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Discovery of new candidate biomarkers in Breast cancer tissue by multiomic analysis

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Table of Contents

INTRODUCTION

A traditional cancerogenic model requires that the tumour cell evolves from a somatic cell that acquires genetic and epigenetic mutations to activate oncogene and downregulate oncosuppressor.

In this environment, we can describe the six Hallmarks of cancer, which are

1. Sustained proliferative signalling
2. Evasion from growth suppression
3. Activation on invasion and metastasis
4. Enabling replicative immortality
5. Induced angiogenesis
6. Cell death resistance

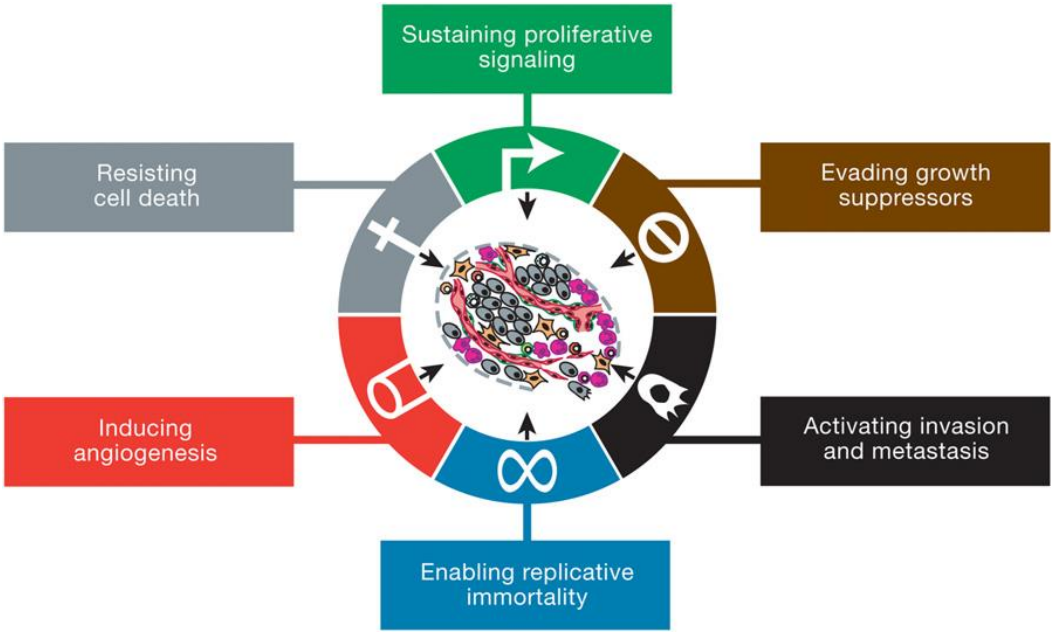


Figure 1. Hallmarks of cancer originally proposed in 2000 (Hanahan & Weinberg, 2011)

Sustained Proliferative Signalling and bypassing growth suppressors

In normal tissue, the controls of the production and the release of growth-promoting signals for the progression, cell growth and division cycle to maintain homeostasis and cell number are well balanced. These signals are represented by different growth factors that bind cell-surface receptors containing intracellular tyrosine kinase domains. These receptors

transduce the signal through intracellular signalling pathways that regulate cell growth and progression in the cell cycle. To date, controlling the release of these mitogenic pathways is poorly described because these mechanisms are complicated by paracrine signalling and by the action of a complex network of proteases, sulfatases and other enzymes.

In contrast, in cancer cells, the signalling is better understood some mutations lead to the acquisition of an unlimited proliferative potential, forming a heterogeneous tumoral mass, where every single cell can maintain a new tumoral mass or expand in metastasis.

Tumoral cells can enhance the production of growth factor ligands and relative receptors, resulting in autocrine proliferative stimulation, or can send signals to normal cells to stimulate them to supply cancer cells with growth factor ((Cheng et al., 2008); (Bhowmick et al., 2004)).

Another mechanism that increases proliferation is the bypassing of negative feedback mechanisms. This type of regulation is used by Ras oncoprotein; in fact, no mutation increases the power of Ras activity, but it works within a negative feedback mechanism that ensures the transitory signalling.

The evasion from growth suppressor pathways, like RB (retinoblastoma-associated), TP53 proteins and TGF-β, is critical to enhance the proliferation. The RB proteins receive the signal from extracellular and intracellular sources, deciding whether or not the cell can proceed to the division cycle (Burkhart & Sage, 2008). Defection in the RB pathway allows persistent cell proliferation. TP53 receives signals from stress sensors like genome damage or low glucose and oxygen levels and blocks cell-cycle progression. Mutations for this protein lead to non-implementation of blocking mechanisms.

Cell death resistance

Cell death is a natural barrier against cancer development; normal cells undergo apoptosis triggered by various physiologic stresses, and tumoral cells can bypass this signalling in multiple manners. The apoptotic mechanism comprises an upstream and downstream regulator; these regulators are divided into two clusters: one that receives and processes extracellular death-inducing signals and the other that senses various signals. The end is the caspases 8 and 9, which start a cascade of proteolysis; in this way, the cell is disassembled, and slowly cell death occurs. Against this process is a family of proteins called Bcl-2 that

acts as an apoptosis suppressor, inhibiting two BH3 motif proteins, Bax and Bak. These two proteins destroy the outer mitochondrial membrane, causing the release of cytochrome c, which activates the caspases that activate the apoptotic program (Adams & Cory, 2007; Willis et al., 2005) The tumoral cells evolve strategies to limit this process; one is the increasing production of Bcl-2, which acts as a survival signal.

Autophagy, which is evaded from cancer cells, is regulated as apoptosis. Recent studies have revealed that autophagy and apoptosis are connected, e.g., in the signalling pathway that involves PI3 kinase, Akt, and mTOR, kinases are downregulated when survival signals are insufficient (Levine & Kroemer, 2008)

Invasion and Metastasis

Metastasis is a crucial aspect of cancer progression. It occurs when cancer cells spread from the tumor's original (primary) site to other parts of the body, forming new tumors. This process typically happens through the bloodstream or lymphatic system. Metastasis enables cancer to invade distant tissues and organs, often leading to more severe health complications and decreased chances of successful treatment.

The best-characterized alteration in metastasis is the loss of expression of E-Cadherin in tumoral cells. E-cadherin plays a critical role in cell adhesion, particularly in epithelial tissues. E-cadherin forms adherents junctions between adjacent epithelial cells as a type of cell adhesion molecule. These junctions help to maintain the structural integrity and organization of epithelial tissues, contributing to their function as barriers and facilitating their role in tissue architecture. One of the critical functions of E-cadherin is to mediate calcium-dependent cell-cell adhesion. E-cadherin molecules from neighbouring cells bind through its extracellular domain, forming homophilic interactions. This binding stabilizes cell-cell contacts and facilitates the formation of adherent junctions. The intracellular domain of E-cadherin interacts with various proteins involved in cytoskeletal organization, helping to anchor the cadherin complex to the actin cytoskeleton within the cell (Gumbiner, 1996).

In contrast, N-Cadherin is upregulated in many invasive carcinomas, is often found in mesenchymal cells and is involved in various processes such as neuronal development,

cardiac morphogenesis, and muscle differentiation; otherwise, N-cadherin promotes tumor cell migration, invasion, and metastasis by facilitating interactions between cancer cells and stromal cells and promoting EMT and tumor cell dissemination (Jeanes et al., 2008; Wheelock et al., 2008).

The invasion-metastasis cascade is a complex series of steps that cancer cells undergo to spread from the primary tumor site to distant organs in the body. This process involves multiple stages, each presenting significant challenges for the cancer cells (Talmadge & Fidler, 2010)

1) **Local Invasion**: Cancer cells escape from the primary tumor mass by loosening their connections with neighbouring cells and degrading the surrounding extracellular matrix (ECM). This allows them to invade nearby tissues and blood vessels;
2) **Detachment and embolization**: tumor cell aggregates, which may be increased in size via interaction with hematopoietic cells within the circulation;
3) **Intravasation**: Cancer cells enter the bloodstream or lymphatic system, often facilitated by increased motility and changes in cell adhesion molecules. Once in circulation, they are known as circulating tumor cells (CTCs);
4) **Survival in Circulation**: CTCs face numerous challenges in the bloodstream, including shear forces and immune surveillance. Some CTCs evade these obstacles and survive in circulation;
5) **Extravasation**: CTCs exit the bloodstream by adhering to the endothelial cells lining blood vessels at distant sites. They then penetrate the vessel walls and enter the surrounding tissue, often aided by interactions with immune cells and the ECM;
6) **Proliferation of tumoral cells**: Cancer cells proliferate and establish micrometastases once in the new tissue microenvironment. These small clusters of cancer cells may remain dormant for extended periods or continue to grow and invade nearby tissues;
7) **Angiogenesis**: Micrometastases recruit blood vessels to provide oxygen and nutrients for further growth. This process, known as angiogenesis, facilitates the expansion of metastatic lesions;

8) **Macrometastasis Formation**: Over time, micrometastases may develop into larger, clinically detectable macrometastases. These metastatic lesions can disrupt normal tissue function and cause clinical symptoms.

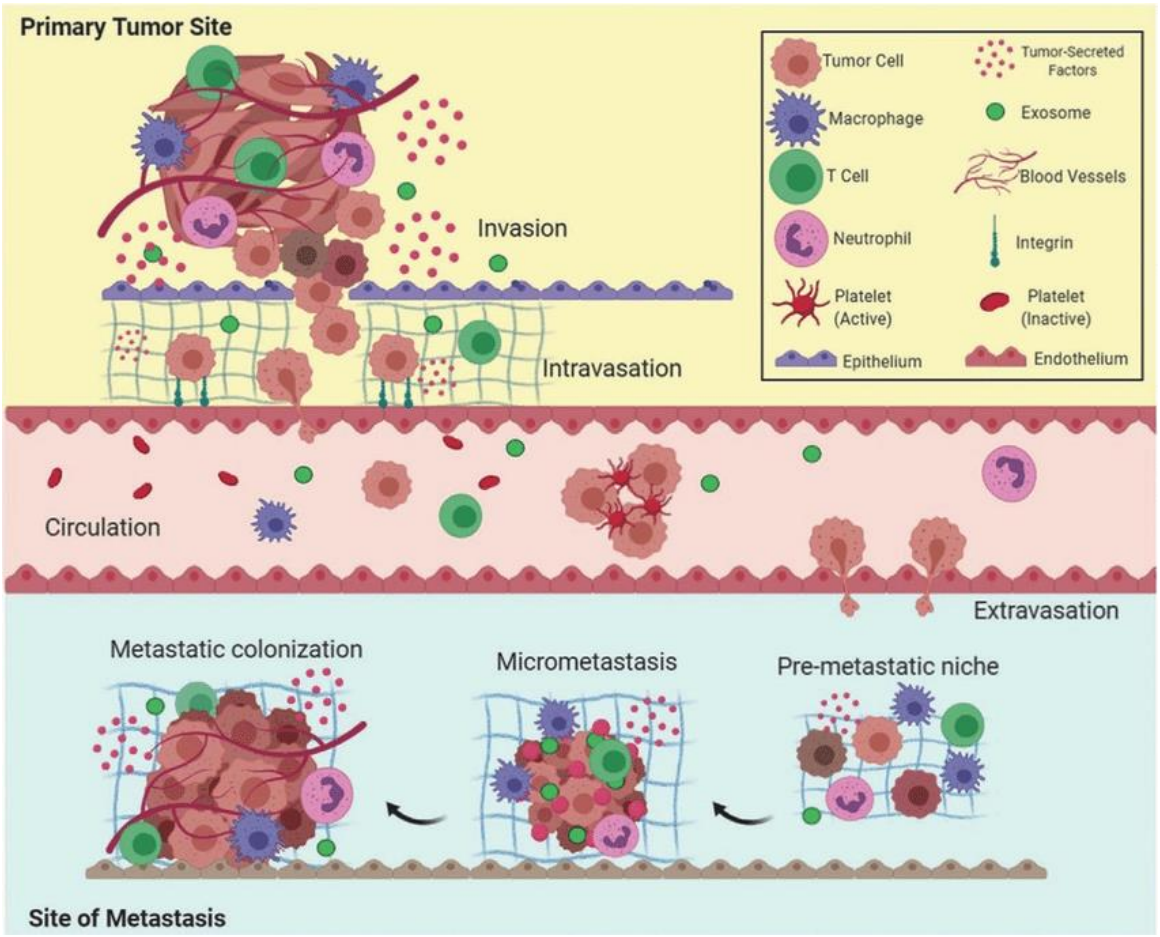


Figure 2. Metastatic mechanism illustration (Fares et al., 2020)

The epithelial-mesenchymal transition (EMT) regulates the metastasis process, which can be transiently or stably activated. Some transcription factors are involved, like Snail, Slug, Twist and Zeb1/2, these are expressed in combination in many tumors (Sadlecki et al., 2018). Some can directly repress E-cadherin (Peinado et al., 2004).

EMT is typical of "mesenchymal" invasiveness. Still, we can find two other types of invasion: the collective invasion typical of squamous cell carcinomas and the ameboid form, which is unclear(Hanahan & Weinberg, 2011).

Enabling Replicative immortality

After some cycle of propagation, normal cells propagated in cell culture start to exhibit senescence signs, and the majority of the population dies. This phenomenon is due to the progressive shortening of telomeres. The telomers, composed of a six-nucleotide repeats sequence, in cancer cells are protected by the activity of telomerase that protects the ends of chromosomes, adding the repeats to the 3' strands of the chromosomes. In this way, in cancer cells, the barriers to proliferation and apoptosis are bypassed so the cells can outgrow and form neoplastic tissue.

Inducing Angiogenesis

Angiogenesis is the formation of new blood vessels that play a critical role in tumor growth and metastasis. Tumor tissue needs a nutrient boost to grow, so the development of new vessels is largely stimulated and maintained, facilitating their proliferation and survival.

A complex interplay of proangiogenic and anti-angiogenic factors regulates angiogenesis. A well-known angiogenesis inhibitory inducer pair is represented by the Vascular Endothelial Growth Factor-A (VEGF-A) and the Thrombospondin-1 (TSP-1), respectively. Tumor cells secrete various angiogenic factors, creating a proangiogenic environment conducive to blood vessel formation; the vessels produced from cancerous cells are typically aberrant and are characterized by excessive branching, distorted vessels, unbalanced blood flow and leakiness (Baluk et al., 2005).

VEGF-A gene, a PDGF/VEGF growth factor family member, encodes a heparin-binding protein that exists as a disulfide-linked homodimer, it induces proliferation and migration of vascular endothelial cells, essential for physiological and pathological angiogenesis, its upregulation is observed in many known tumors, and its expression correlates with tumor stage and progression. On the other hand, the TSP-1 gene, encoding for a subunit of a disulfide-linked homotrimeric protein, an adhesive glycoprotein that mediates cell-to-cell and cell-to-matrix interactions and binds transmembrane receptors displayed in endothelial cells, suppressing proangiogenic stimuli (Kazerounian et al., 2008). Other factors, such as Fibroblast Growth Factor (FGF), Platelet-Derived Growth Factor (PDGF), and angiopoietins, also contribute to angiogenesis (Baeriswyl & Christofori, 2009).

An emerging role in tumoral cell proliferation is represented by the differential expression of different Micro-RNAs (miRNAs) and Long non Coding RNAs (lncRNAs). The first one is a class of highly conserved small oligonucleotide (19-25 nucleotides) non-coding RNAs that bind to specific mRNAs, resulting in gene silencing; lncRNAs are long oligonucleotide (200 nucleotides) that are non-translated into protein lncRNAs can promote or inhibits translation variously.

Some miRNAs, like miR-17-92, interfere with the expression of E2F protein translation, inhibiting the positive feedback mechanism of c-myc (Coller et al., 2007). This miRNA has been observed in B-cell lymphomas, the overexpression of miR17-92 promotes cell proliferation (He et al., 2005).
For example, high expression of the lncRNA HULC is correlated with hepatocellular carcinoma, this lncRNA regulates the expression of the P18 protein, which inhibits CDK4 and CDK6 (Du et al., 2012). Other lncRNAs are involved in different tumors in different ways, like LINCRNA-21, that inhibits CDK2 in hepatocellular carcinoma (Martino et al., 2021) or PTENP1 role in squamous cell carcinoma (Liu et al., 2017)

Immune evading and inflammation

Emerging roles in cancer initiation and development are represented by evasion from immune response and inflammation. In fact, around 20% of cancer cases are preceded by inflammation (Grivennikov et al., 2010). Inflammation may aid cancer initiation, this is due to the action of macrophages and neutrophils that, during inflammation, produce reactive oxygen species (ROS) and nitrogen species (RNI), these can increase mutagenesis and DNA damage (Greten & Grivennikov, 2019). In cancerous cells, pro-survival pathways are expressed, especially that pathway mediated by NF-KB, STAT 3 (Grivennikov et al., 2010), this pathway also contributes to tumor progression as demonstrated in studies on myeloid cells (Greten et al., 2004). In this context, other cytokines' expressions can contribute to tumor progressions like IL-6, IL-7, and IL-11, increasing proliferation rates. Not only initiation and progression are coadjuvant by inflammation, but also metastasis. Cytokines like TNF and IL1-Beta can induce the expression of transcription factors such as Twist and Slug (Suarez-Carmona et al., 2017). Another interleukin, like IL-11, is involved in metastasis events by

recruiting TGF-β producing fibroblast able to support invasion and immune evasion in colon and breast cancer (Calon et al., 2012; Marusyk et al., 2016).

An emerging Hallmark is the reprogramming of energy metabolism

During the transition from normal to malignant, cells change morphology, lose important regulative functions, and modify their energetic metabolism (Hanahan & Weinberg, 2011). Normal tissues metabolize glucose by oxidative phosphorylation into pyruvic acid (in the presence of oxygen) through glycolysis and then, inside the mitochondria, oxidize all pyruvic acid to CO2. Oxygen is needed as a final electron acceptor to oxidize glucose completely during this process. In the absence or deficiency of oxygen, cells can use pyruvic acid to generate lactate. During the anaerobic glycolysis, this lactate allows continuing glycolysis but with a limited production of ATP. Warburg observed that cancerous cells tend to convert most of the glucose into lactate regardless of oxygen. This process is the same in tumoral and normal proliferative cells, but the production of ATP by aerobic glycolysis is less efficient than oxidative phosphorylation (Vander Heiden & DeBerardinis, 2017).In vivo and in vitro studies have shown that tumours are highly hypoxic due to inefficient vascularization—adaptative response to hypoxia results in the increase in glucose uptake and the production of lactic acid. There must be a cellular alteration that causes the aerobic glycolytic phenotype. The oncogenic pathway can stabilize HIF-1α expression; this mechanism stabilizes tumoral aerobic glycolysis in normoxic conditions (Warburg effect). Other oncogenes, like MYC, are overexpressed in many tumours and directly transactivate some glycolytic pathway genes (HK2, ENO1, LDH), binding to their promotors in hypoxic conditions (Dang and Semenza 1999).

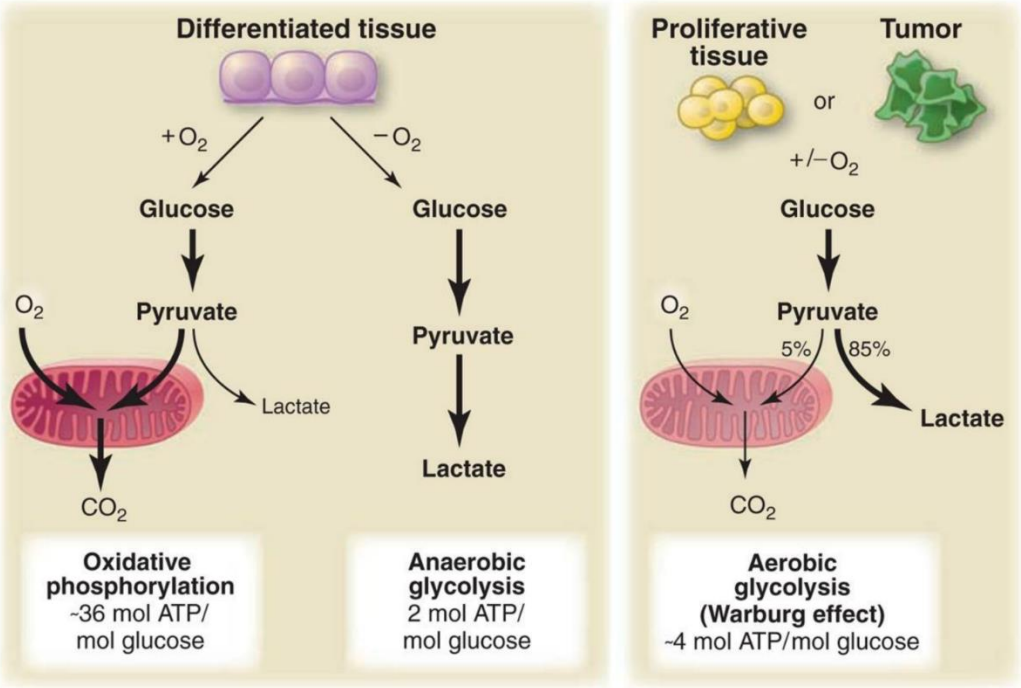


Figure 3. Warburg effect mechanism illustration showing the difference between oxidative phosphorylation (OXPO), aerobic glycolys and aerobic glycolysis (Vander Heiden MG et al., 2009*)*

This autonomous oncogenic glycolysis seems to sustain the Warburg effect; however, higher levels of glycolytic enzymes resulting from this effect may contribute to tumour adaptation through the non-glycolytic alternative functions of these enzymes. Metabolic differences observed between tumours and normal tissues could be the basis for possible therapeutic applications (Kim & Dang, 2006)

Some enzymes have a non-metabolic function that contributes to cancerous cellular transformation. Some non-metabolic functions of these enzymes can guide the proliferation in contrast to apoptosis (Snaebjornsson & Schulze, 2018)

In this general context, the metabolic differences observed between tumor and normal tissues could be the basis of possible prognostic and therapeutic applications (Dang et al., 2008).

BREAST CANCER

Breast cancer is a disease that affects millions of individuals worldwide. It is the most common form of cancer in women, comprising approximately 25% of all cancer diagnoses among females. Breast cancer is a significant global health issue, affecting millions of individuals each year and posing substantial challenges to healthcare systems worldwide. Breast cancer is the most common cancer among women globally; according to the World Health Organization (WHO), there were an estimated 2.3 million new cases of breast cancer worldwide in 2020, making it the most prevalent cancer globally, new cases diagnosed in 2020 alone (Arnold et al., 2022). It is also a leading cause of cancer-related deaths in women, accounting for over 685,000 deaths annually (Bray et al., 2018). While breast cancer incidence rates vary geographically, with higher rates observed in developed countries, it remains a significant public health concern worldwide.

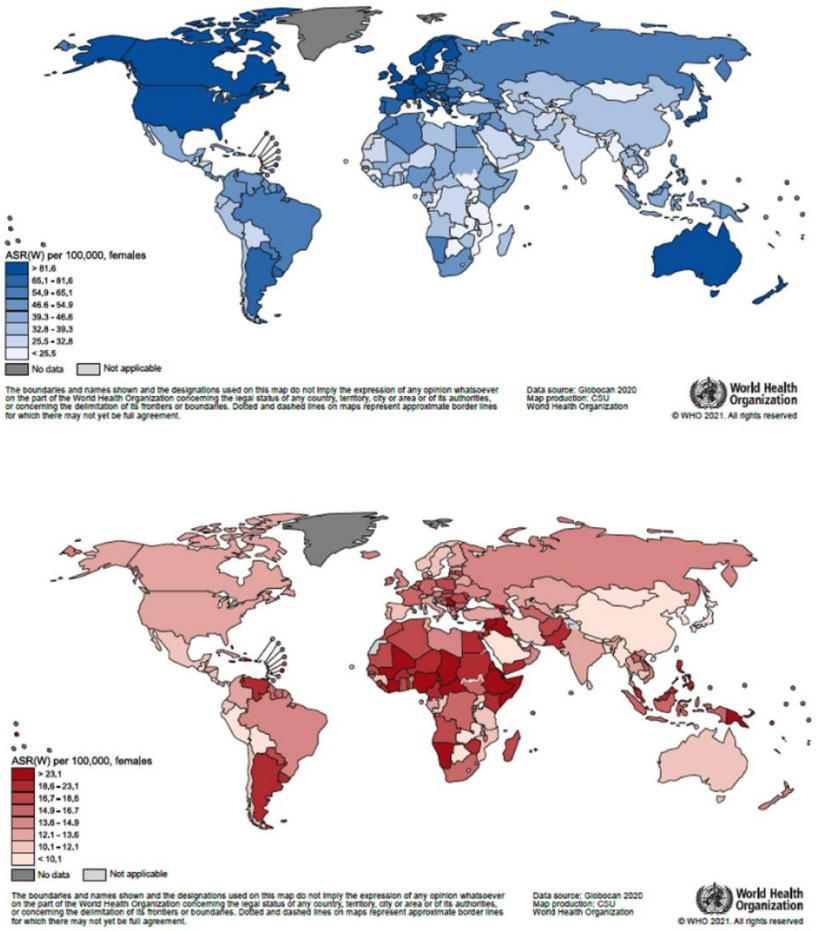


Figure 4. Age-standardized breast cancer incidence (top, blue) and mortality (bottom, red) rates per 100,000 females. Breast cancer cases and deaths by country.

While the exact cause of breast cancer remains elusive, certain risk factors has been identified that may increase an individual's likelihood of developing the disease. These risk factor include age, family history of breast cancer, inherited genetic mutations (such as BRCA1 and BRCA2), hormonal factors (such as early menstruation or late menopause), reproductive history (such as nulliparity or late age at first childbirth), lifestyle factors (such as obesity, alcohol consumption, and lack of physical activity), and exposure to radiation or certain chemicals (Colditz & Bohlke, 2014).

It is essential to enhance our knowledge in the field of breast cancer because, by 2040, the number of newly diagnosed cases could grow by over 40% to about 3 million every year. Deaths from breast cancer could increase by more than 50%, from 685,000 in 2020 to 1 million in 2040 (Arnold et al., 2022).

Breast anatomy and cancer classification

The organ designated for lactation is the breast, which consists of adipose tissue and a set of glandular structures called lobules that are joined together to form a lobe. On average, between 15 and 20 lobules can be found in a breast, with milk reaching the nipple through small ducts called galactophores. In theory, tumors can form from all types of breast tissue, but the most common arises from the epithelial cells or those forming the wall of the ducts.

Breast cancer can be divided into two forms:

- The preinvasive forms
- The invasive forms

Preinvasive forms include the following:

- DICIS: Ductal carcinoma in situ
- LICIS: Lobular carcinoma in situ

Invasive forms, on the other hand, are:

- Ductal carcinoma, which is when the neoplasm exceeds the duct wall. It accounts for 70 to 80 per cent of all forms of breast cancer;

● Lobular carcinoma, which is when the tumor exceeds the lobule wall. It accounts for between 10 and 15% of all cases of breast cancer. It can affect both breasts simultaneously or appear in multiple locations in the same breast;

● Other forms of carcinoma: tubular, papillary, mucinous, and cribriform carcinoma. They have a favourable prognosis and are less frequent forms.

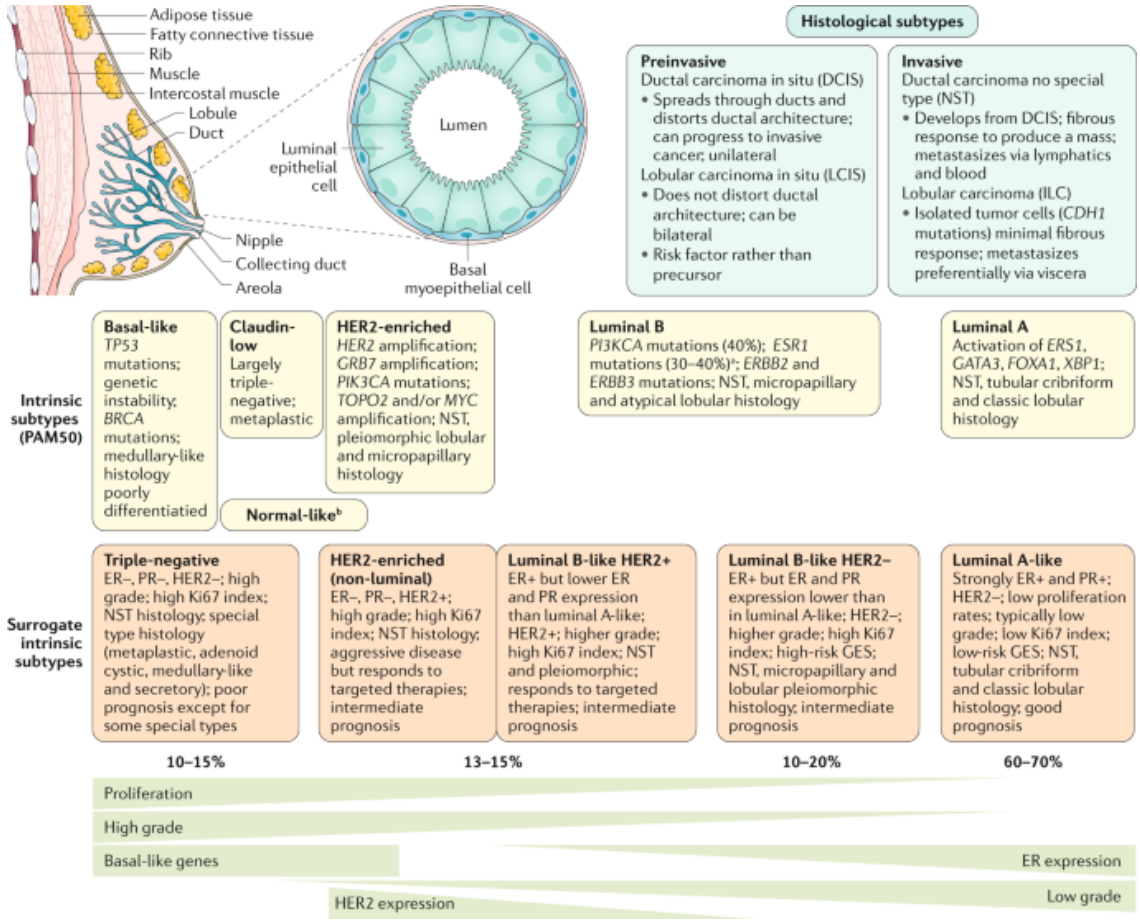


Figure 5. Histological and molecular classification in Breast Cancer (Harbeck et al., 2019)

Recently gene expression studies have shown heterogeneity of breast cancer and several molecular subtypes have been identified that can help clinical practice to establish the best treatment. However, to date, it has not been determined whether these molecular characteristics would influence unequivocally the clinical management of breast cancer (Harbeck et al., 2019).

Evolution of breast cancer

Breast cancer is classified into five stages.

- Stage 0: It is also called carcinoma in situ and can be of two types:
    - Lobular carcinoma in situ: this is a nonaggressive tumor but is a risk factor for the future formation of a malignant lesion;
    - Ductal carcinoma in situ (DCIS): affects duct cells and is considered a precancerous form rather than a true tumor. In most cases, it does not evolve into a true tumor but regresses spontaneously by the action of the body's defence mechanisms.
- Stage I: is an early-stage tumor, with a diameter of less than 2 cm and without lymph node involvement;
- Stage II: can be either an early-stage tumor with a diameter of less than 2 cm, which, however, has already involved lymph nodes present under the axilla, or a tumor with a diameter greater than 2 cm without lymph node involvement;
- Stage III: is a locally advanced tumor of variable size with involvement of lymph nodes located under the axilla or tissues close to the breast (e.g., skin);
- Stage IV: is an already metastasized cancer that has involved other organs outside the breast.

The Union for International Cancer Control (UICC) proposes the classification, which parallels the TNM classification system (Tumor, Node, Metastasis) proposed and updated by the American Joint Committee on Cancer (AJCC). The eighth edition was published in early 2018, it includes an anatomic classification based on anatomical features (e.g., the extent of the primary tumor [T], metastasis absence or presence and extent to regional lymph nodes [N], metastasis absence or presence of distant M), and a prognostic classification (Prognostic Stage Group) that includes, in addition to anatomical variables (TNM), tumor grade, the status of hormone receptors (ER and PR), and HER2 receptor status. However, relapses vary between 9 and 30 per cent of cases, depending on the therapy carried out. Conversely, if the lymph nodes are positive and contain cancer cells, the five-year survival is 75%. In metastasized cancer, where the latter has already affected other

organs outside the breast (usually the lungs, liver, and bones), the average survival of patients treated with chemotherapy is two years, with cases of much longer survival, even up to ten years (https://www.aiom.it/ 2018).

Diagnostic Methods

Different diagnostic methods are used to detect and diagnose breast cancer, including clinical breast examinations, imaging tests (such as mammography, ultrasound, and magnetic resonance imaging), and biopsy procedures (such as fine-needle aspiration, core needle biopsy, or surgical biopsy) to confirm the presence of cancer, determine its subtype and stage, and guide treatment decisions.

Treatment Options

Treatment for breast cancer depends on several factors, including the type and stage of the cancer, as well as the individual's overall health and personal preferences.

Common treatment modalities may include:

- Surgery (such as lumpectomy or mastectomy)
- Radiation therapy
- Drug therapy:
  - Hormone therapy
  - Chemotherapy
  - Targeted therapy and immunotherapy.

Unfortunately, most women with diagnosed breast cancer have to undergo surgery, regardless of stage, that involves:

- Portion of the breast; this technique is called quadrantectomy;
- A more significant portion of the breast with a segmental or partial mastectomy;
- The entire breast or total mastectomy.

In all cases, after surgery, the patient undergoes radiation therapy intending to protect the remaining gland, when present, from the risk of local recurrence and the appearance of a new neoplasm. Additionally, in some cases, removal of the sentinel lymph node(s) is performed.

Early detection of breast cancer through routine screening mammography and clinical breast examinations can significantly improve prognosis and survival rates. Furthermore, healthy lifestyle habits, such as maintaining a healthy weight, engaging in regular physical activity, limiting alcohol intake, and avoiding tobacco use, can help reduce the risk of developing breast cancer (World Cancer Research Fund/American Institute for Cancer Research, 2018).

Hormone therapy (endocrine therapy)

It is the primary choice for the treatment of women affected by receptor hormone-positive breast cancer for the hormone estrogen (ER) and progestin (PR).

It includes the use of two different strategies.

- Aromatase inhibitors: these molecules reduce estrogen production, which in women affected by breast cancer, works as a stimulating of the proliferation of cancer cells;
- Anti-estrogen therapies that bind the receptor to block the action of the estrogen directly to the cells.

Chemotherapy

Antitumoral drugs (such as doxorubicin) have strong side effects because they are not targeted, so they can attack all cells. For this problematic side effect, chemotherapy can be administered to:

- Patients with fast-growing tumors;
- To patients with non-hormone-responsive and Her2- negative tumors;
- To patients no longer responsive to hormone treatment.

Target therapy and immunotherapy

This therapy is aimed at specific targets of cancer, like crucial development proteins like HER2, the product of the ERBB2 gene (Epidermal growth factor receptor 2), CDK4/6 (Cyclin Dependent Kinase 4/6) and VEGF (Vascular Endothelium Growth Factor).

Therapies aimed against HER2

HER2 tightly binds to other ligand-bound EGF receptors to form a heterodimer, stabilizing ligand binding and enhancing kinase-mediated activation of downstream signalling pathways, such as those involving mitogen-activated protein kinase and phosphatidylinositol-3 kinase.

It is possible to use monoclonal antibodies (pertuzumab and trastuzumab), which act by binding HER2 outside the tumor cells; HER2 inhibitors, which bind inside the tumor cells; or anti-HER2 antibodies conjugated with a chemotherapeutic agent, such as T-DM1, which binding to the HER2 receptor, delivers the chemotherapeutic within HER2-positive cells and it has been shown to be a feasible first-line therapeutic option for metastatic HER2-positive BC (Monteiro et al., 2024)

Therapy aimed against CDK4/6

CDK4 (Cyclin Dependent Kinase 4) is a protein that works as a catalytic subunit of the protein kinase complex, which is essential for cell cycle G1 phase progression. The activity of this kinase is restricted to the G1-S phase, which is controlled by the regulatory subunits D-type cyclins and CDK inhibitor p16 (INK4a).

CDK6 (Cyclin Dependent Kinase 6) this kinase is a catalytic subunit of the protein kinase complex that is important for cell cycle G1 phase progression and G1/S transition. The activity of this kinase first appears in the mid-G1 phase, which is controlled by the regulatory subunits, including D-type cyclins and members of the INK4 family of CDK inhibitors.

CDK4/6 inhibitor therapy: this is a new class of drugs that can inhibit cyclin-dependent kinases involved in cancer cell replication. The combination of these drugs with hormone therapy represents a new treatment option for patients with advanced breast cancer and hormone receptor-positive and HER2-negative breast cancer.

Therapy aimed against VEGF

VEGF exists as a disulfide-linked homodimer. This growth factor induces proliferation and vascular endothelial cell migration, essential for both physiological and pathological angiogenesis.

The bevacizumab is a monoclonal antibody against VEGF; this inhibits angiogenesis, which is overexpressed in tumoral cells.

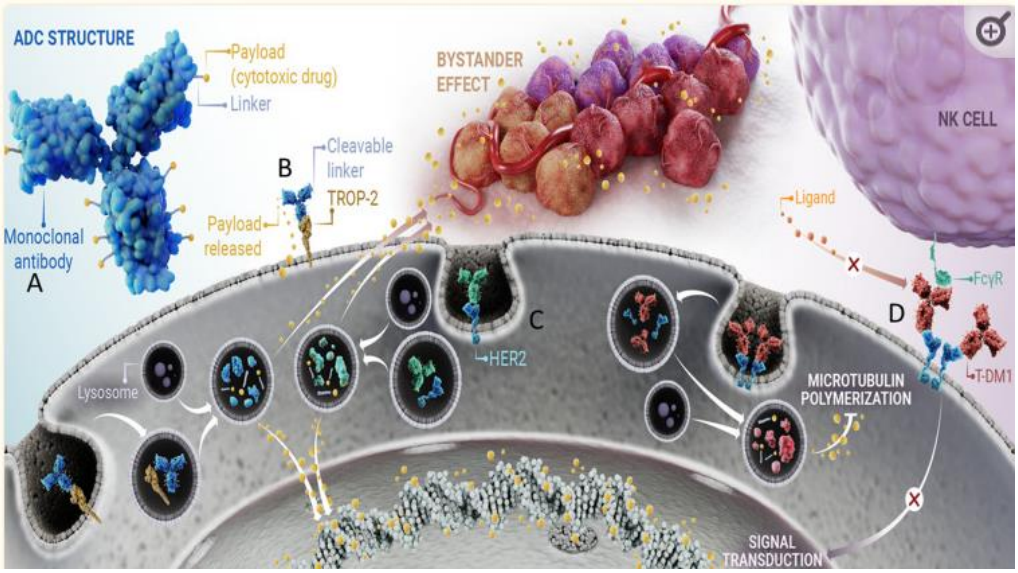


Figure 6. Structure and Mechanism of Action of Antibody-Drug Conjugates in the Treatment of Breast Cancer (from Monteiro et al., 2024).

Alpha-enolase and Myc promoter-Binding Protein 1 - MBP-1

In the context of the energy metabolism reprogramming and in the sustenance of the Warburg effect, a key role is done by α-enolase, encoded from the gene ENO1. Enolases are glycolytic enzyme that catalyses the reversible conversion of 2-phosphoglycerate to phosphoenolpyruvate, an essential step in the glycolytic pathway. Alpha-enolase gene is ubiquitously expressed in various tissues and plays a crucial role in cellular metabolism by contributing to the generation of ATP. Beyond its metabolic function, α-enolase is a multifunctional protein involved in several non-glycolytic processes such as extracellular matrix degradation, growth control and hypoxia tolerance (Díaz-Ramos et al., 2012; Huang et al., 2022).

Alpha-Enolase plays a significant role in cancer, primarily through its contributions to metabolic reprogramming, cell proliferation, migration, invasion, survival and angiogenesis induction (Huang et al., 2022).

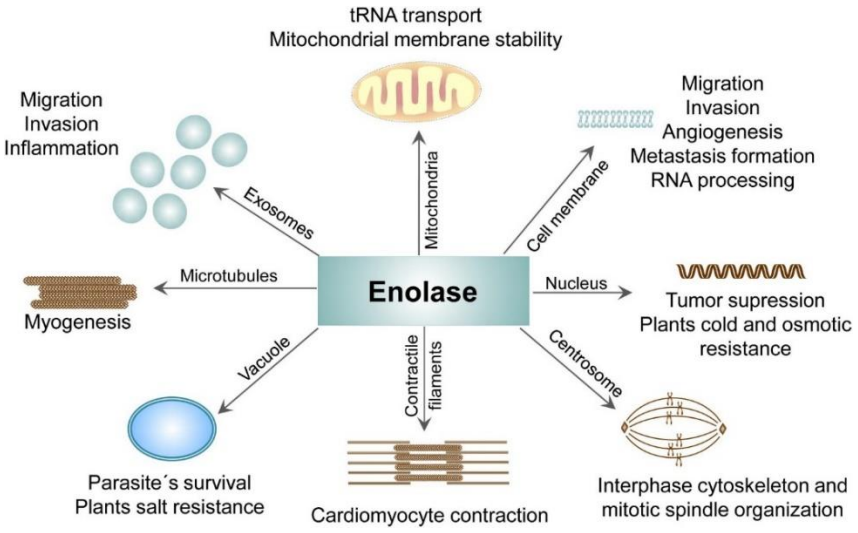


Figure 7. Enolase functions diagram in different cell compartments (Didiasova et al., 2019).

In tumoral cells, ENO1 promotes uncontrolled proliferation, for example, in breast cancer SKBr3 cells, the silencing of ENO1 blocked the cell in the G2/M phase (Zhang et al., 2020). ENO1 can promote invasion via the plasminogen pathway, α-Enolase can bind to plasminogen on the cell surface, facilitating plasminogen activation to plasmin, degrading the extracellular matrix and promoting tumour invasion and metastasis (Hsiao et al., 2013). ENO1 is also capable of inducing EMT; in fact, a study has demonstrated its role in lung cancer (Maureen Chen et al., 2020). As shown by a study on nude mice ENO1 is also involved in angiogenesis, mice injected with breast cancer cells transfected withENO1 small interfering RNA (siRNA) reduced angiogenesis and proliferation (Gao et al., 2013).

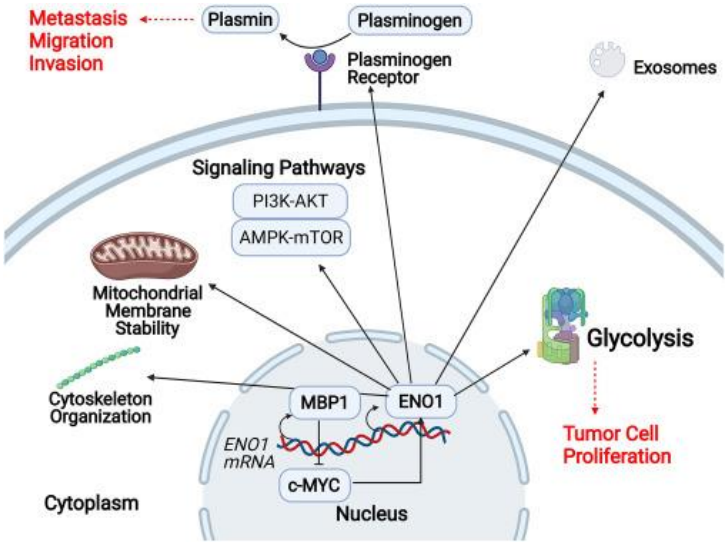


Figure 8. ENO1 localization and function (Huang et al., 2022).

ENO1 encodes not only for α-enolase but also for a shorter form called Myc promoter-Binding Protein (MBP1). Mainly localized in the nucleus, MBP-1 acts as a competitor of TBP (TATA-binding protein) on the P2 TATA-box of the MYC gene, resulting in the transcriptional repression of the gene (Chaudhary & Miller, 1995; Ray & Miller, 1991).

Many studies confirmed that MBP-1 originate from the ENO1 gene transcript by alternative translational starting at the internal AUG 97 compared to AUG 1 for alfa-enolase, and lacking the first 96 amino acids of ENO1 glycolytic enzyme (Chaudhary & Miller, 1995; Feo et al.2000; Lung et al., 2010; Maranto et al. 2015).

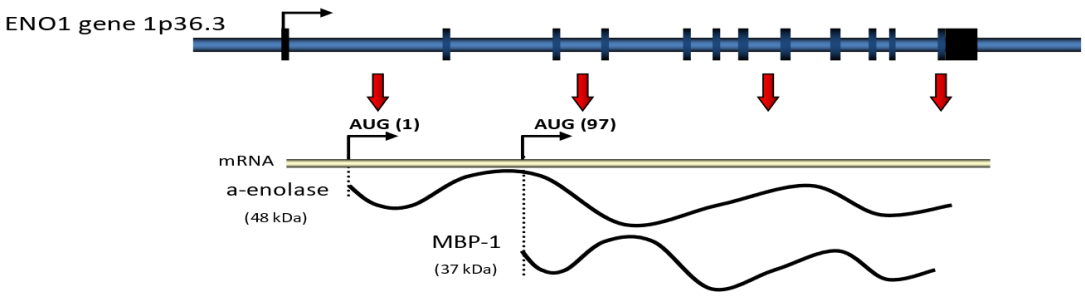


Figure 9. Schematic representation of α-enolase and MBP1 translation

Exogenous expression of MBP-1 inhibits the growth of breast cancer in naked mice (Ray et al. 1995), induces cell death in neuroblastoma cells (Ejeskär et al., 2005), suppresses proliferation in lung cancer cells and induces the block of G0-G1 transition in chronic myeloid leukaemia cells (Pal et al. 2011). In addition to the MYC gene, other oncogenes have been identified as direct targets of MBP-1: the COX-2 gene in gastric tumoral cells (Hsu et al. 2009), ERBB2 in breast tumoral cells (Contino et al. 2013), NMYC in neuroblastoma cells (Ejeskär et al., 2005) and FOXP3 in T cells (De Rosa et al., 2015).

MBP-1 can exert its function by cooperating with other factors: MIP-2/sedlin (Ghosh et al. 2001), the histone deacetylase 1 (HDAC1) (Ghosh et al., 1999, Contino et al., 2013), the kelch protein NS1-BP (Perconti et al., 2007) and the intracellular domain of the Notch 1 receptor (Hsu et al., 2008).

Consistent with its oncosuppressor role as a negative regulator on cell growth, the endogenous level of MBP-1 in tumor cells is relatively low, and glucose concentration, hypoxia and oxidative stress, have been reported to modulate MBP-1 expression, thereby affecting cell proliferation (Sedoris et al., 2007; Sedoris et al., 2010, Maranto et al., 2015). Therefore, MBP-1 appears to be one of the factors controlling cell growth and proliferation, and alterations in its expression level induced by the tumor microenvironment may contribute to cancer development (Maranto et al., 2015; Cancemi et al., 2019). MBP-1 is expressed in normal mammary epithelial cells, however, a loss of expression occurs in most primary invasive ductal carcinomas (IDC). In addition, the expression of MBP-1 is inversely related to the expression levels of Erbb2 and Ki67 proteins (Lo Presti et al., 2010), suggesting a direct functional link between MBP-1 and the ERBB2 gene in human breast cancer.

Principal Aims of the research

The main aim of thesis studies is to find MBP-1 new candidate targets to better understand the oncosuppressor activity of MBP-1 protein and their use as prognostic or therapeutic targets.

One of the problems is that no cancer cell model are available that express MBP1, probably because this protein is almost or entirely downregulated in most cancer cell lines. The first aim is to obtain a cell model that expresses MBP-1. To achieve this, SKBr3, MDA-MB231, MCF-7, and HeLa (as reference) cancer cell lines will be transiently transfected with a plasmid for the expression of MBP-1 under the control of a strong promoter like CMV.

Once the transfected cells will be obtained, protein extract will be analysed for transfected MBP-1 expression using Western blot analysis. Moreover, whole genome analyses of the RNA will be performed using the Agilent Micro Array platforms to better understand how gene expression changes in the presence of MBP-1 protein. This solution presents some limits:

i) transient transfection is an appropriate technique to study localization, interactions, and other characteristics of transfected proteins;

ii) efficiency, max. 60-70% of transfected cells for some cell types;

iii) analyses beyond 48-72 hours from the transfection.

These limits do not allow the study of phenomena like inductions or inhibitions of small magnitude that could be significant in cellular homeostasis or kinetic experiments to check a protein's stability, turnover or interaction. For these reasons, the second aim will be the attempt to obtain tumor cell lines with conditionally expression of MBP-1 by using the Clontech Tet-On®3G Inducible Expression System.

There are two crucial elements in the Tet-On 3G system: i) the Tet-On 3G Transactivator Protein and ii) the inducible PTRE3G promoter. When these two elements are combined in a mammalian cell, the expression of cloned gene cloned under the PTRE3G promote it is modulated by exposure to different concentration of Doxycycline.

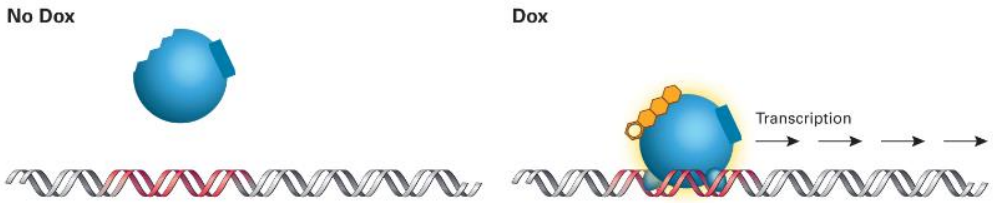


Figure 10. Diagram of the operation of the Tet-On® 3G InducibleExpression Systems (Tet-On® 3G InducibleExpression Systems User Manual).

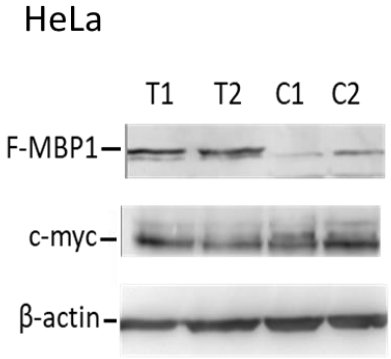
RESULTS AND DISCUSSION

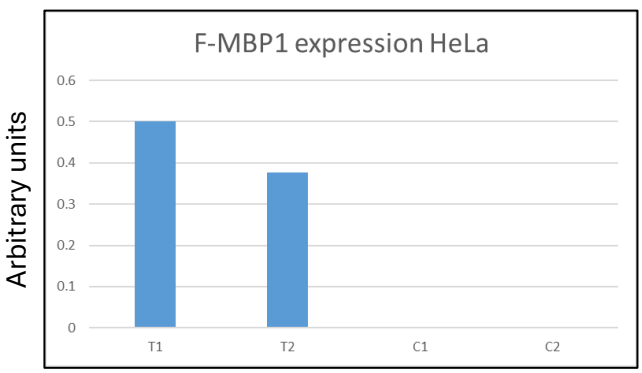
MBP-1 transient transfection in cancer cells

For transient transfection experiments, we decided to work with four different cell types:

- **SKBr3** is a human breast cancer cell line with amplified ERBB2 and MYC genes. SKBr3 cells are used as a model of tumors of the ErbB2-positive molecular subtype, characterized by high expression of ErbB2 and negative for the estrogen receptor (ER) and progesterone receptor (PR).
- **MCF-7** is a cell line derived from a human breast adenocarcinoma with characteristics of differentiated breast epithelium. MCF-7 cells are representative of the molecular subtype called Luminal A and are characterized by being negative for the expression of ERBB2 and positive for the expression of ER and PR receptors.
- **MDA-MB-231** is a cell line derived from a highly aggressive human breast adenocarcinoma characterized by the lack of expression of the ER, PR and ERBB2 receptors, by the over-expression of the EGF receptor (EGFR) and by the presence of a mutated form of p53. This line is representative of the molecular subtype of Basal-like tumos and so-called triple-negative breast tumors (TNBC).
- **HeLa** is a cell line derived from an ovarian cancer that is a standard reference for genomic studies due to the several report and analyses freely available.

Transient transfection experiments on cell lines have been performed using the plasmid pFlag-MBP1 that encodes the protein MBP-1 fused with the FLAG tag from Sigma. Two independent transfections for each cell line were performed.





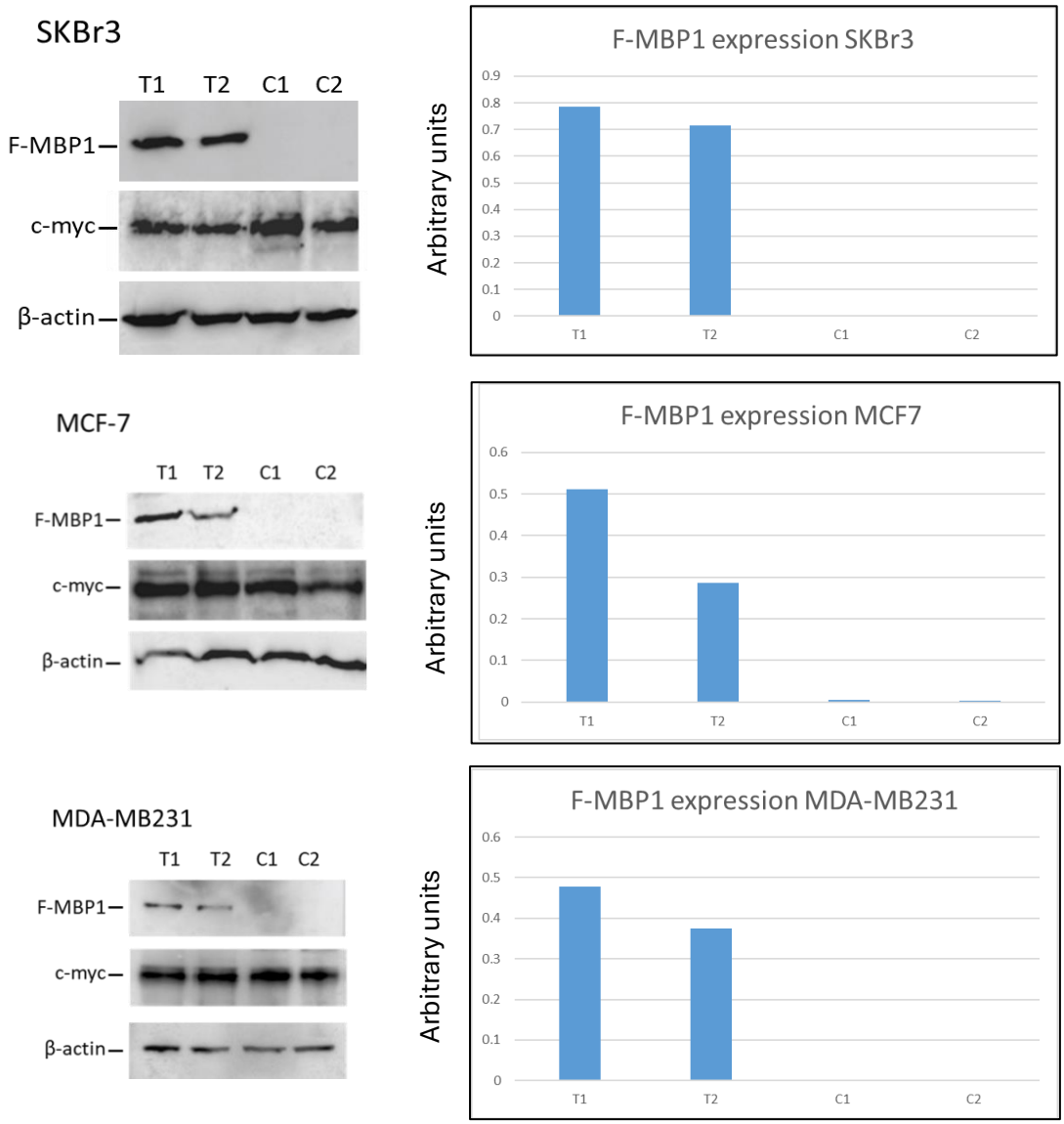


Figure 11. Western blot analyses the different cell lines, and the graph illustrates the relative band density for the different proteins analysed. Anti-FLAG (F3165, Sigma, St. Louis, MO, USA), anti-c-myc (#18583, Cell Signaling,) and anti-β-Actin (sc-47778, Santa Cruz, Dallas, TX, USA) were used as primary antibody for the detection.

After transfection and Western blot analyses, as described in material and methods, we observed a good expression of the Flagged-MBP1 (F-MBP1) in all transfections with the pFlag-MBP1 (T1 and T2 in figure 11) comparted to control transfection (C1 and C2 in figure 11). Westerns with anti-c-myc antibodies show that the best downregulation of the gene was obtained in the SKBr3 cells, that contain an amplified c-myc gene. For this reason, this cell line has been chosen for stable transfection together with HeLa cell line, chosen as standard reference.

These two cell lines were initially transfected with the plasmid pEF1α-TET3G, which encodes the transactivating protein TET3G. This plasmid also includes a gene for the resistance to geneticin (G418) to facilitate the selection of stably transfected cells.

Several clones were obtained from both cell lines, and they were screened by Western blot analysis for the expression of TET3G (figure 12 and 13).

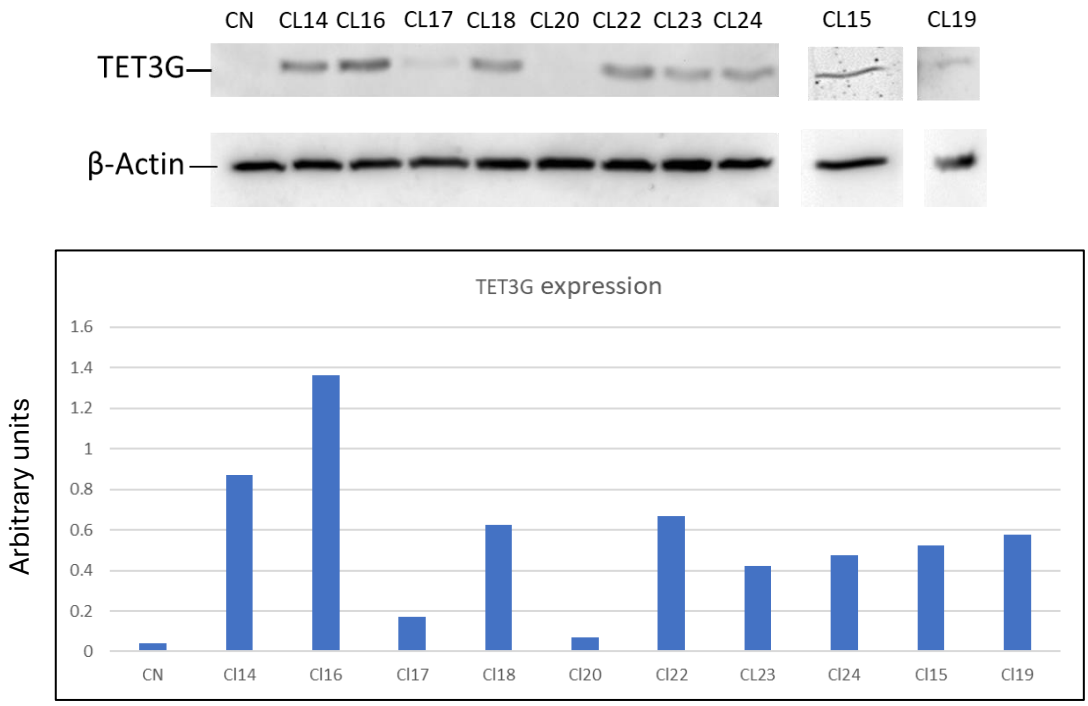
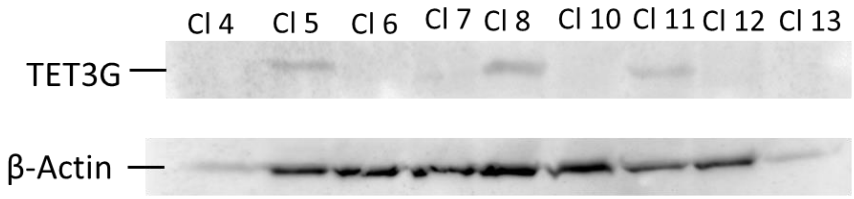


Figure 12. Western blot analyses for the expression of TET3G Transctivator protein, and graph illustrating the relative band density for the different selected clones of SKBR3.



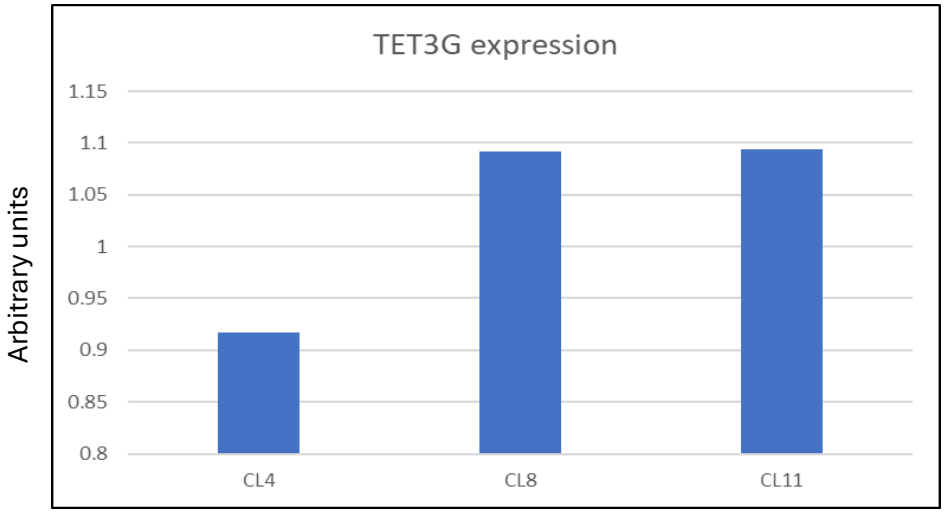


Figure 13. Western blot analyses for the expression of TET3G Transctivator protein, and graph illustrating the relative band density for the different closes selected from HeLa

Many colonies expressed the TET3G protein. Clone Cl8 from HeLa cell line and clone Cl16 from SKBr3 were selected for further experiments due to their higher expression levels of the transactivator protein.

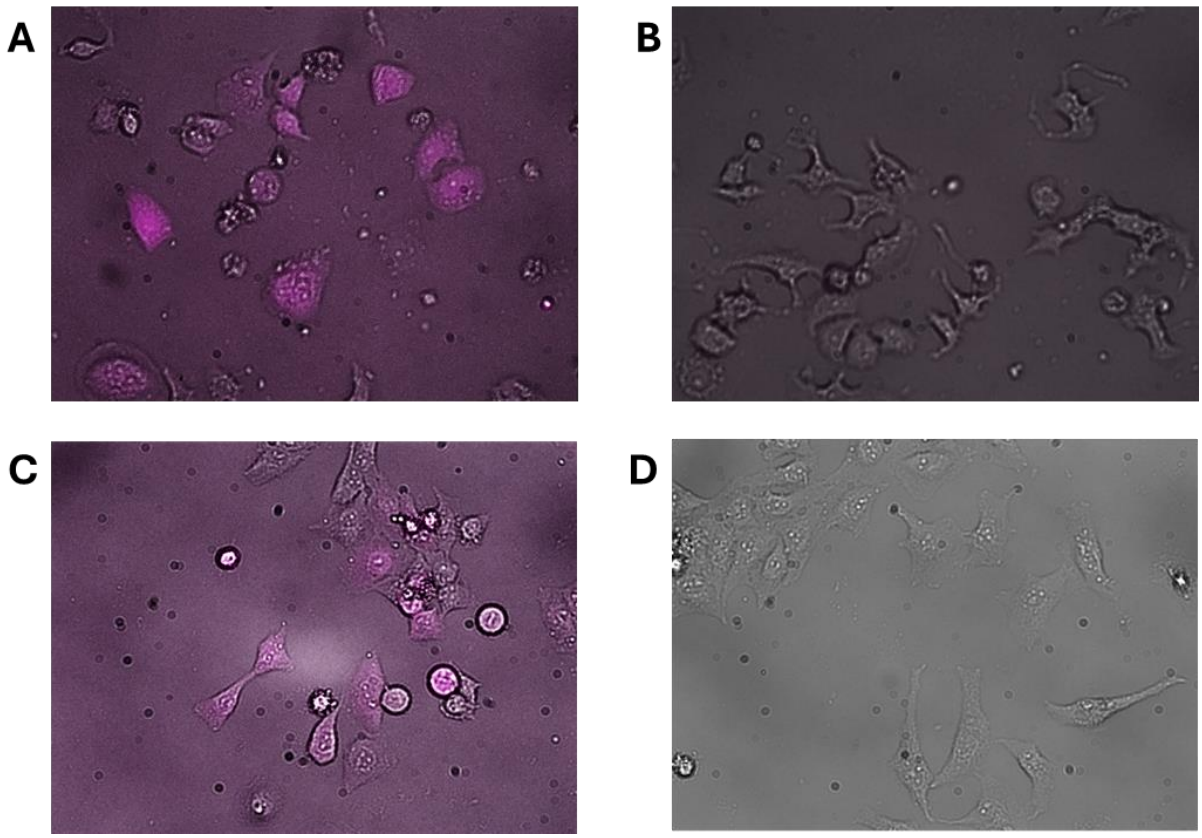


Figure 14. Fluorescent microphotography for the expression of mCherry in cell transfected wih pmC-F-MBP1 induced with Doxycycline (A) SKBR3 Cl16 induced, (B) SKBR3 Cl16 not induced, (C) HeLa CL8 induced, (D) HeLa CL8 not induced

Before proceeding to the second stable transfection, a transient transfection was performed to validate the system's functionality. HeLa Cl8 and SKBr3 Cl16 cells were transfected, as described in material and methods, with the plasmid pmC-F-MBP1. This plasmid contains a bidirectional promoter, which, under the control of doxycycline, drives the transcription of both F-MBP1 and the reporter gene mCherry.

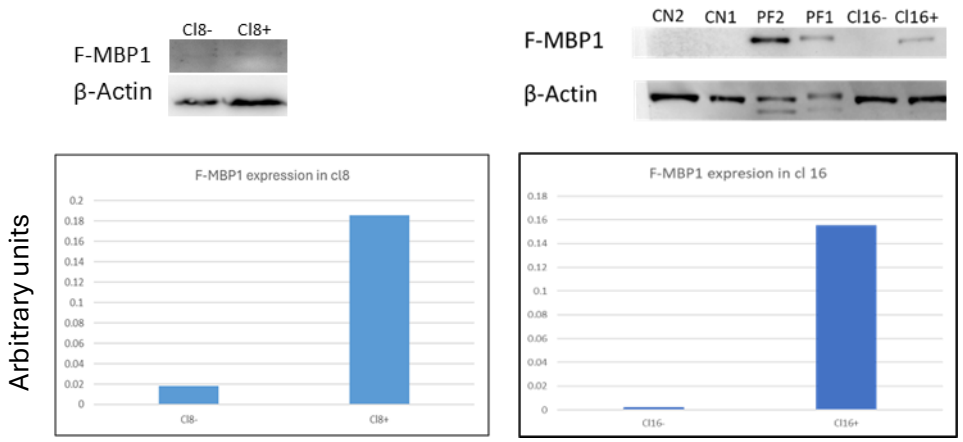


Figure 15. Western blot analyses for the expression of F-MBP1, and graph illustrating the relative band density for the different in SKBr3 Cl16 and SKBr3 Cl14 in induced with DOX(+) and non induced with DOX (-) sample.

After transfection the clones were scored, by fluorescence microscopy, for mCherry expression (figure 14) and by Western blot for F-MBP1 expression (figure 15). SKBr3 Cl16 cells show a good expression of mCherry and F-MBP1, whereas the HeLa CL8 cells show a weaker expression of both proteins. The results confirm an inducible expression of the reporter gene mCherry and F-MBP1 in both clones, confirming the system's functionality. Given that the stable transactivation protein lines (SKBr3 Cl16 and HeLa Cl8) effectively induced F-MBP1 and mCherry in transient transfections, stable transfection with the plasmid pmC-F-MBP1 was performed on the SKBr3 Cl16 and HeLa Cl8 cell lines. Since pmC-F-MBP1 lacks a resistance marker, it was co-transfected with a linear DNA fragment containing the puromycin resistance gene, allowing for the selection of stably transfected cells. In the first attempt, no stable lines were obtained for SKBr3 Cl16, while a stably transfected line was established for HeLa Cl8. However, DOX-dependent regulation and expression were lost after several passages in culture.

Newer attempts were performed only on SKBr3 Cl16; this choice was made because these cells gave the best results in transient transfection with PMC-F-MBP1 (figure 15). After selection many puromycin-resistant colonies were initially selected, but a few showed expression for both mCherry and F-MBP1. First of them were the colonies called SKBr3 CL6 DT and SKBr3 CL9 DT, these colonies showed resistance to puromycin and expression for mCherry (figure 16); no expression for F-MBP1 was revealed in Western blot analyses.

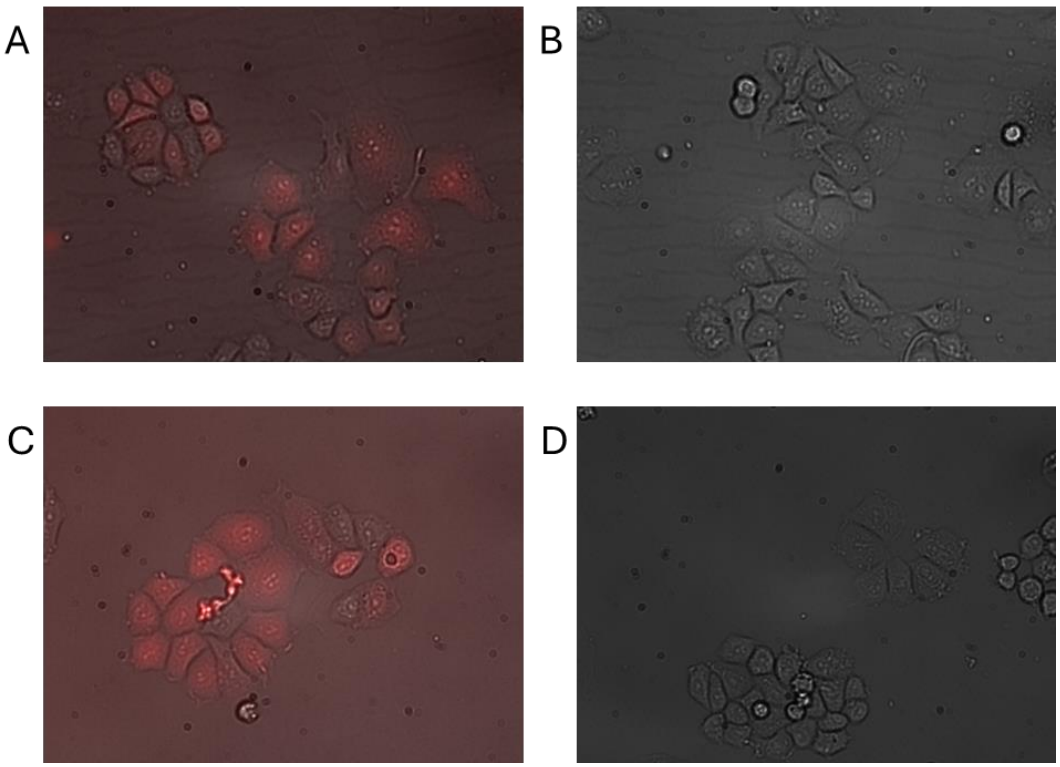


Figure 16. Fluorescent microphotography for the expression of mCherry in cell transfected with pmC-F-MBP1 induced with Doxycycline (A) SKBR3 Cl6 DT induced, (B) SKBR3 Cl6 DT not induced, (C) SKBR3 Cl9 DT induced, (D) SKBR3 Cl9 DT not induced

Several PCRs, with oligos for F-MBP1 were conducted on DNA and RNAs from selected clones to investigate integrity and lack of F-MBP1 expression. The results suggested (not shown) that the plasmid pMC-F-MBP1 likely integrated in CL9 and CL6 clones DNA by disrupting the F-MBP1 sequence, preventing the transcription of the gene. In a final experiment, various colonies geneticin- and puromycin-resistant, expressing mCherry were isolated. However, all the colonies died after induction with DOX. As shown in figure17 the DOX-induced cells exhibited altered morphology, including large vacuoles on their surfaces, and even when grown in DOX-free media, the cells did not survive.

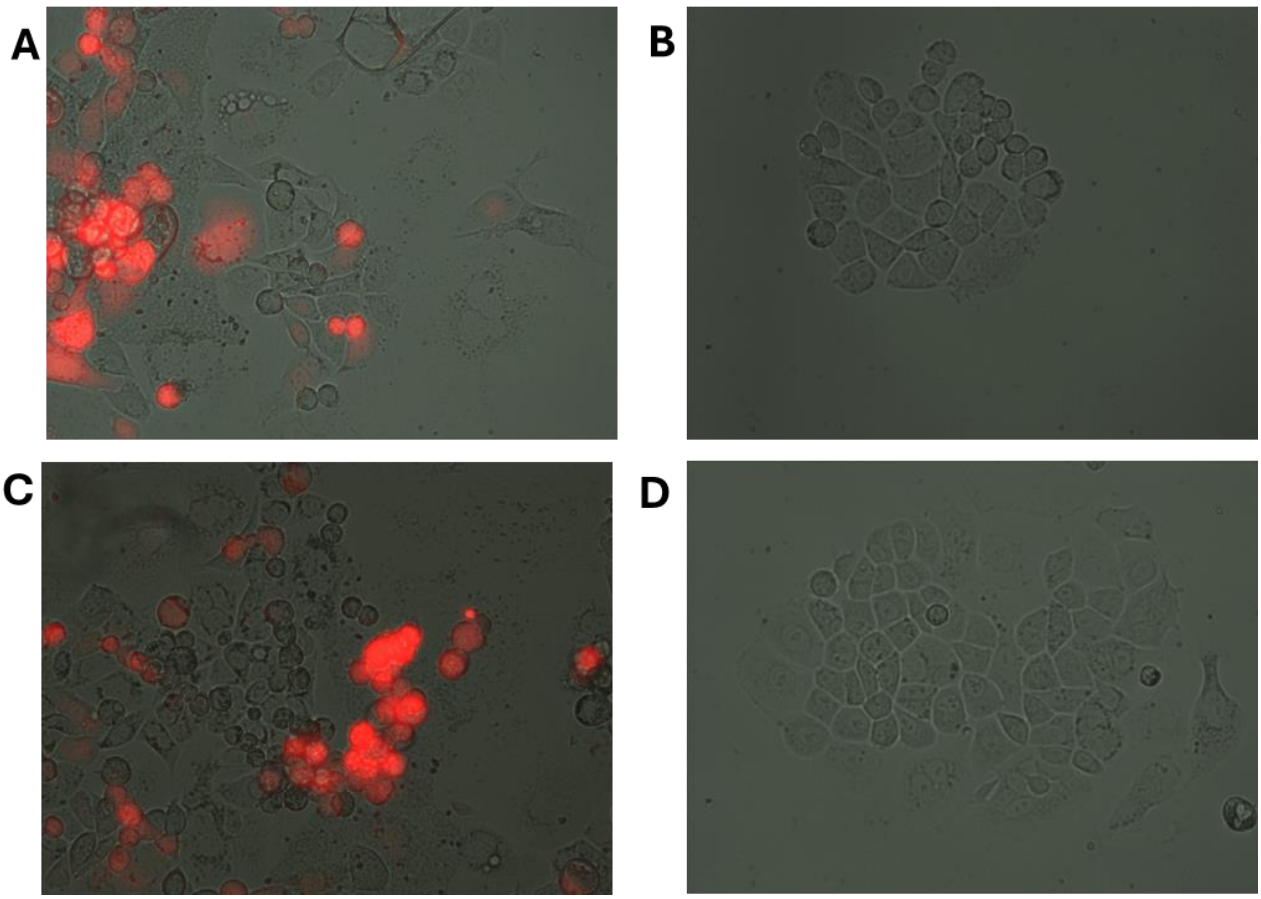


Figure 17. Examples of induced with DOX (A and C) and not induced with DOX (B and D) colonies

All isolated clones died after several passages, making it impossible to expand them sufficiently for Western blot and other analyses. Additionally, the clones exhibited poor growth in culture, likely due to a malfunction in the inducible system. Probably the system failed to completely block the expression of theF-MBP1 gene even without induction. Up to now, just one colony seems to survive. This colony has been called SKBr3 Cl5 DT.

Microarrays Analysis of cancer cells

After filtering on flag to remove all not detected entities present in the arrays, algorithms for the analysis of principal components (PCA) were applied on data obtained from the 4x44 K platform and the 8x60 K platform to reduces dimensions without considering class labels and transform correlated variables into linearly uncorrelated principal components that capture most of the data variance (figure 18). The same data were hierarchical clustered by a Euclidean similarity measure algorithm and Ward's linkage rule with no cluster conditions (figure 19).

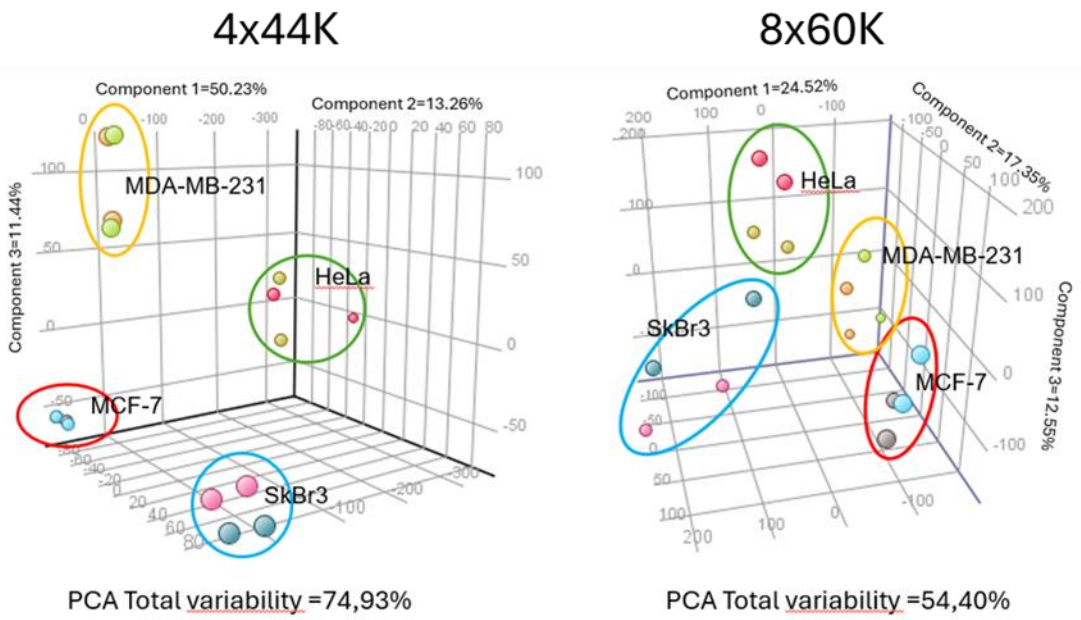


Figure 18. Graphical view of the principal component analysis (PCA) of the flag filtered entities obtained from the 4x44 K array platform (34.043) and the 8x60 K platform (58.112).

In the light of the results obtained from PCA and clustering analyses, we decided to follow two different procedures for further data analysis:

- Identify differentially expressed genes (DEGs) common to the three breast cancer cell lines, excluding HeLa cells as they are very different in terms of global expression profile, and search for common regulatory pathway;
- Analyse each cell line independently by looking at cell line specific DEGs, and search for cell specific functional pathway.

First we did a direct comparison averaging the values obtained from all the three transfected BC cell lines against that of the all control cells (Mock) with p values <0.05 and an FC of ± 2.0. These filters led to the selection of 37 entities corresponding to only 26 annotated differentially expressed genes (DEGs). This low number of DEGs between the two conditions (MBP1+ vs Mock) is probably due to big variability in gene expression between cells, as previously shown by clustering analysis (figure 19). Therefore, we decide to look at common DEGs in all analysed cells using a differ statistic metric using Gene Set Enrichment Analysis (GSEA), as shown afterward.

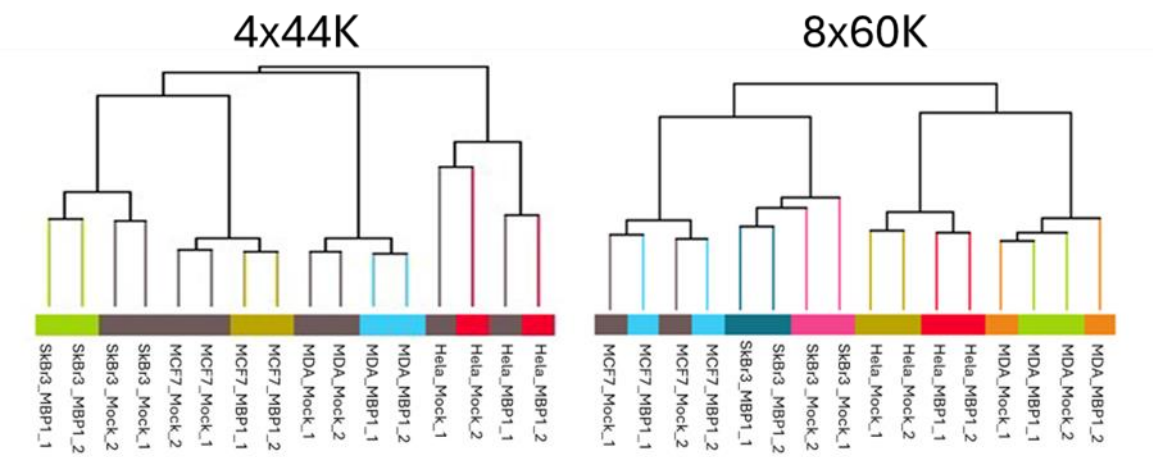


Figure 19. Unsupervised hierarchical clustering on flag filtered entities obtained from the 4x44 K array platform (34.043) and the 8x60 K platform (58.112).

We proceed with the second proposed procedure looking for unique DEGs expressed in each cell line. The most interesting results were obtained looking at DEGs up- and down- regulated in MDA cells after F-MBP1 transfection. The selected genes were further analyzed for significant enrichment of specific biological pathways in GO analysis by Funrich software, as described in material methods.

As shown in figure 20 overexpression of F-MBP1 results in an increase of gene involved in membrane remodeling and cell communication (E-cadherin functions and L1 and L3 functions, respectively).

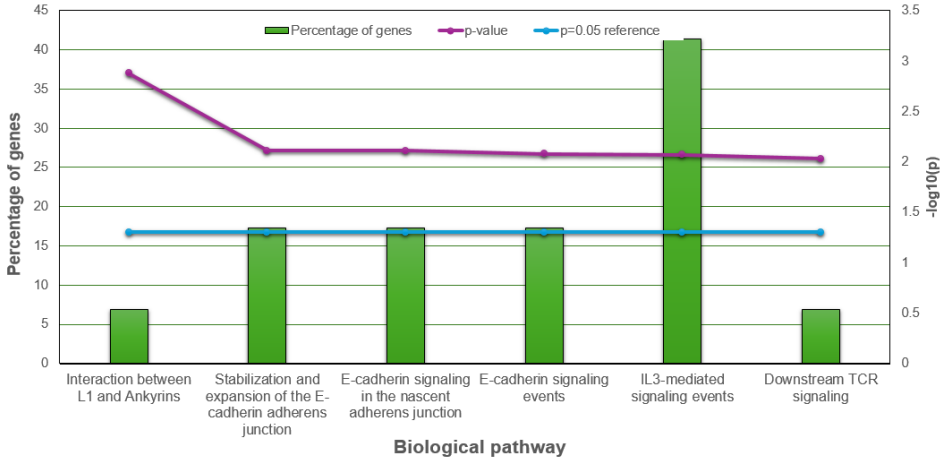


Figure 20. The most enriched biological pathways obtained using DEGs up-regulated in MDA cells upon F-MBP1 transfection.

On the contrary, in figure 21, are indicated the pathway that result down-regulated in F-MBP1-transfected MDA cells. A significant downregulation is observed in DEGs that are involved in EMT and signal transduction (EMT regulation and ATF-2 and PERK networks, respectively).

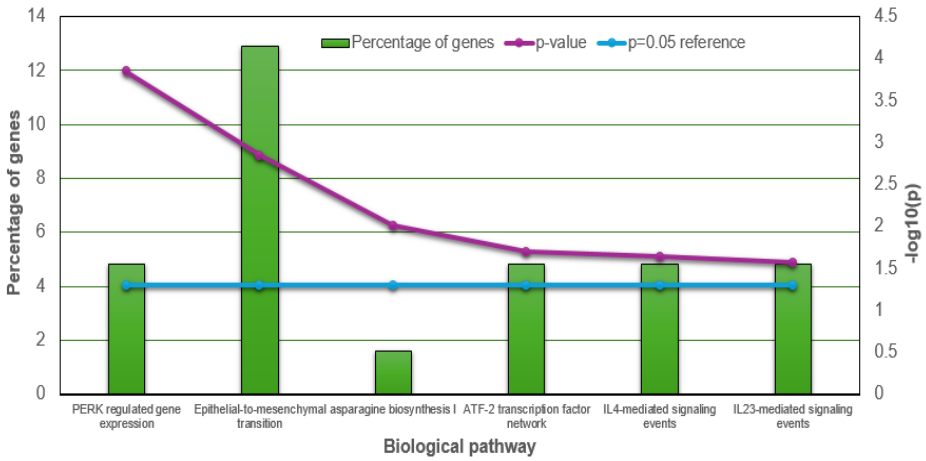


Figure 21. The most enriched biological pathways obtained using DEGs down-regulated in MDA cells upon F-MBP1 transfection.

We selected three genes (GDF15, ARAP2 and SGR2), among the most downregulated genes in MDA, enriched in the EMT biological pathway. The three identified genes were analyzed, with combined probability, for their correlation with prognosis in breast cancer tumors using the Kaplan Meier-plotter (http://kmplot.com/analysis/). An increase in the probability of survival is observed, for low expression of the genes, in TNBC while no significant difference is observed in all subtypes of BC tumors (figure 22).

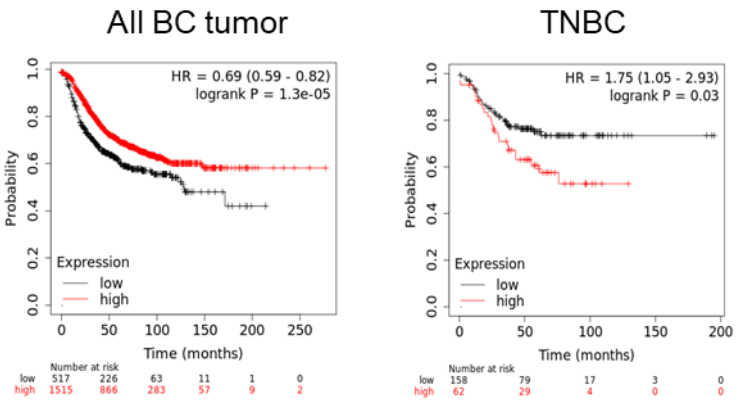


Figure 22. Plot of the results of combined Kaplan Meier analysis with the genes: GDF15, ARAP2 and SGR2 in all subtypes of BC tumors and TNBC.

It has been shown that silencing GDF15 inhibits the proliferation of tumor cells and increases their sensitivity to paclitaxel in vitro and in vivo, whereas the treatment of purified GDF15 protein confers breast cancer cells with chemoresistance ability. Moreover, GDF15 activates protein kinase B (AKT)/mammalian target of rapamycin (mTOR) signalling, inhibition of AKT or mTOR reverses the prosurvival effect of GDF15 and enhances the antitumor efficacy of paclitaxel in TNBC cells (He et al., 2023) AGR2 has a role in relieving endoplasmic reticulum stress, promoting the dissemination of metastatic tumor cells, and stimulating cell survival and proliferation (Salmans et al.,2013).

ARAP2 has been identified as one of the strongest candidate mediators of chemoresponse and chemoresistance in ErbB2-negative tumors (Al Amri et al.,2020.)

Altogether, these studies, suggest a role of MBP-1 in the transcriptional regulation of these genes and in repressing their tumor-promoting activities. Further study on the molecular mechanisms at the basis the transcriptional repression may represent new potential targets for TNBC therapy.

Gene Set Enrichment Analysis (GSEA)

Microarray data were further analyzed with the GSEA bioinformatics software (Subramanian et al., 2014) to identify gene sets significantly up or down regulated in MBP1-expressing cells with respect to control cells (Mock transfected).

GSEA is a computational method that determines if a priori defined set of genes indicates statistically significant differences between two phenotypes. In GSEA, genes are ranked by their correlation with phenotype and every enrichment gene set will get an enrichment score (ES). After collapsing the entities to gene symbols and ranking for each contrasted phenotype (MBP1+ vs MOCK) significant differentially expressed genes (figure 22), analysis was done to identify significative gene enrichment in GSEA Halmarks geneset database (https://www.gsea-msigdb.org/gsea/msigdb/human/collections.jsp#C2).

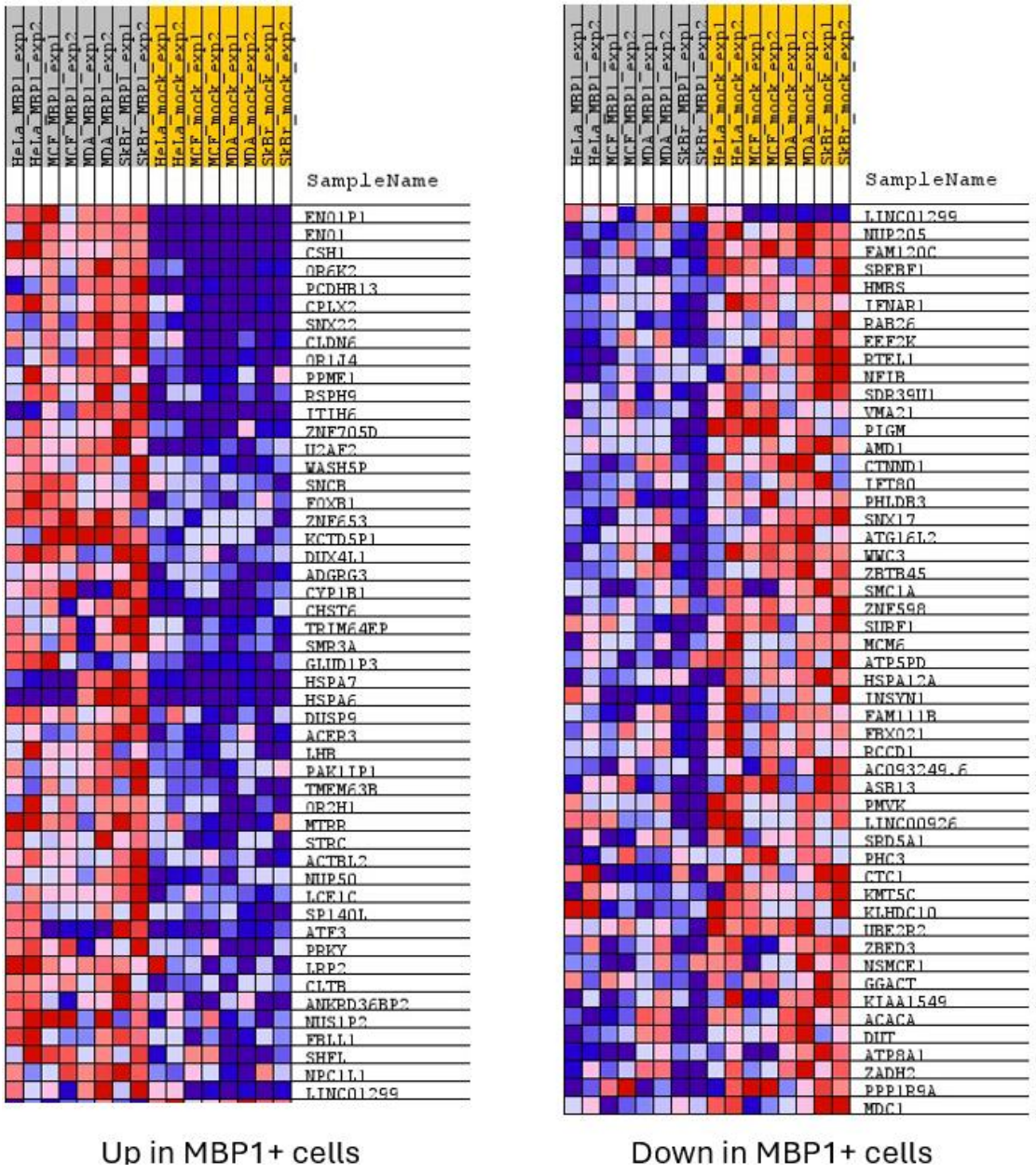


Figure 23. Heat Map of the top 50 DEGs collapsed to gene symbols after the ranking for each contrasted phenotype (MBP1+ vs MOCK).

This analysis revealed a pattern of pathways and networks being up- or down-regulated in all four MBP1 overexpressing cell lines compared to control cells (Mock transfected). Table 2 shows the most significant (FDR q-value < 0.25) up- and down- regulated gene sets.

In figure 23 are shown same representative enrichment plots of significant enriched gene sets shared by the four MBP1-transfected cell lines. Apoptosis and_Apical_Junction gene sets were positively correlated (ES = 0.41 and 0.27 respectively) with the MBP1 expression. Conversely, the Mitotic_Spindle and E2F_Targets gene sets were negatively correlated (ES = -0.38 and -0.39 respectively) with the control (Mock transfected) cells. It is noteworthy that on top of the upregulated genes are present ENOP1 and ENO1 genes. This confirms that in all transfected cell lines there was a significant increase of signal from probes for ENO1 and ENO1P1, in the arrays, that cross hybridize with the transfected F-MBP1 RNA, validating the transient transfection efficiency.

Table 2

UP -regulated Hallmarks genesets	SIZE	ES	NES	p-Value	FDR q-value
APOPTOSIS	155	0.41000	0.00000	0.00000	0.00176
TGF_BETA_SIGNALING	49	0.47000	0.00000	0.00000	0.00197
REACTIVE_OXYGEN_SPECIES_PATHWAY	48	0.47000	0.04587	0.00000	0.08194
HYPOXIA	187	0.39000	0.01375	0.04587	0.10059
KRAS_SIGNALING_UP	195	0.31000	0.02852	0.01375	0.11437
P53_PATHWAY	190	0.33000	0.03604	0.02852	0.12994
APICAL_JUNCTION	188	0.27000	0.06100	0.03604	0.14013
UV_RESPONSE_UP	150	0.31000	0.03346	0.06100	0.14493
IL2_STAT5_SIGNALING	189	0.25000	0.04174	0.03346	0.15041
MYC_TARGETS_V2	56	0.44000	1.04000	0.04174	0.15984

DOWN-regulated Hallmarks genesets	SIZE	ES	NES	NOM p-val	FDR q-val
MITOTIC_SPINDLE	191	-0.38437	-1.08030	0.00756	0.11385
OXIDATIVE_PHOSPHORYLATION	190	-0.39595	-1.06144	0.03404	0.11548
BILE_ACID_METABOLISM	106	-0.23469	-1.02758	0.01240	0.13554
ESTROGEN_RESPONSE_EARLY	188	-0.31995	-1.02216	0.00148	0.14623
E2F_TARGETS	193	-0.39296	-1.01499	0.03236	0.16717
G2M_CHECKPOINT	193	-0.34716	-0.87783	0.03153	0.16950
WNT_BETA_CATENIN_SIGNALING	39	-0.29046	-0.87190	0.02413	0.16991
NOTCH_SIGNALING	30	-0.28826	-0.84545	0.12477	0.17682
ESTROGEN_RESPONSE_LATE	190	-0.26157	-0.82244	0.05247	0.17737
PI3K_AKT_MTOR_SIGNALING	98	-0.23010	-0.81165	0.04618	0.18309
EPITHELIAL_MESENCHYMAL_TRANSITION	195	0.24000	0.82000	0.04481	0.09120

It is interesting to observe that repeating the analysis using the “Only Breast Hallmarks” genesets (data not shown) we found enriched genes in similar genesets (Up regulated:

Myc_Target_V2, Apoptosis and down regulated Mitotic_Spindle and EMT). These observations substantiate the close correlation in breast cancer between the expression of MBP1 and the up regulation of genes mainly linked to Apoptosis and the increase to cellular stress such as the UV_response and to p53 and KRAS signaling pathways. Similarly, the down regulation of gene involved in cell proliferation, like Mitotic_Spindle and E2F_Targets, and EMT, were observed.

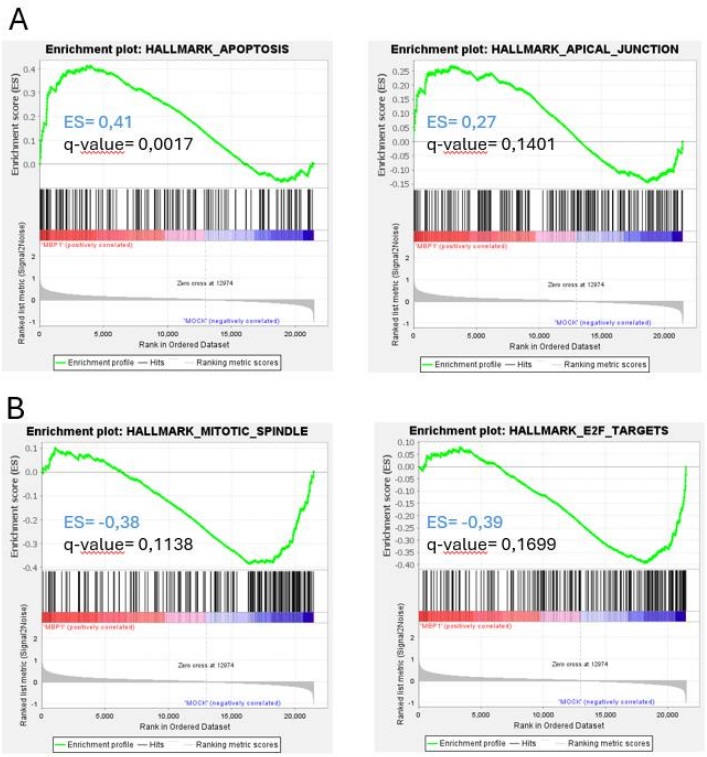


Figure 24. Representative enrichment plots of significant enriched gene sets shared by the three MBP1 transfected cell line: MCF7, MDA and SKBr3. The y-axis shows the enrichment scores (ES) and the x-axis shows gene members for the gene sets. The green line connects points between ES and genes. ES represent the amount to which a gene is over-represented in a gene set. The colored bands represent positive (red) or negative (blue) degrees of correlation of genes with the MBP1-positive phenotype. Significance threshold set at q-value <0.25.

All the data, together, indicate a complex and pleiotropic effect of MBP1 in different cellular functions. The enrichment of some gene sets may be the consequence of the primary activity of MBP1 on repressing MYC and ErbB2 in breast cancer (MYC_Target_V2, Apoptosis, PI3K_AKT_MTOR_Signaling, Mitotic _Spindle). On the other end we have identified gene sets that for their function are not correlated to MYC or ERBB2 activity and can be direct target of MBP1 transcriptional repression.

Among the gene sets down-regulated in the cells transfected with MBP1, the genes enriched in the (EMT) gene sets were scored for their prognostic value.

Gene expression-based studies have identified at least six different triple- negative breast cancer (TNBC) molecular subtypes (Harbeck er al., 2019).

Our study provided several candidate genes, including COL5A1, GDF15 and SCGB2A1 that might be clinically relevant to prognostic evaluation and to the personalized treatment of TNBC.

As shown in figure 25, The expression of the three genes is downregulated in the cells transfected with F-MBP1 particularly in MDA cells, representative of the TNBC tumor subclass.

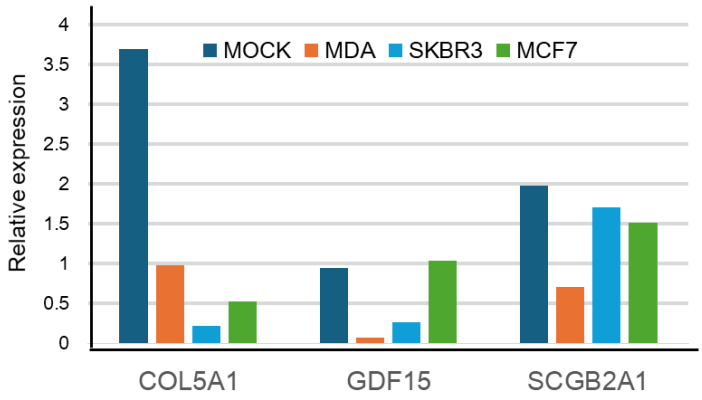


Figure 25. Relative expression from the microarrays data analysis of three genes in control (Mock) and F-MBP1 transfected cells.

The three identified genes were analyzed for they correlation with prognosis in breast cancer tumors using the Kaplan Meier-plotter (http://kmplot.com/analysis/). An increase in the probability of survival is observed, for low expression of all three genes, in TNBC while no difference is observed in all subtypes of BC tumors.

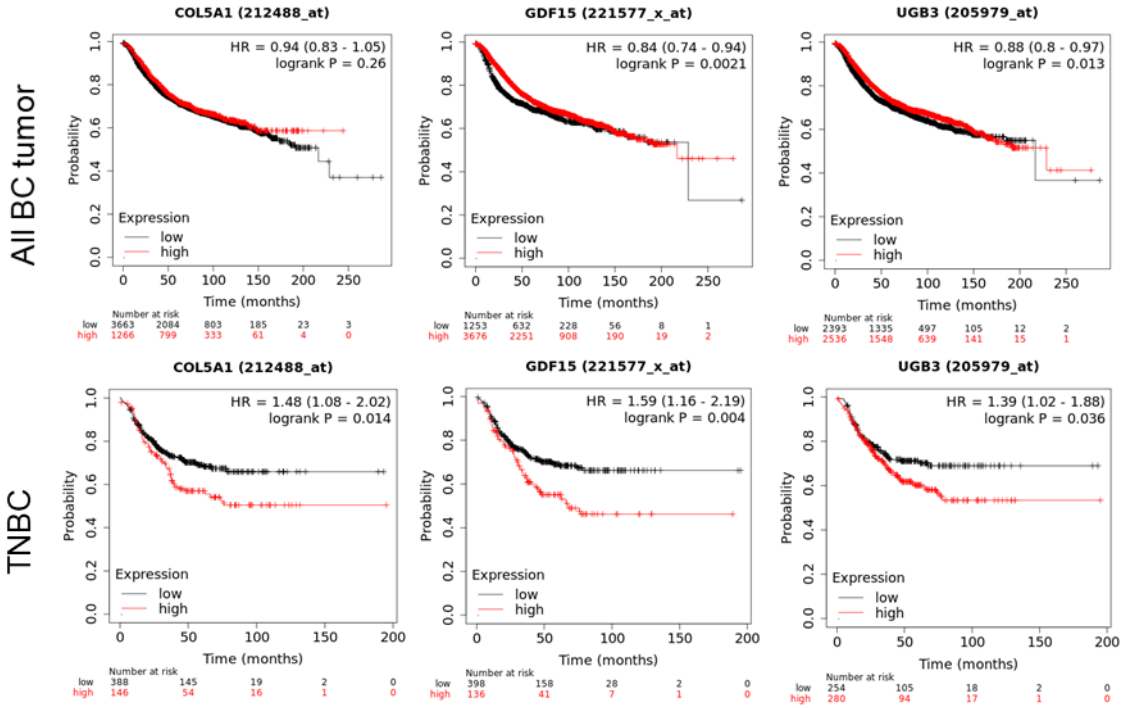


Figure 26. Plot of the results of Kaplan Meier analysis with the genes: COL5A1 GDF15, and UGB3 in all subtypes of BC tumors and TNBC.

CONCLUSION

The presented data, all together, indicate a complex and pleiotropic effect of MBP1 in different cellular functions. The enrichment of some genesets may be the consequence of the primary activity of MBP1 on repressing MYC and ErbB2 in breast cancer (MYC_Target_V2, Apoptosis, PI3K_AKT_MTOR_Signaling, Mitotic_Spindle).

On the other end we have identified gene sets that for their functions are not correlated to MYC or ERBB2 activities and can be direct targets of MBP1 transcriptional repression.

Gene expression based studies have identified at least six different TNBC molecular subtypes (Harbeck er al., 2019), our study provide several candidate genes, that might be clinically relevant to prognostic evaluation and to the personalized treatment of TNBC.

MATERIALS AND METHODS

Cell culture

SKBr3, MCF-7, MDA-MB231 and HeLa cell lines were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). All cell lines were cultured in DMEM high glucose medium (Corning, Corning, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Corning, Corning, NY, USA) and 1% Penicillin Streptomycin.

Plasmid construction

The Flag-MBP1 (pF-MBP1) plasmid, expressing MBP- fused in frame at the NH2-Terminus with the FLAG epitope, was generated by PCR with appropriate oligonucleotides by amplification of the Δ-NH2 (aa 97–433) enolase coding sequence, using as a template the α-enolase cDNA clone pH48 (Giallongo et al., 1986). The PCR fragment was digested with HindIII and XbaI enzymes and cloned in the same sites of pFlag-CMV-2 vector from Sigma, as previously described (Perconti et al., 2007).

The pCMV-Tet3G (Catalog No.631335) and pTRE3G-BI-mCherry (No. 631333,), were purchased by Clontech (Clontech Laboratories, Mountain View, CA, USA). The pMC-F-MBP1 expression plasmid was obtained by PCR with appropriate oligonucleotides, from the previously described pF-MBP1 plasmid, and cloned after digestion with AvaI and BamHI enzymes, in the AvaI and BamHI sites present in the multiple cloning site of pTRE3G-BI-mCherry vector (No. 631333, Clontech Laboratories, Mountain View, CA, USA).

All the expression constructs were sequenced to verify the correct sequence and open reading frames.

Transient cell transfection

SKBr3, MCF-7, MDA-MB231 and HeLa cells were transfected with Lipofectamine 2000 (MCF-7, MDA-MB231 and HeLa) and Lipofectamine 3000 (SKBr3) reagents in OptiMem Medium as instructed by the manufactured (Invitrogen). $4x10^5$ cells were plated into two six wells plates, one for RNA extraction and one for protein extraction, and transfected (in

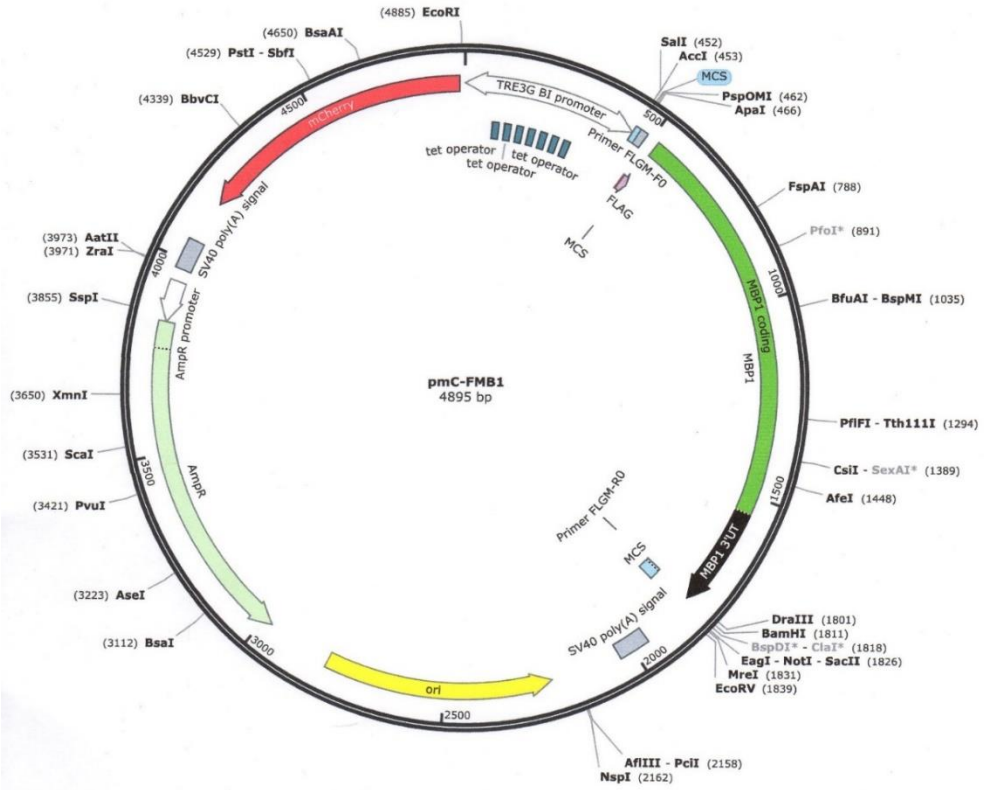


Figure 27. Plasmid Diagram pmC-F-MBP1.

duplicate) with pFlag-MBP1 (5µg) and pFlag-CMV-2 empty plasmid (5µg), as shown in figure 28. Protein and RNA extracts were prepared 48 hrs after transfection. An aliquot of the transfected cell extracts was used for Whole Genome and Western blot analyses with anti-Flag, c-myc, and β-actin antibodies.

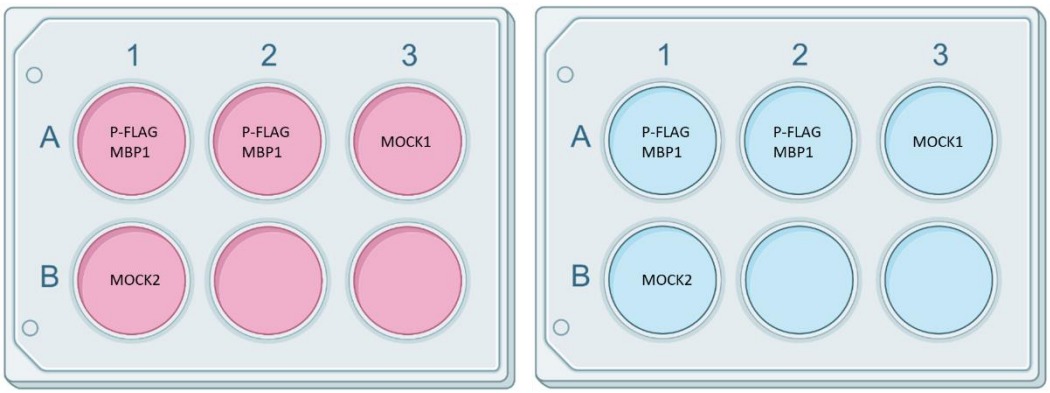


Figure 28. Experimental set for transient transfection

Stable cell transfection

Stable transfection with Clontech Tet-On®3G Inducible Expression System is a two-step transfection:

1)First Transfection

SKBr3 and HeLa cells were transfected using Xfect Transfection Reagent as instructed by the manufacturer (Takara). Clontech Tet-On ® 3G Inducible Expression System was used. $4x10^5$ cells were plated in a single well of a six wells, 24h after plating, the cells were transfected using **pEF1α-TET3G;** this plasmid contains the sequences for the expression of the transactivation protein (TET-On 3G) and the resistance to Geneticin. 48 h after transfection, cells were propagated into four 100mm dish plates. After 48 h, a dose of 600 µg/ml of Geneticin (Gibco, Cat n.101310-27) was added to select positive colonies.

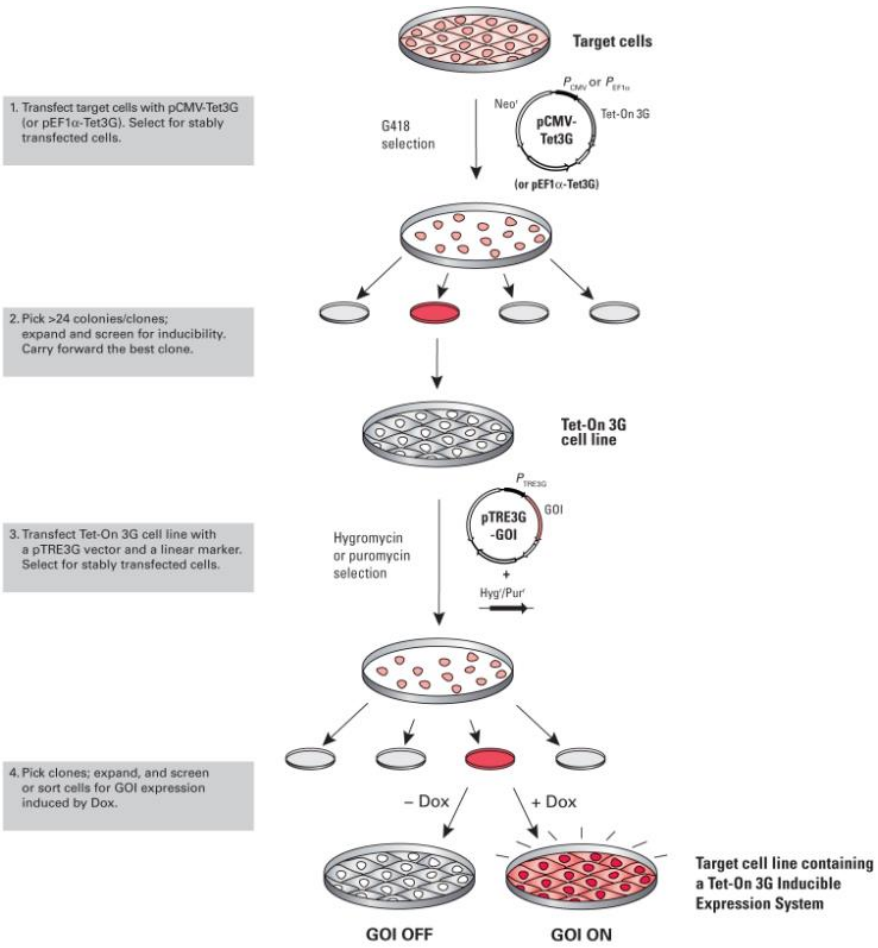


Figure 29. Workflow for stable transfection using Clontech Tet-On ® 3G Inducible expression system.

After selection, positive colonies were scanned by Western blot analysis for the expression of TET-On 3G protein using specific antibodies against this protein TetR Monoclonal Antibody (Clone 9G9). The best colonies have been chosen for the second transfection.

Transient transfection on selected Cells

Before moving to the second transfection, to verify if the system works correctly, selected cells (SKBr3) were transfected with Lipofectamine 3000 reagents in OptiMem Medium as instructed by the manufacturer (Invitrogen). $4x10^5$ cells were plated in six-well plates and transfected using PmC-F-MBP1 (3µg) plasmid. 48h after transfection, induction for the expression of F-MBP1 and mCherry was performed using one µg/ml of DOXOCYCLINE for 48h; the cells were scanned for the expression of the mCherry reporter protein by fluorescence microscopy and the expression of F-MBP1 by Western blot analysis using antibodies against the FLAG.

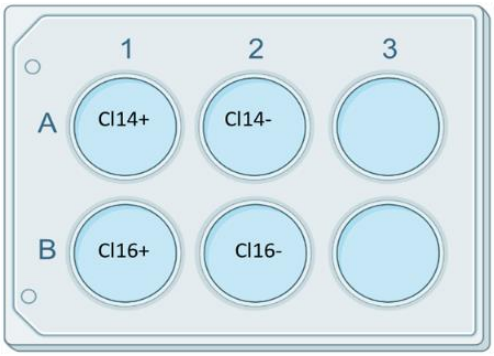


Figure 30. Example of experimental set for transient transfection on selected cell. For each colonies an induced and non-induced sample has been collected.

2)Second Transfection

Selected cells (SKBr3) were co-transfected using Xfect transfection reagent as instructed by the manufacturer (Clontech). Into a single well of a 24 wells plate $1x10^5$ cells were plated. After 24h from plating cells were co-transfected with PmC-F-MBP1 plasmid (2,5 µg/well) and Linear Hygromycin/Puromycin Marker (Clontech) in 1:20 molar ratio to PmC-F-MBP1. After 48h, the cells were propagated into two 100 mm plates and a dose of 600 µg/ml of Geneticin (Gibco, Cat n.101310-27) and 0.3 µg/ml of Puromycin (sigma) were added, 48h later to select for positive transfected colonies. Once the colonies were selected and expanded, induction for the expression of F-MBP1 and mCherry proteins was performed incubating with 1.0 µg/ml of Doxocicline (Clontech). After 24h cells were scored for the expression of the mCherry reporter

protein by fluorescence microscopy and for the expression of F-MBP1 protein by Western blot analysis using antibodies against the FLAG.

Western blot analysis

Total cell proteins from transfected cells were extracted using RIPA lysis buffer (50 mM TrisHCl pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) and protease inhibitor mixture (P2714, Sigma, St. Louis, MO, USA,). The extract protein concentration was determined by the Bradford microassay method (Sigma) using bovine serum albumin as a standard (Bradford MM., 1976) on a D30 Biophotometer (Eppendorf). From 25 to 40 µg of whole-cell protein extracts were electroforetically separated in a 10% SDS–polyacrylamide gel and electroblotted to a ECL nitrocellulose membrane (Thermo Fisher, Waltham, MA, USA). After blocking for 1h in 5% nonfat dry milk in TBST buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.2% Tween 20) the membranes were incubated overnight with mouse monoclonal anti-TetR (clone 9G9, Clontech), anti-FLAG (F3165, Sigma, St. Louis, MO, USA), anti-c-myc (#18583, Cell Signaling,) and anti-β-Actin (sc-47778, Santa Cruz, Dallas, TX, USA). Peroxidase conjugated anti-rabbit (W401B) or anti-mouse (W402B, Promega, Fitchburg, WI, USA) were used as secondary antibodies. The antigen-antibody complexes were visualized by chemiluminescence using the SuperSignal™ West Femto Maximum Sensitivity substrate (Thermo Fisher, Waltham, MA, USA) on a Chemidoc XSR image system and analysed with the Quantity One software (BioRad Laboratories, Hercules, CA USA). Quantification of the chemiluminescent signals was performed with the ImageJ software (https://imagej.net/ij/) using the β-actin signals to normalize the samples.

Lipofectamine 2000 was used as a transfection reagent for MDA-MB231 and MCF-7 while Lipofectamine 3000 was used as a transfection reagent for SKBR3. To identify the F-MBP1 by Western blot, an anti-Flag (1:3000, Mouse MAbs F3165 SIGMA) has been used as primary Ab and an anti-Mouse-HRP (1:3000, W-4028 Promega) as secondary Ab. For c-myc anti-c-myc (1:1000, in Rabbit, Cell Signaling #18583) has been used as primary Ab and anti-Rabbi-HRP (1:3000, W-4018 Promega) as secondary Ab. For β-actin anti-β-actin (1:1000 in Mouse sc-47778) as primary Ab and Anti-Mouse-HRP (1:3000, W-4028 Promega) as secondary Ab.

To identify the TET3G, anti-TET3G (1:1000, in Mouse Clone9G9 Takara) has been used as primary and anti-Mouse-HRP (1:3000, W-4028 Promega) as secondary Ab. β-actin was detected as previously described.

MicroArray Analysis

Total RNA from transfected cells was extracted on six-well plates, using 500 µl of TRIzol per well as instructed by the manufacturer (Invitrogen). RNA quality and integrity (RIN, RNA integrity Number) were performed using Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA) with the RNA 6000 nano chip kit (Agilent, Santa Clara, CA, USA). Only samples with a RIN >8 were used for subsequent analyses. All samples had two independent biological replicates, and each replicate was labelled and hybridized with two colour protocol (for a total of four replicate for sample). Cyanine-3 (Cy3) or Cyanine-5 (Cy5) labelled cRNA was prepared from total RNA using the Low Input QuickAmp Labelling Kit according to the manufacturer's instructions (Agilent, Santa Clara, CA, USA). Dye incorporation and cRNA yield were checked with the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Hybridization and washing were performed using the in-situ Hybridization Plus Kit following the manufacturer's instructions (Agilent protocol: Gene Expression Two Colour ver. 6.9.1, August 2015). Briefly, labelled cRNAs were purified using the RNeasy RNA isolation kit (Qiagen, Hilden, Germany) and 300 ng of Cy3- or Cy5-labeled cRNA for 8x60K slide and 825 ng of Cy3- or Cy5-labeled cRNA for 4x44K slide (specific activity > 9.0 pmol Cy/µg cRNA) was fragmented at 60°C for 30 min in a reaction volume of 25 µl (8x60K slide) or 55 µl (4x44K slide) containing 1× fragmentation buffer and 2× blocking agent. On completion of the fragmentation reaction, 25 µl (8x60K slide) or 55 µl (4x44K slide) of 2× hybridization buffer was added to the mixture and hybridized to Whole Human Genome Microarray 8x60K (Agilent G4851C) or 4x44K (Agilent-G4845A) for 17 h at 65°C in a rotating hybridization oven. After hybridization, microarrays were washed 1 min. at room temperature with Agilent wash buffer 1, another minute at 37°C with Agilent wash buffer 2 and then dried immediately. Slides were scanned on the Agilent DNA Microarray Scanner (G5761A) using two colour scan setting for 8x60K array (slide area 61 × 21.6 mm, scan resolution 3 µm, dye channels PMT set to 100%) and 4x44K array (slides area 61 × 21.6 mm, Scan resolution 5 µm, dye channels PMT set to 100%,). The scanned images were analysed with Feature Extraction Software 11.5.1.1 (Agilent, Santa Clara, CA, USA), using default parameters (protocol: GE2-v5_95_Feb07 and Grid: 072363_D_F_20150612) to obtain background subtracted, dye normalized and spatially detrended processed signal intensities.

Gene expression profiling and data analysis

Statistical data analysis, background correction, normalization and summary of expression measure were conducted with GeneSpring GX ver. 14.9.1 (Agilent, Santa Clara, CA, USA). Data were filtered using two steps procedure: first the entities were filtered based on their flag values P (present) and M (marginal) and then scored for their signal intensity values. Statistically significant differences were computed by oneway Anova or moderate t-test, for pairwise comparisons, and the significance level was set at $p<0.05$. Benjamini-Hochberg (FDR) was applied as multiple test correction method. Unsupervised hierarchical clustering was performed using the Euclidean distance and Ward's linkage method. Differentially expressed genes (DEGs) were selected by a supervised approach with a contrast fold change of at least ±1.5 and an FDR corrected p-value <0.05 was used to perform multiple pairwise comparisons between each class (FMBP-1 expressing SKBr3, MCF-7, MDA-MB231 and HeLa cells) and the control (Mock transfected). To Translate gene lists into biological insights DEGs common to all cell lines or in a single cell line were subjected to GeneOntology (GO) and biological pathway enrichment analysis using FunRich tool (http://www.funrich.org/) against human FunRich background database and/or using the WEB-based Gene Set Analysis Toolkit (https://www.webgestalt.org/) against the human Agilent wholegenome database.

Gene set enrichment analysis (GSEA)

Normalized expression data for all the probesets obtained by microarray analysis of SKBr3, MCF-7, MDA-MB231 and HeLa cells transfected with FMBP-1 or controls (Mock) plasmids were further analysed by using the Gene Set Enrichment Analysis (GSEA) tool (Subramanian et al.,2014). One thousand gene permutations were used to generate a null distribution for ES, and give to each gene set/pathway a normalization enrichment score (NES). H:Hallmark, and C5:BP:GO biological process (including more than 7000 different genesets) were scored as genesets database (https://www.gsea-msigdb.org/gsea/msigdb/). Genesets were considered significantly enriched with $q\text{-value}<0.25$.

Statistical analysis

Unpaired two-tailed student's t-test for comparison of two independent groups were performed with GraphPad software (GraphPad Software Inc., La Jolla, CA, USA). Quantitative data are represented as the mean ± standard deviation or SEM of at least three independent experiments.

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