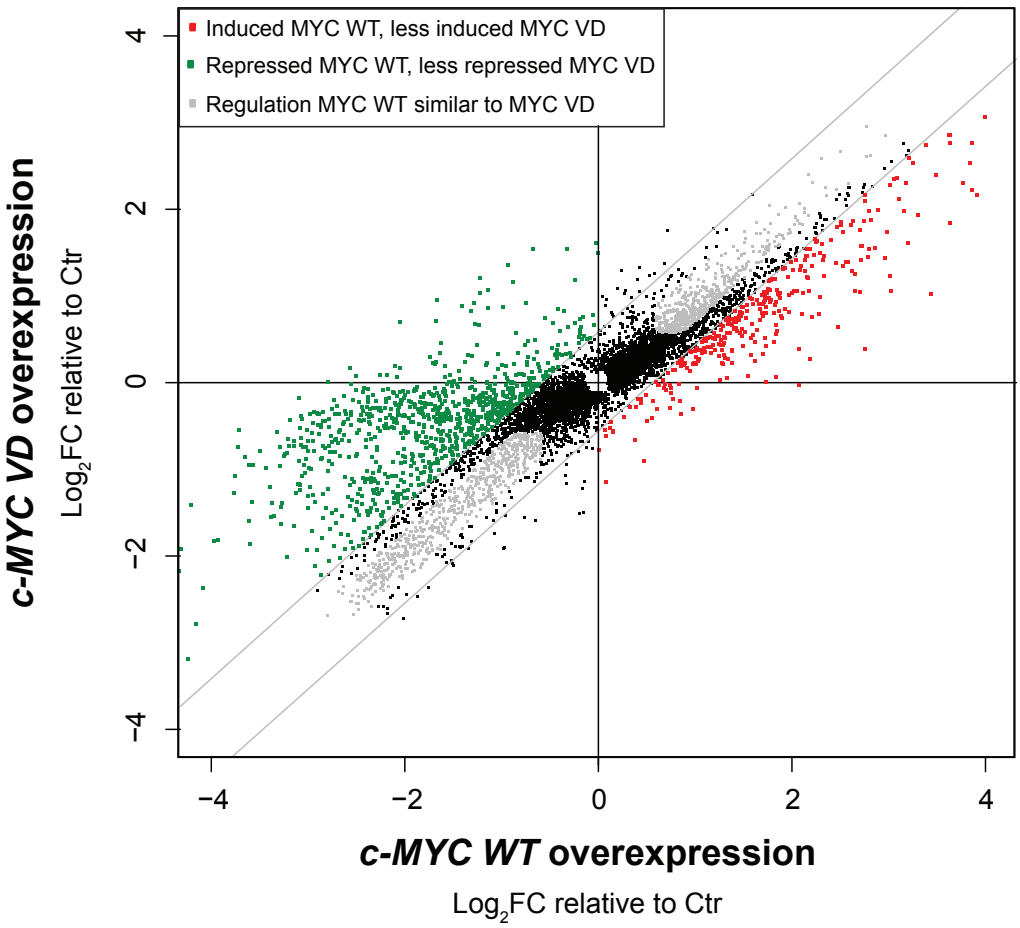

Figure 39: Genes differentially regulated between *c-MYC WT* and *c-MYC VD* overexpressing primary mammospheres. The scatters plot shows all the 8445 genes regulated in a statistically significant manner (q-value ≤ 0.01; FDR ≤ 1%) in *c-MYC WT* or in *c-MYC VD* overexpressing cells respect to control. Each dot is a gene and it is positioned on the basis of its changes in expression in *c-MYC WT* (x axes) and *c-MYC VD* (y axes) samples relative to the control (expressed as Log_2 fold change). In particular: in green are highlighted the genes repressed by MYC WT and, less repressed/induced by MYC VD (888 genes), in red are highlighted genes induced by MYC WT and less induced/repressed by MYC VD (305 genes). The grey dots are those genes that are regulated in a similar manner in the two systems ($|Log_2FC$ WT - Log_2 FC VD$|<0.3$). Considering only the significant genes, the correlation between the two datasets, calculated by Pearson coefficient, is good: $\rho_{XY}= 0.86$, pvalue < 2.2e-16.

After defining these functional categories, we performed Gene Ontology analysis for each of them. We started from the 888 genes differentially repressed by MYC WT and VD. The top scoring GO terms are listed in Table 7.

Among the molecular function enriched in these DEGs there are terms related to cell adhesion and motility.

GO ID	Term	p-value
GO:000268	positive regulation of immune system process	1.19E-24
GO:0009611	**response to wounding**	5.72E-22
GO:0009986	**cell surface**	8.03E-17
GO:0001817	regulation of cytokine production	2.27E-11
GO:0030695	GTPase regulator activity	5.30E-10
GO:0042330	**taxis**	4.51E-09
GO:0006909	phagocytosis	8.47E-09
GO:0010941	regulation of cell death	5.10E-07
GO:0007155	**cell adhesion**	1.12E-04
GO:0010648	**negative regulation of cell communication**	1.18E-04

Table 7: The top ten Gene Ontology categories of differentially repressed genes between MYC WT and VD. The individuated 888 genes repressed by MYC WT and less repressed/induced by MYC VD were submitted to Gene Ontology analysis (http://david.abcc.ncifcrf.gov). The top ten scoring GO categories are listed with the corresponding p-value.

Similar GO categories were found enriched also in the genes similarly repressed between MYC WT and VD (Table 8).

GO ID	Term	p-value
GO:0016327	**apicolateral plasma membrane**	**7.14E-20**
GO:0007155	**cell adhesion**	**2.14E-07**
GO:0060429	**epithelium development**	**9.19E-07**
GO:0030855	**epithelial cell differentiation**	**5.18E-06**
GO:0045860	positive regulation of protein kinase activity	1.08E-04
GO:0048598	embryonic morphogenesis	2.90E-04
GO:0030030	cell projection organization	3.36E-04
GO:0060284	**regulation of cell development**	**3.79E-04**
GO:0014033	neural crest cell differentiation	4.36E-04
GO:0005604	**basement membrane**	**2.30E-03**

Table 8: The top ten Gene Ontology categories of negatively regulated genes by both MYC WT and VD. The individuated 640 genes repressed both by MYC WT and MYC VD were submitted to Gene Ontology analysis (http://david.abcc.ncifcrf.gov). The top ten scoring GO categories are listed with the corresponding p-value.

Thus, we found that Myc/Miz1 complex contribute to part of the negative regulation due to Myc overexpression of genes involved in extracellular matrix interaction, migration, cell adhesion and epithelial morphogenesis.

The same kind of analysis was performed on the 305 genes differentially induced between Myc WT and VD. The GO categories scored belong to biological functions such as extracellular region, cell adhesion, cell-cell signaling, gland development (Table 9).

GO ID	Term	p-value
GO:0044421	**extracellular region part**	**4.87E-11**
GO:0007155	**cell adhesion**	**1.48E-10**
GO:0005509	calcium ion binding	1.10E-06
GO:0001871	**pattern binding**	**5.17E-04**
GO:0007267	**cell-cell signaling**	**6.86E-04**
GO:0004175	endopeptidase activity	3.00E-03
GO:0008083	growth factor activity	4.00E-03
GO:0006029	proteoglycan metabolic process	1.00E-02
GO:0043062	**extracellular structure organization**	**1.70E-02**
GO:0048732	**gland development**	**1.90E-02**

Table 9: The top ten Gene Ontology categories of differentially induced genes between MYC WT and VD. The individuated 305 genes induced by MYC WT and less induced/repressed by MYC VD were submitted to Gene Ontology analysis (http://david.abcc.ncifcrf.gov). The top ten scoring GO categories are listed with the corresponding p-value.

These categories were enriched in the genes differentially induced between MYC WT and VD, less in genes commonly induced by the two proteins among which we scored gene regulating the interaction with the extracellular matrix and the metallopeptidase activity, together with genes belonging to the classical Myc induced classes, such as cell cycle, DNA metabolic processes, spindle regulation and purine nucleoside binding (Table 10).

GO ID	Term	p-value
GO:0000278	mitotic cell cycle	1.82E-14
GO:0005819	spindle	3.53E-05
GO:0001883	purine nucleoside binding	6.04E-05
GO:0006259	DNA metabolic process	1.78E-04
GO:0043169	cation binding	9.10E-04
GO:0016208	AMP binding	9.98E-04
GO:0007017	microtubule-based process	1.60E-03
GO:0031012	**extracellular matrix**	**1.80E-03**
GO:0007268	synaptic transmission	3.50E-03
GO:0008237	**metallopeptidase activity**	**4.00E-03**

Table 10: The top ten Gene Ontology categories of positively regulated genes by both MYC WT and VD. The individuated 504 genes induced both by MYC WT and MYC VD were submitted to Gene Ontology analysis (http://david.abcc.ncifcrf.gov). The top ten scoring GO categories are listed with the corresponding p-value.

This could be due to the fact that the Miz1 binding to Myc can regulate, more likely indirectly, also the activation of specific classes of genes, in particular those regulating cell adhesion, interaction with the extracellular matrix and gland development. Myc overexpression, and above all the repressive activity of Myc/Miz1, may determine a new cellular organization; in these conditions, some targets could be induced simply because of the new phenotype.

Considering previous observations that verify the influence of Myc/Miz1 complex only in the repressive part of Myc transcriptional program (Walz et al., 2014), we surmise that the differences observed in the transcriptional activation by MYC WT and VD were indirect. In the end, we hypothesize that the mechanism by which MYC WT can induce the increment of self-renewal in epithelial cells could be mostly mediated by the repression of some of these genes. Thus, the MYC VD reduced self-renewal ability observed could be due to the dampening of this transcriptional repression.

3.6.2 microRNA-seq analysis

Myc modulates gene expression not only through regulation of transcription but also post-transcriptionally (Kress et al., 2015). In particular, Myc regulates the

expression of several microRNAs (miRNAs) and in this way indirectly controls the stability and translational efficiency of their target mRNAs (Bui & Mendell, 2010). Either activating or repressing the expression of specific miRNAs, Myc coordinates numerous functions pivotal in tumorigenesis and in stem cells differentiation/self-renewal (Chang et al., 2007; Frenzel, Loven, & Henriksson, 2010; C.-H. Lin et al., 2009). Given the importance of the miRNAs in breast cancer development (Dvinge et al., 2013), we decided to evaluate the effects of MYC (WT or VD) overexpression on miRNAs regulation in mammospheres. MYC WT and VD overexpressing mammospheres, together with control, were subjected to small RNA profiling by miRNA-seq. For each condition three independent samples were sequenced obtaining around 1 million reads aligned (see Material and Methods). The different species were divided in: canonical miRNA, 3'-modified (nucleotides added to the 3' end of the reference miRNA), trimmed (the 5' or 3' dicing site is upstream or downstream from the reference miRNA) and 5'-modified (nucleotides added to the 5' end of the reference miRNA) (Nicassio, 2014). The results are listed in Table 11.

	Reads			
IsomiR	**Canonical**	**3'-modified**	**Trimmed**	**5'-modified**
Ctr (1)	515767	271050	276926	5104
Ctr (2)	511400	269882	28064	5621
Ctr (3)	506783	278376	278246	5800
Myc WT (1)	498877	418136	186305	7406
Myc WT (2)	487606	453814	172732	7634
Myc WT (3)	513686	358163	214669	6785
Myc VD (1)	513645	363591	210553	6463
Myc VD (2)	531021	338449	209984	6732
Myc VD (3)	517521	285124	265063	5774
Tot. N.	197	550	164	44

Table 11: List of counted miRNAs in Ctr, MYC WT and MYC VD primary mammospheres. After filtering for expression, miRNAs were subdivided in categories of IsomiRs: canonical miRNA, 3'-modified, trimmed or 5'-modified. The reads of each category were counted for all the three replicates of each experimental group (Ctr, MYC WT and MYC VD overexpressing mammospheres). The total number of miRNAs mapped for each category is indicated in the last row.

It is noteworthy that in MYC-overexpressing samples (more WT than VD) the 3'-modified isomiRs reads are more abundant respect to the controls. Since it has been demonstrated that 3' modifications can be a mechanism for altering miRNAs stability and function (Li Guo et al., 2011) and miRNAs isoforms abundance can be useful to distinguish breast cancer subtypes (Telonis, Loher, Jing, Londin, & Rigoutsos, 2015), we will investigate more in depth this difference. Since the canonical miRNAs are the most characterized forms, we decided to focus our analysis at first on the 197 canonical miRNAs mapped in our samples. We identified 65 miRNAs differentially regulated between Myc overexpressing (both WT and VD) and control cells with significant statistical difference (p-value ≤ 0.05; FDR ≤ 5%) and $|Log_2$ fold change$|>0.5$ (Figure 40). The MYC overexpressing cells respect to the control cells repressed miRNAs regulating myogenic differentiation (mir-133, mir-1, mir-206), while induced miRNAs did not enrich for the regulation of a specific function.

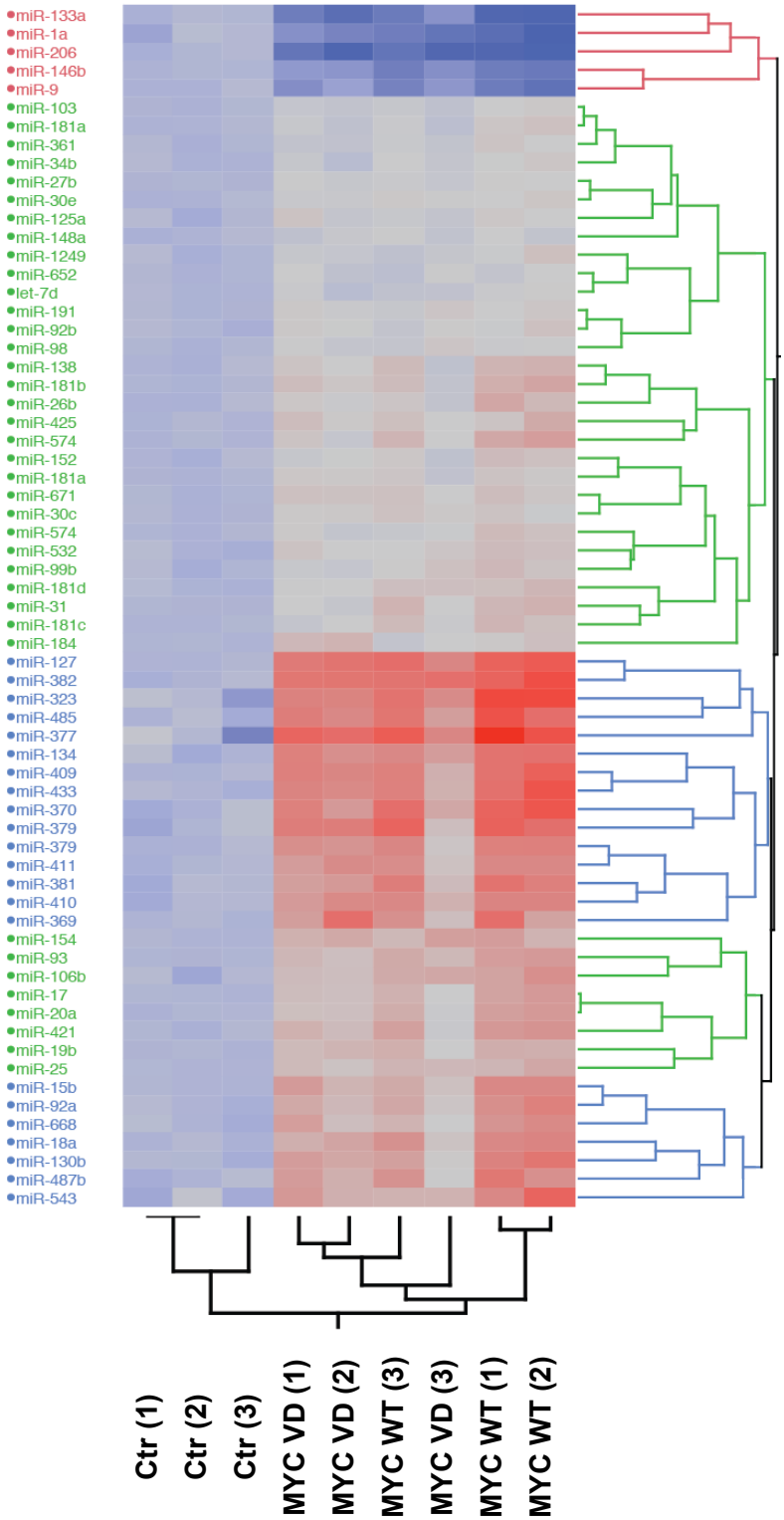

Figure 40: Differentially regulated miRNAs between Ctr and *c-MYC* (WT and VD) overexpressing primary mammospheres. The heatmap shows all the 65 miRNAs differentially regulated in a statistically significant manner (q-value ≤ 0.05; FDR ≤ 5%) in both *c-MYC WT* and *c-MYC VD* overexpressing cells respect to the Control cells. On the bottom the clusterization of the samples is shown.

Instead 57 miRNAs were differentially regulated between MYC WT and MYC VD overexpressing cells (Figure 41).

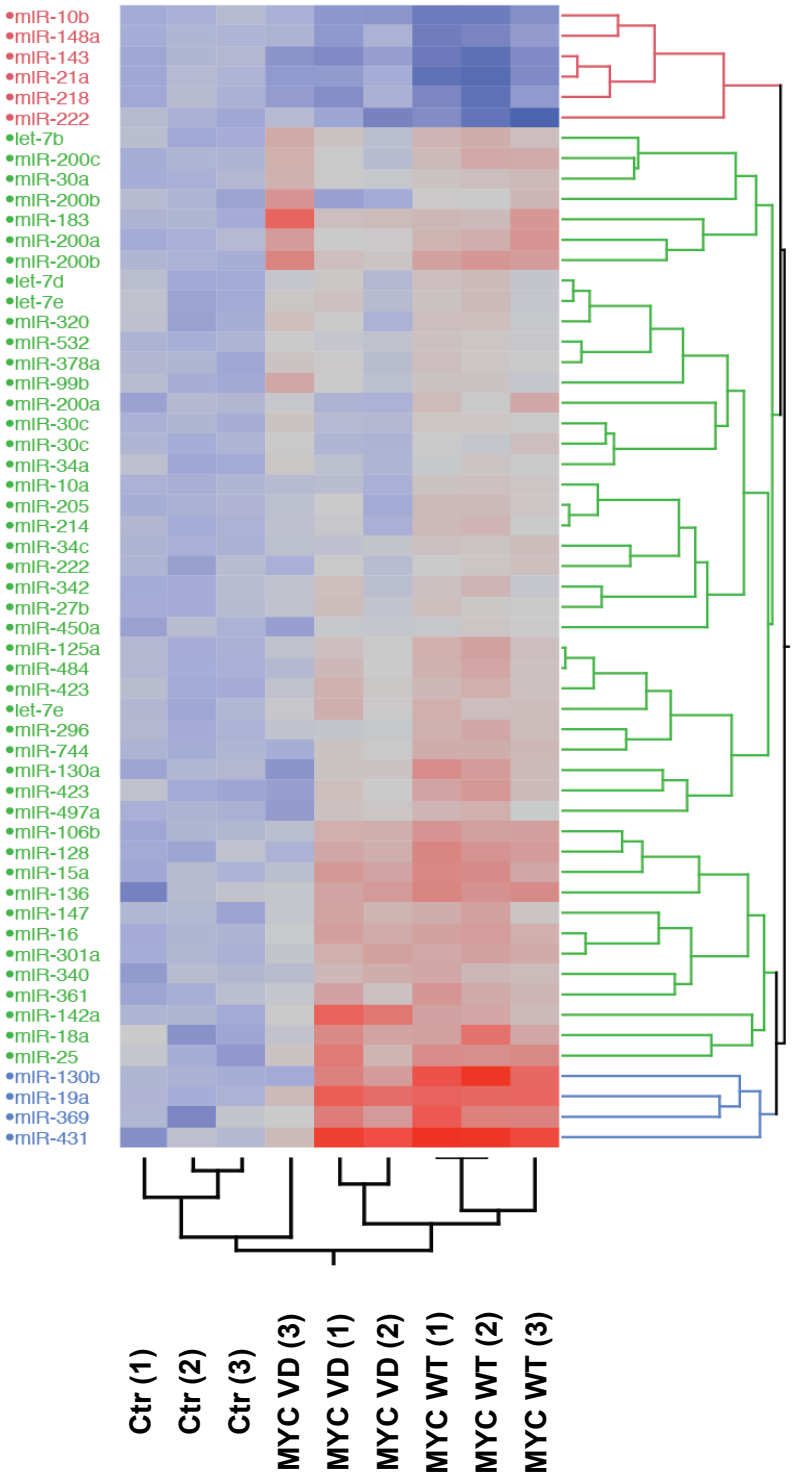

Figure 41: Differentially regulated miRNAs between *c-MYC* WT and *c-MYC* VD overexpressing primary mammospheres. The heatmap shows all the 57 miRNAs differentially regulated in a statistically significant manner (q-value ≤ 0.05; FDR ≤ 5%) between *c-MYC* and *c-MYC VD* overexpressing cells. On the bottom the clusterization of the samples is shown

Also in this case, MYC VD samples are positioned between MYC WT and Ctr samples. It seems that MYC VD mutant acts as a less strong regulator of miRNAs, either in induction or in repression, respect to the MYC WT.

The evaluation of the relevance of the differentially regulated miRNAs for the observed phenotype is still ongoing along with a detailed scrutiny of the available literature.

3.7 Myc/Miz1 repressed genes in mammospheres: functional screening setting

We hypothesized that the genes differentially repressed between MYC WT and VD overexpressing mammospheres could be part of the mechanism that underlies the different self-renewal capacity of the two cell populations. Reasoning that a more stem-like phenotype is also a feature of more aggressive and invasive breast cancer (Pece et al., 2010), we are thus planning to functionally characterize those genes in order to identify critical mediators of MYC tumorigenic activity. In particular, along the lines adopted in a published screening in primary cells (Sheridan et al., 2015), we decided to perform a drop-in screen in mammospheres (Figure 42). In this way we plan to identify those genes whose knockdown is facilitating mammospheres self-renewal and expansion, and thus whose repression by Myc could be critical in mediating a more tumorigenic and aggressive phenotype.

For the feasibility of the screening in primary cells, in which we observed the phenotype, we decided to shorten the list of candidates that we can screen. Indeed, we took into account the following considerations: for each sample we can infect 2×10^6 mammospheres cells (considering that from each mouse we obtain about 2×10^5 epithelial cells), the transduction frequency would be of 40% (in order to have only one shRNA/cell), 0.85% of cells will give rise to mammospheres, the coverage will be 200 fold for each shRNA (200 cells harbouring the same shRNA),

we want to use at least 3 validated shRNAs sequences for each genes and do five biological replicates, to be sure about the significance of any shRNA enrichment. On this basis we decided that we could handle a total of 180 shRNAs, divided in 4 pools of 45 shRNAs thus 60 genes.

For these reasons, we decided to restrict our list of potential candidates on the basis also of their biological importance, starting from expression data from breast cancer patients and published datasets.

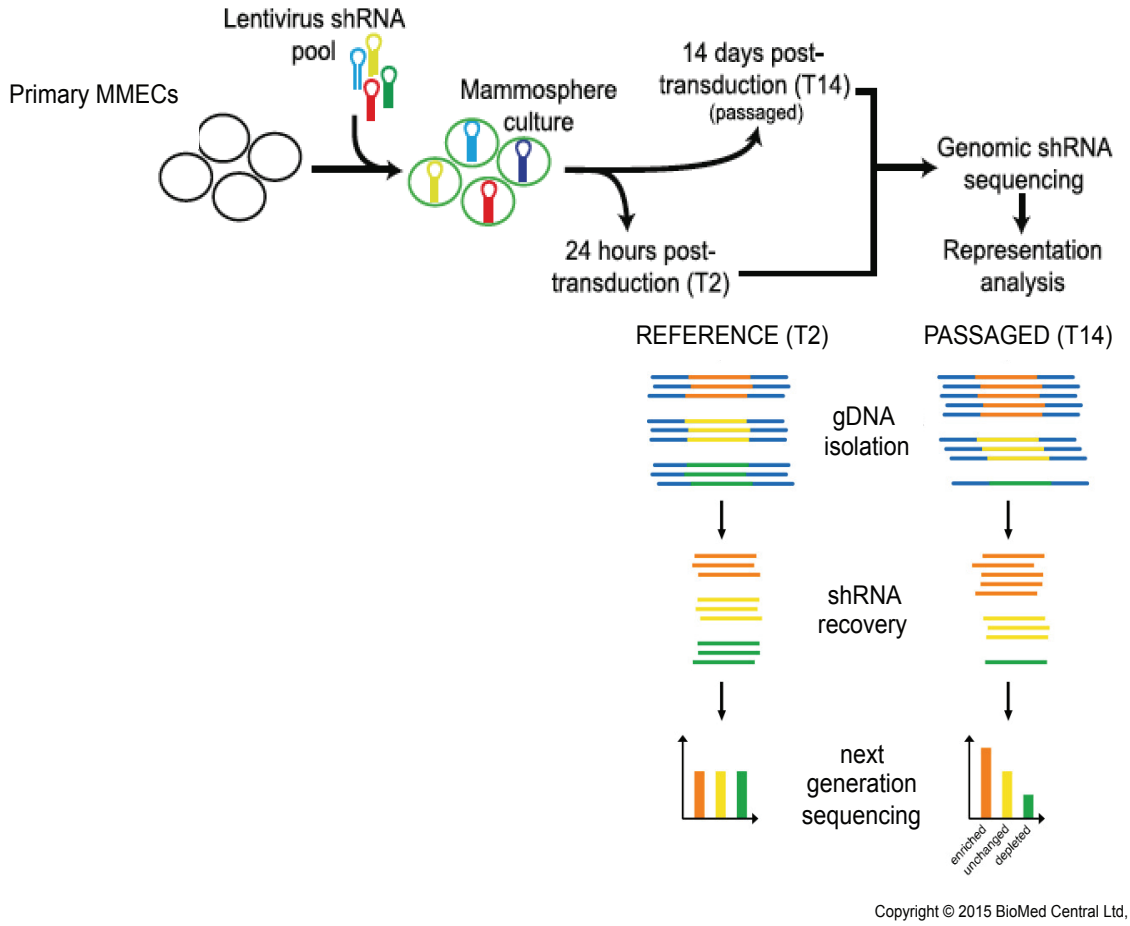

Figure 42: Functional screening of candidate genes in mammospheres. Primary MMECs will be transduced with lentiviral shRNAs pools, cloned in a ZIP-SFFV vector (Transomic). The cells, grown as mammospheres and selected by Puromycin or ZsGreen marker, will be harvested after 24 hours and 14 days, from the transduction. shRNAs representation will be evaluated by: gDNA isolation, shRNA recovery and next generation sequencing. The shRNAs enriched at 14 days respect to 24 hours, whose knockdown gives a selective advantage to the mammosphere growth, will be the candidate targets by which Myc can induce self-renewal (modified from Sheridan et al., 2015 and J. Wolf et al., 2013b)

3.7.1 Breast cancers (TCGA)

Starting from the idea that the stem cells presence is pivotal in tumorigenesis (Stingl & Caldas, 2007), we speculate that the MYC repression of some targets,

and the resulting increase in self-renewal ability, can be one of the oncogenic mechanisms operated by MYC in breast tumors. Hence, looking for genes that could have a role in MYC-driven breast cancer, we analysed the expression of the individuated Myc/Miz1 repressed genes in a panel of breast cancers. First, data from 1070 human tumors present in The Cancer Genome Atlas (TCGA) database were obtained and classified in subcategories (Luminal A, Lumina B, Luminal unknown, Triple Negative Breast Cancer or TNBC, HER2+) on the basis of the available information about estrogen receptor (ER), progesterone receptor (PR), epidermal growth factor receptor 2 (HER2) and Ki67 expression (Voduc et al., 2010). The expression data were defined as Log_2 fold change respect to the median of 112 normal breast tissue samples, a *t*-test analysis was used to define genes regulated in statistically significant way in tumors respect to the normal counterpart (p-value ≤ 0.05; FDR ≤ 5%). Out of 888 genes of our signature of interest, we could univocally assign a human counterpart to 754 that were used for further analysis. 23% (177/754 genes) of the genes belonging to the Myc/Miz1 repressed signature were repressed also in all the human breast cancer samples analysed relative to the normal counterpart; while up to 35% (265/754 genes) were repressed specifically in the most aggressive Triple Negative Breast Cancer (Figure 43). Moreover, we consider the amplification status of the MYC locus in these tumors. If we also consider this information and we select just the tumors harbouring MYC amplification: 12% (91/754) of the genes belonging to the Myc/Miz1 repressed signature was repressed in all the human breast cancer samples bearing *MYC* amplification, while 22% (165/754) were repressed specifically in Triple Negative Breast Cancer with amplified MYC.

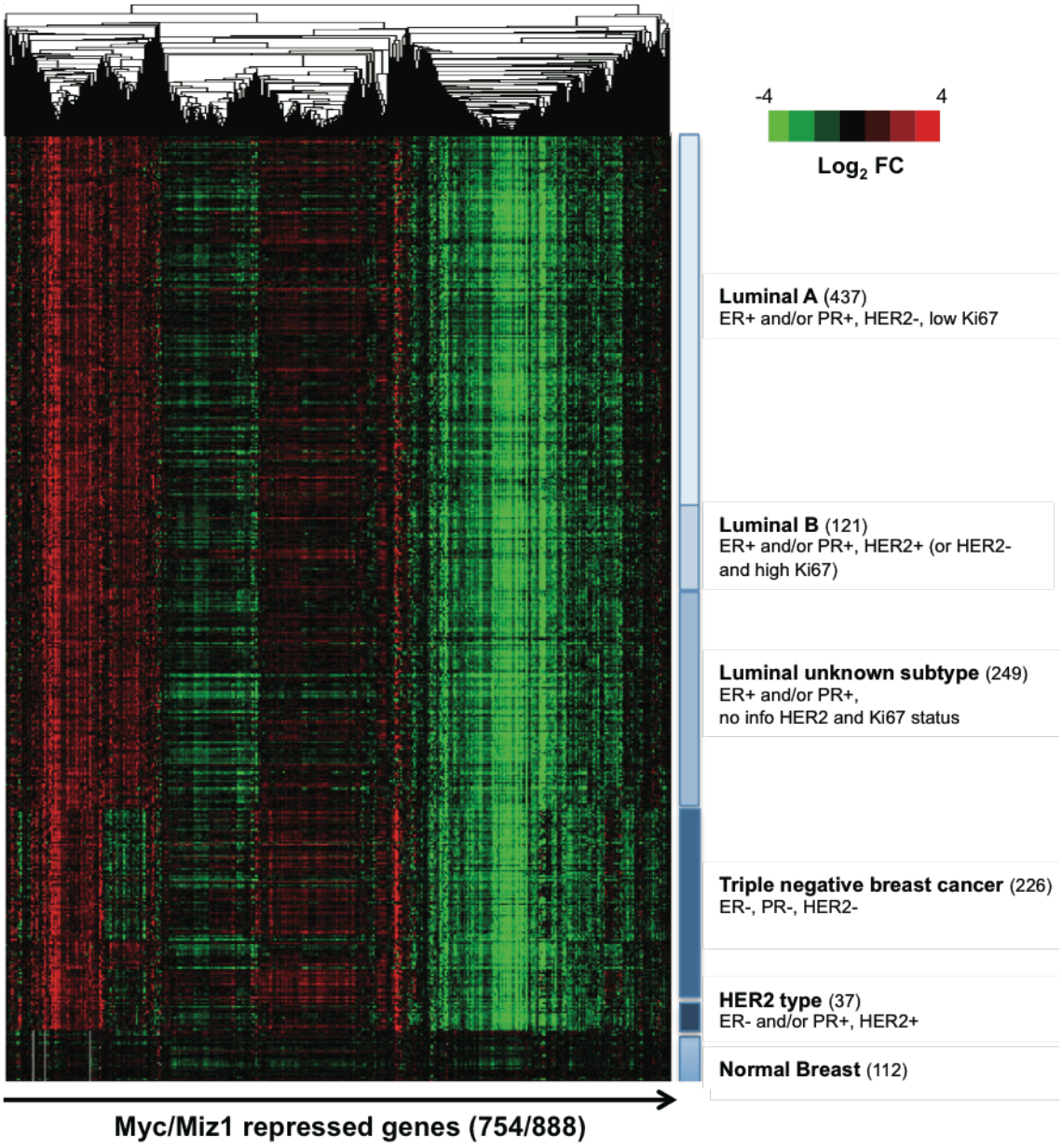

Figure 43: Expression data of Myc/Miz1 repressed signature in a panel of human breast cancer (TCGA). Expression of Myc/Miz1 repressed genes in a panel of human breast tumors. Transcriptional data from TCGA (1070 tumor samples) were divided in subcategories (Luminal A, Luminal B, Luminal unknown, Triple Negative Breast Cancer (TNBC), Her2-type) on the basis of available information. The analysis has been done for the 754 (out of 888) genes differentially repressed by MYC WT and VD in mouse mammospheres for which a human counterpart could be identified. Expression data are calculated as Log_2 fold change respect to the median of Normal breast tissue (112 sample).

3.7.2 Published datasets

With the intent to further shortening the list of genes belonging to the Myc/Miz1 repressed signature selecting those genes which could be biologically relevant and on which it will be worth pursuing a more functional analysis, we plan to

analyse them also in several published transcriptional datasets. The criteria of selection will be the following:

- Datasets of transcriptional analysis of mouse or human epithelial cells subpopulations in the mammary gland (Stromal, Mature luminal, Luminal progenitors and Mammary Stem Cells), sorted by surface markers (Kendrick et al., 2008; Lim et al., 2009; 2010; Sheridan et al., 2015).
- Dataset of transcriptional analysis of human breast tumors in which are compared CSCs (sorted for surface markers: CD44+ and CD24- or for sphere-forming ability) resistant to chemotherapy and the other cancer cells of the primary bulk invasive cancer (Creighton et al., 2009).

Only those genes of the Myc/Miz1 repressed signature down regulated in breast cancers overexpressing MYC compared the normal counterpart (previous paragraph) and coherently regulated in each dataset (down-regulated in MaSCs vs. other subpopulations, down-regulated in CSCs vs. other tumors cells) will be taken into account for the functional screening.

4. Discussion

The *MYC* oncogene is a signaling hub in cancer (Dang, 2012) and as such an ideal candidate for therapy. Several strategies have been developed to target MYC, trying to demonstrate the feasibility to inactivate a molecule that was thought to be untouchable owing to its many roles in normal cellular processes (Ponzielli, Katz, Barsyte-Lovejoy, & Penn, 2005; Soucek et al., 2008; Vita & Henriksson, 2006).

MYC is overexpressed in a wide range of human tumors (Dang, 2012). In breast, in particular, MYC is frequently deregulated in cancer cells and preferentially detected in the most aggressive tumors (Chen & Olopade, 2008; Xu, Chen, & Olopade, 2010). We thus decided to set up a system to profile the specific transcriptional response governed by Myc in mammary epithelial cells, in order to find new downstream targets, through which it may regulate pivotal functions in tumor initiation and maintenance such as adhesion, migration and self-renewal.

4.1 Myc negatively controls the expression of genes involved in extracellular matrix interaction, cell adhesion and epithelial morphogenesis in mammary epithelial cells

We defined the core of Myc regulated transcriptional program in immortalized murine mammary epithelial cells (MMECs) through genome-wide transcriptional analysis, upon either deletion or super-activation of Myc. The main biological processes promoted by Myc-induced genes are proliferation, biosynthetic metabolism, ribosomal biogenesis, RNA processing and apoptosis as also described in several other models (Zeller, Jegga, Aronow, O'Donnell, & Dang,

2003; Herkert & Eilers, 2010; Kress, Sabò, & Amati, 2015). On the contrary Myc negatively regulates genes involved in lysosome and vesicle-mediated transport, GTPase regulator activity, extracellular matrix interaction, epithelial development and morphogenesis. These signatures endorse the role of Myc in regulating functions that shape the morphology of the cells and their interplay with the microenvironment (cell adhesion, interaction with extracellular matrix, cytoskeleton organization) (Coller et al., 2000; Shiio et al., 2002, Elkon et al., 2015), the effects of which are still not completely clear and seem to be highly context-dependent. Indeed, in some contexts (e.g. keratinocytes, HSCs), by modulating the interaction with the local microenvironment, Myc induces cell differentiation and depletes the stem cell reservoir, due to an inadequate retention in the niche (Frye, 2003; Gebhardt, 2006; Wilson, 2004). In other cases, the repressive activity of Myc on cell adhesion, has been observed to accompany transformation processes (Akeson & Bernards, 1990) and contribute to a more aggressive and metastatic phenotype (Wolfer & Ramaswamy, 2011), also dramatically changing cell morphology by acquisition of mesenchymal features (Bin Cho, Cho, Lee, & Kang, 2010; Cowling & Cole, 2007; Smith et al., 2008; Trimboli et al., 2008). This topic is of particular interest since loss of the typical epithelial features of adhesion, morphology and differentiation has been shown to be one crucial step in the acquisition of a stem-like status, which correlates with the capacity to act as cancer stem cells (Mani et al., 2008; Morel et al., 2008). However, even within the same tissue, in this case the mammary epithelium, the evidences regarding the effects of Myc on cell adhesion and migration appear to be discordant. Indeed, Myc is an activator of cell motility and invasiveness (Wolfer & Ramaswamy, 2011), but in some cases it acts as suppressor of these processes (Cappellen, Schlange, Bauer, Maurer, & Hynes, 2007; Liu et al., 2012). The divergent role of Myc in different epithelial cell lines could be due to its interaction with distinct cofactors

(Patel, Loboda, Showe, Showe, & McMahon, 2004) that leads to a differential transcriptional regulation, highlighting the importance of the specific cellular context.

Our experiments show that in immortalized mammary epithelial cells Myc negatively regulates the expression of genes involved in extracellular matrix interaction, cell adhesion and epithelial morphogenesis, leaving open the question of whether this may affect in some way the balance between cell differentiation and self-renewal. The answer to this question will be particularly relevant in the case of aberrant Myc activation in the mammary gland (Liao & Dickson, 2000), due to the emerging hypothesis of the cancer stem cells.

4.2 Myc enhances self-renewal in primary mammary epithelial cells through interaction with Miz1

Among the factors cooperating with Myc in its repressive function, Miz1 appears to be one of the most relevant (Peukert et al., 1997). Indeed, Miz1 is essential in the down-regulation of 25-40% of Myc repressed genes (Walz et al., 2014), and is very likely to be involved in co-regulation of adhesion and self-renewal related genes (Gebhardt, 2006; Herkert et al., 2010; Kerosuo et al., 2008). With the aim to further investigate the mechanisms of transcriptional repression by Myc in mammary epithelial cells, and in particular the role of the Myc/Miz1 interaction, we took advantage of a Myc mutant (MYC V394D, here MYC VD) impaired in its interaction with Miz1 (Herold et al., 2002). In order to address the role of Myc/Miz1 binding in promoting self-renewal, primary mammary epithelial cells conditionally overexpressing MYC WT or VD, have been grown in non-adherent conditions in a clonal sphere-forming assay (Dontu et al., 2003). In general, MYC overexpressing cells showed increased self-renewal compared to control cells. However, cells with

MYC VD up-regulation exhibited a milder effect relative to their MYC WT counterpart.

Since Myc is pivotal in cell cycle regulation (Amati, Alevizopoulos, & Vlach, 1998; Oster, Ho, Soucie, & Penn, 2002) and its interaction with Miz1 is essential for the induction of apoptosis (Patel & McMahon, 2006), we checked both proliferation and cell death in MYC-overexpressing mammospheres, to clarify the role of these phenomena in the observed phenotype. In our hands, MYC up-regulation in mammary epithelial cells grown as mammospheres induced proliferation but, differently from what expected, decreased apoptosis. The latter result could be due to the low levels of MYC overexpression, below the threshold required for cell death induction (Murphy et al., 2008). Furthermore, since cells in non-adherent conditions undergo massive death due to anoikis, a mild MYC overexpression could increase the resistance to anoikis. In this context, MYC proliferative and apoptotic potential seems to be mainly independent from Miz1, since MYC WT and VD up-regulation had very similar effects.

The difference between MYC WT and VD in supporting self-renewal has been further confirmed *in vivo* by cleared fat pad reconstitution assay, in which stem cell frequency (evaluated by mammary outgrowths) was higher in MYC overexpressing cells relative to control cells, with MYC VD overexpressing cells yielding intermediate values. Our results corroborate previous indications about the positive role of MYC in promoting mammary stem cell and progenitor functions (Moumen et al., 2012; Pasi et al., 2011) and, at the same time, suggest that Miz1 is involved in this effect. This is in line with what previously pointed out in neurospheres, in which Myc enhances self-renewal via Miz1, (Kerosuo et al., 2008).

The fact that the highest differences in sphere-forming ability between MYC-overexpressing and control cells were observed at the first two passages (M2-M3)

could suggest that MYC acts mainly by increasing the proliferation of the progenitor population, which has a finite lifespan (Visvader, 2009). While plausible, this scenario would not explain the self-renewal long-term phenotype, which requires an effect on the stem cells pool either through an expansion of the stem cells themselves or through reprogramming of progenitors to a stem-like status. Our results do not currently allow distinguishing between these two possibilities, both scenarios being compatible with the results obtained in mammospheres and in transplantation assays. To discriminate between the two hypothesis, one should induce MYC overexpression only in selected sub-populations, identified by surface marker staining (Stingl, 2009) or PKH26 dye retention (Cicalese et al., 2009); since only stem/early progenitor cells are able to regenerate mammospheres and to reconstitute cleared fat pad, MYC overexpression specifically in purified progenitor/differentiated cells could be useful to verify the hypothesis of MYC reprogramming of these cells. Data obtained in this way in Pier Giuseppe Pelicci's group suggest that Myc up-regulation could expand the stem cell compartment both by inducing symmetric division of the stem cells and by reprogramming the progenitors cells (personal communication). Repeating this kind of experiments with MYC WT and MYC VD would help to dissect the specific contribution of Myc-mediated transcriptional repression to each of these processes.

4.3 The Myc/Miz1 interaction is necessary for repression of genes involved in extracellular matrix interaction, cell adhesion and epithelial morphogenesis in primary mammary epithelial cells

To investigate the transcriptional program that controls self-renewal, we performed whole-genome expression analysis in primary non-adherent mammary epithelial cells overexpressing MYC WT and VD. Both forms of MYC were able to induce an

overlapping set of approximately 600 genes, which was enriched in genes related to cell cycle, DNA metabolic processes, spindle regulation or purine nucleoside binding. A smaller set of genes (ca. 300) was induced preferentially in MYC WT overexpressing mammospheres: since the VD mutation does not affect Myc/Max association and transcriptional activation (Walz et al., 2014), defects in gene activation by MYC VD were most likely indirect and probably they reflect the phenotype of enhanced self-renewal ability in MYC WT overexpressing cells. The genes included in this list were related to extracellular region organization, cell adhesion, cell-cell signaling and gland development. On the other side, loss of Myc/Miz1 interaction mainly affected MYC mediated transcriptional repression, with more than 800 genes repressed by MYC WT more effectively than by MYC VD. While this effect is most likely a direct consequence of loss of Miz1 binding by MYC VD mutation, ChIP experiments mapping MYC WT, MYC VD and Miz1 biding to the genome, along with Miz1 loss of function experiments, would be needed to formally prove which are the direct and indirect transcriptional effects. The main gene ontology category describing the genes differentially repressed by MYC WT and VD were related to extracellular matrix organization, cell adhesion and epithelial morphogenesis. Similar categories are enriched in genes (approximately 500 genes) repressed by both forms of MYC. The repression of the genes mediating these functions could be crucial in MYC -induced increase in self-renewal and Myc/Miz1-mediated repression can contribute to this process.

For a thorough characterization of MYC transcriptional program, we also profiled the expression of microRNAs in primary mammary epithelial cells grown as mammospheres. Indeed, MYC can modulate gene expression at different levels and the microRNAs are an effective system by which it may tune the stability and translation of large numbers of transcripts (Bui & Mendell, 2010). Moreover,

several of the known MYC-regulated miRNAs control genes involved in differentiation and self-renewal (Lin, Jackson, Guo, Linsley, & Eisenman, 2009). We first noticed that MYC-overexpressing cells have an increment of the 3'-modified isomiRs. Since these miRNAs isoforms have been described to be endowed with different stability and function respect to the canonical miRNAs (L. Guo et al., 2011), and have been proposed as biomarkers to diagnose and classify breast tumors (Telonis, Loher, Jing, Londin, & Rigoutsos, 2015), this effect will be further investigated along with a detailed scrutiny of the available literature to identify among the differently regulated miRNAs possible relevant mediators of the observed phenotype.

4.4 Myc/Miz1 repressed genes in mammospheres: new mediators of Myc pro-self-renewal activity in mammary epithelial cells

At present, our working hypothesis is that Myc enhances self-renewal in mammary epithelial cells by repressing some of the identified genes. Hence MYC VD, losing this repressive activity, would exert a milder effect. To verify the proposed mechanism, we will perform an shRNAs screen to identify genes, among those repressed by MYC WT, whose knockdown gives a selective advantage to the cells grown as mammospheres (drop-in screen). In order to perform this screen in the same primary cells in which we observed the phenotype, we are forced to limit the list of screened candidates. In particular considering number of epithelial cells obtained from each mouse, transduction frequency, percentage of cells giving rise to mammospheres, library coverage, number of shRNAs/gene and number of biological replicates we decided that is feasible to screen about 50-100 genes. Our selection criteria are based on the biological relevance of the genes relative to our aim. In particular, we will select: (I) genes repressed in MYC-amplified breast

tumors compared to normal tissue (TCGA data, http://cancergenome.nih.gov), (II) genes that show low expression in stem cells compared to differentiated epithelial cells (Kendrick et al., 2008; Lim et al., 2009; 2010; Sheridan et al., 2015), or (III) in cancer stem cells relative to the bulk tumor population (Creighton et al., 2009), (IV) genes repressed by Myc/Miz1 in other datasets (Wiese et al., 2015). We also tried to compare the regulated genes in mammary epithelial cells in adhesion or grown as mammospheres, but we scored a limited overlap that should be due to context-dependency of Myc transcriptional responses. We are currently setting up the experimental conditions for the functional screen, thereby we hope to individuate and validate new downstream targets of Myc relevant in mediating its capacity to induce self-renewal. Since this ability is closely linked to the tumor aggressiveness (Pece et al., 2010), inhibiting Myc repression of these targets or rescuing their activity could be useful for reducing the malignancy of MYC-addicted breast cancers.

Appendix

This study started with the aim to profile Myc and TGFβ transcriptional programs in mammary epithelial cells, to verify if these two pathways can crosstalk and eventually cooperate in tumor formation. The initial hypothesis was that Myc could be one of the crucial molecular players in the switch from tumor suppressor to tumor promoter of TGFβ. Given that idea, we performed genome-wide transcriptional analysis in the immortalized mouse mammary epithelial cells (MMECs) with Myc loss/gain of function described above also in the presence or in the absence of TGFβ treatment, at a time point (two hours) that we had previously chosen on the basis of the peak of transcriptional induction of known TGFβ targets such as *Snail* and *PAI1* (Figure 44A). However, TGFβ treatment in our cellular model produced only modest transcriptional changes, even if it has been confirmed by Gene Ontology analysis that the transcriptional activation reflects a typical TGFβ response. Indeed the number of differentially expressed genes in response to TGFβ was an order of magnitude smaller than that in response to Myc modulation (hundreds compared to thousands) (Figure 44B). Moreover, at least in this model, almost all the TGFβ transcriptional targets did not show a different regulation upon Myc modulation (Figure 44C). Given these results, we decided to focus our attention on the transcriptional response to Myc itself and that become the main topic of the project as also described above.

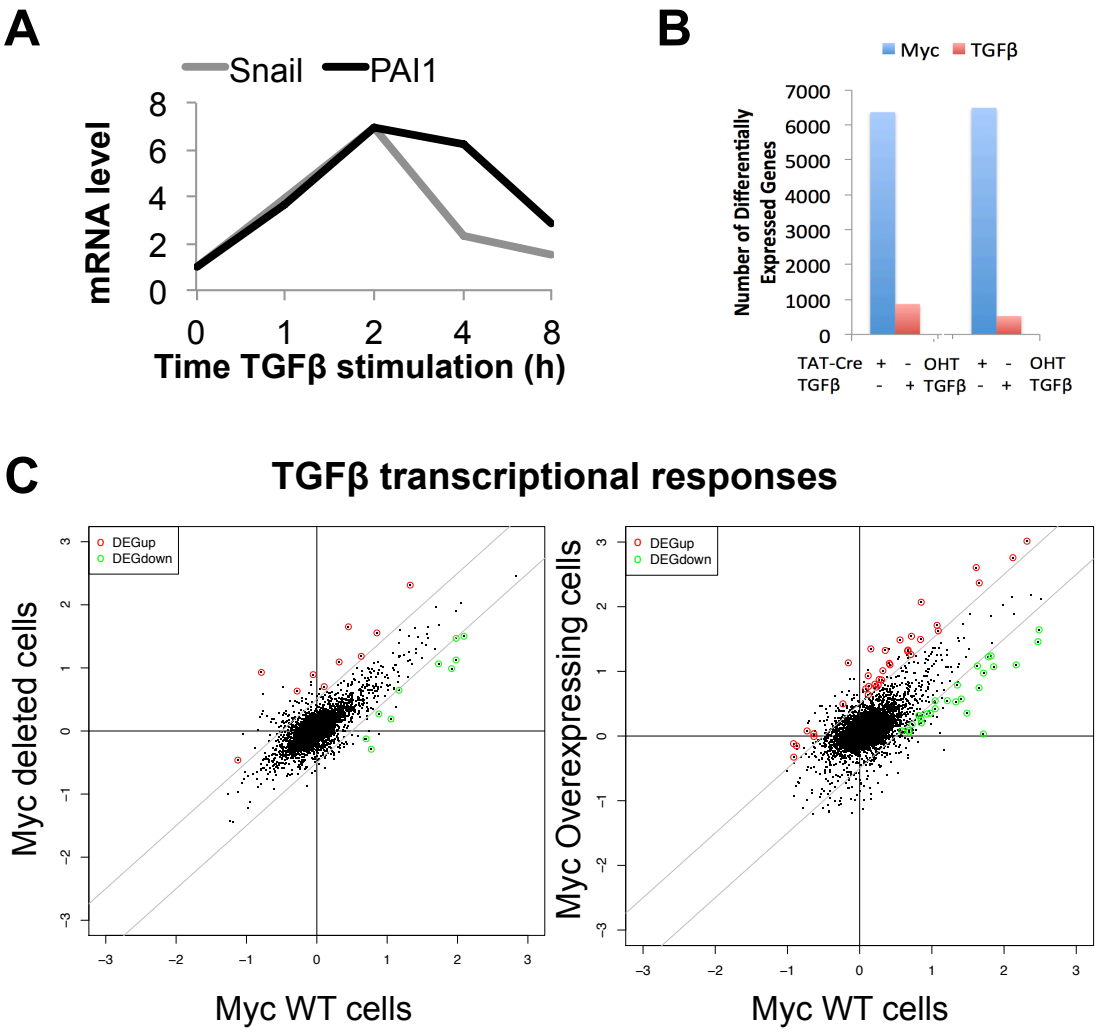

Figure 44: Analysis of Myc and TGFβ transcriptional responses in MMECs. (A) Time course of TGFβ induction in MMECs, the transcriptional activation of TGFβ pathway is verified by two of its known targets (*Snail* and *PAI1*). The mRNA level is normalized on *Rplp0* housekeeper gene. (B) The number of differentially expressed genes in Myc loss (+TAT-Cre) and gain (+OHT) of function samples and in TGFβ-treated samples. The genes whose expression is Myc-dependent are about six thousands; in comparison the TGFβ-dependent genes are about five-eight hundreds (C) The TGFβ transcriptional responses in the absence or in the presence of overexpression of Myc. The significant differentially expressed genes are highlighted: the up-regulated in red, the down-regulated in green.

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