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C-MYC PROMOTER BINDING-PROTEIN 1 (MBP-1) AS A NOVEL MARKER TO IDENTIFY NEW SUBTYPES OF ERBB2 NEGATIVE BREAST TUMORS

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INTRODUCTION

Breast Cancer

Breast cancer is the most prevalent non-skin cancer in the world and the second leading cause of cancer deaths in American women. Despite increased awareness and earlier detection, approximately 40,000 women in the United States die from metastatic disease each year (Robinson 2004). Malignant breast cancer is a complex molecular disease in which alterations take place in the genes that govern cell growth and proliferation (Sledge and Miller 2003; Ingvarsson 2004). Sporadic breast cancer is the predominant form of this kind of tumor , in which oncogenes – which are initially mutated – lead to uncontrolled cell proliferation (Kenemans et al 2004). Other genetic mutations, especially in tumor suppressor genes (TSGs), are then thought to lead to malignancy. Hereditary or familial breast cancer, which represents only 5%–10% of breast cancer cases, is controlled by inheritable mutations to susceptibility genes, among other genes (Pavelic and Gall-Trošelj 2001; Margolin and Lindblom 2006; Walsh and King 2007).

The etiology of most breast cancer cases is unknown. However, numerous risk factors for the disease have been established (table 1) However, except for female gender and increasing patient age, these risk factors are associated with only few breast cancers (Carlson 2009).

Relative Risk	Factor
>4.0	• Female • Age (65+ versus <65 years, although risk increases across all ages until age 80) • Certain inherited genetic mutations for breast cancer (BRCA1 and/or BRCA2) • Two or more first-degree relatives with breast cancer diagnosed at an early age • Personal history of breast cancer • High breast tissue density • Biopsy-confirmed atypical hyperplasia
2.1-4.0	• One first-degree relative with breast cancer • High-dose radiation to chest • High bone density (postmenopausal)
1.1-2.0	
Factors that affect circulating hormones	• Late age at first full-term pregnancy (>30 years) • Early menarche (<12 years) • Late menopause (>55 years) • No full-term pregnancies • Never breastfed a child • Recent oral contraceptive use • Recent and long-term use of hormone replacement therapy • Obesity (postmenopausal)
Other factors	• Personal history of endometrium, ovary, or colon cancer • Alcohol consumption • Height (tall) • High socioeconomic status • Jewish heritage

Adapted with permission from Hulka et al, 2001.

Table 1: Factors that increase Relative Risk for Breast Cancer in Women

The progression from normal to malignant breast tissue has not completely established today,

although some identified events allowed to develop therapies that target the molecular changes that occur during breast carcinogenesis (Osborne et al 2004; Schulz 2006). Traditional chemotherapy for treatment of cancer suffers from two major problems. First, it is non-specific in that the drugs used to treat patients cannot distinguish between tumor and normal cells. This inability to distinguish between the two types of cells leads to a second problem – toxic side effects that are often more debilitating than the disease. A new approach to treatment – targeted therapy – attempts to resolve these problems by the rational design of drugs that specifically target cancer cells (Segota and Bukowski 2004; Seynaeve and Verweij 2004; Garrett 2005; Pegram et al 2005; Sledge 2005; Sharkey and Goldenberg 2006). Over the past decade, targeted therapy has offered particularly promising results in treating breast cancer (Bange et al 2001; Sledge 2001; Kaklamani and O'Regan 2004; Osborne et al 2004; Gasparini et al 2005; Hobday and Perez 2005; Johnson and Seidman 2005; Tripathy 2005; Muss 2006). There are many potential targets for the therapy, including plasmatic and membrane associated receptors for known or even unknown ligands, tyrosine kinases receptor, gene mutations resulting in defective regulation of apoptosis, epigenetic silencing of tumor suppressor genes, and mechanisms involved in angiogenesis. Protein kinases can be modulated in several ways including the abundant availability of growth factors, overexpression of growth factor receptors, and altered protein kinase levels and/or function. Although many potential targets exist, there are only a limited number of drugs that have resulted in recent changes in treatment paradigms. These include the anti-growth factor receptor antibodies trastuzumab (Herceptin®) (See ErbB2 paragraph) and cetuximab and the VEGF ligand-directed antibody bevacizumab. The tyrosine kinase inhibitors imatinib, gefitinib and erlotinib also constitute very important developments. The consequences of EGFR signalling via the erbB1 receptor include an activation of both the Ras/Raf and MAPK pathways on the one hand, and the PI3K/Akt pathways on the other, leading to an intracellular signalling cascade and activation of all the processes required for tumor growth, including tumor cell survival, proliferation, metastasis and induction of angiogenesis. Cetuximab is a monoclonal antibody known to bind to EGFR. The binding of Cetuximab to EGFR results in the blockade of downstream signaling and inhibition of the above cellular processes. Another important consideration in the development of biologically targeted therapies is vascular endothelial growth factor (VEGF), which is a key mediator of angiogenesis. Potential therapies include VEGF inhibition by the antibody bevacizumab, as well as upstream activators of VEGF synthesis or downstream signaling pathways including the activation of protein kinase C-b (PKC-b) (Zielinski 2006

Breast Cancer Classification

Unlike colon cancers, defining the progression of breast cancer has not been possible due to lack of markers that define hyperplasia (typical and atypical) (Stingl 2007). However, breast cancer can be broadly categorized into in situ carcinoma and invasive (infiltrating) carcinoma. Breast cancer can be categorized in several ways, including clinical features, expression of tumor markers, and histologic type. The two most common histologic types of invasive breast cancer are ductal and lobular carcinomas, accounting for approximately 75 and 15% of all cases in the US, respectively (Li et al, 2003). Classically the most significant risk factors for breast cancer recurrence and overall survival are tumor size and lymph node status (Smart 1997, Rosen 1989). Indeed, the standard for diagnosis and prognosis in breast cancer is the TNM system, in which the stage of disease is determined from the size of the tumor (T), the extent of regional lymph node involvement (N), and the presence or absence of distant metastases (M) (Robinson 2004).

Breast carcinoma in situ is further sub-classified as either ductal or lobular; growth patterns and cytological features form the basis to distinguish between the two types. Ductal carcinoma in situ (DCIS) is considerably more common than its lobular carcinoma in situ (LCIS) counterpart and encompasses a heterogeneous group of tumors. DCIS has traditionally been further subclassified according to the architectural features of the tumor which has given rise to five well recognized subtypes: Comedo, Cribiform, Micropapillary, Papillary and Solid (Fig. 1) (Connolly 2004)

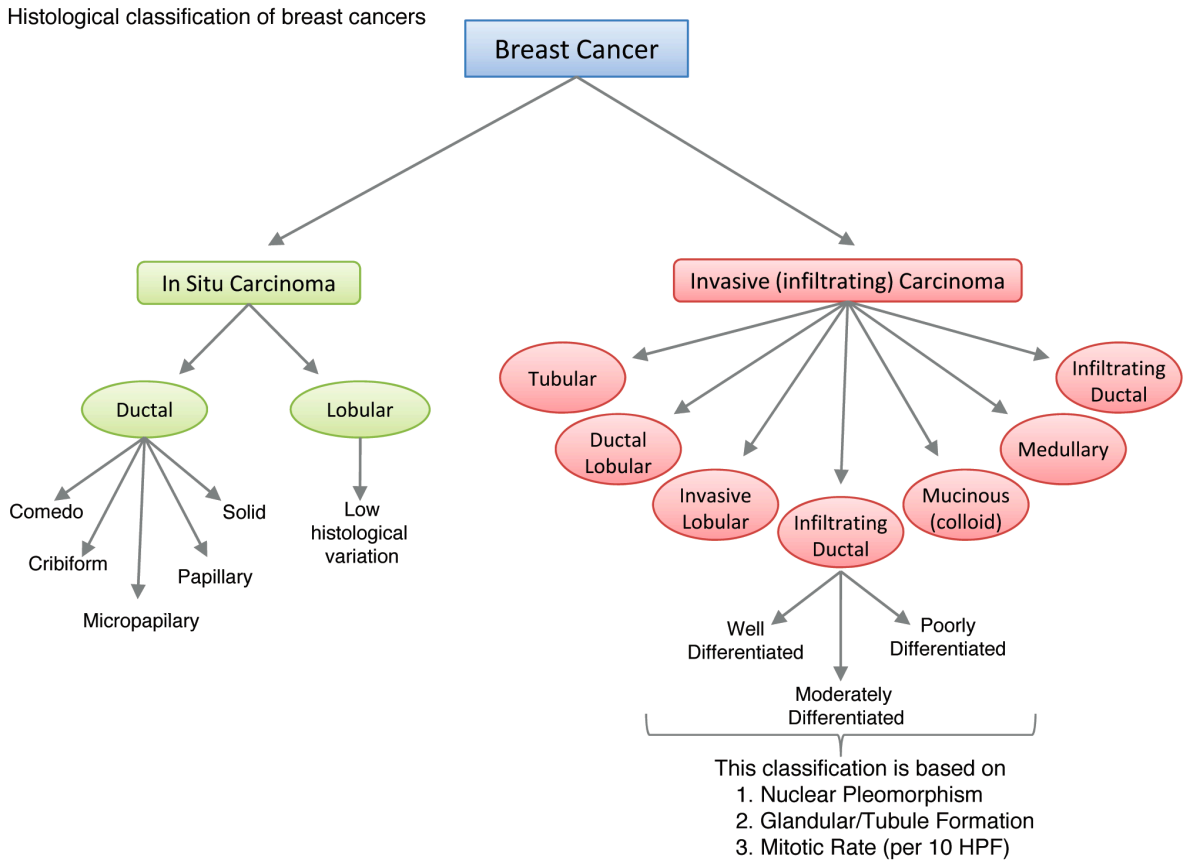

Figure 1.Histological classification of breast cancer subtypes. This scheme, currently used by clinicians, categorizes the heterogeneity found in breast cancer based on architectural features and growth patterns. HPF: high power field.(Malhotra 2010)

While this classification scheme has been a valuable tool for several decades, it relies solely on histology without utilizing new molecular markers that have a proven prognostic significance. In light of surgical advances leading to breast-conserving therapy, it has become necessary to more accurately stratify patients based on relative risk of recurrence or progression. These demands have led to the generation of several newer classification systems that incorporate molecular markers such as ER, PR, ErbB2 (Her2/neu) and p53 (Lagios 1989; Poller 1994, Holland 1994, Silverstein 1995). While the routine use of these markers for DCIS has not been accepted by the larger medical community, it is notable that the National Comprehensive Cancer Network (NCCN) has included determination of ER status as part of their DCIS workup (NCCN 2010). This paradigm shift foreshadows the future of molecular medicine that we have only recently begun to appreciate. Similar to in situ carcinomas, invasive carcinomas are a heterogeneous group of tumors differentiated into histological subtypes. The major invasive tumor types include infiltrating ductal, invasive lobular, ductal/lobular, mucinous (colloid), tubular, medullary and papillary carcinomas (Fig. 1). Of these, infiltrating ductal carcinoma (IDC) is, so far, the most common subtype accounting for 70–80% of all invasive lesions (Li 2005). IDC is further sub-classified as either well-

differentiated (grade 1), moderately differentiated (grade 2) or poorly differentiated (grade 3) based on the levels of nuclear pleomorphism, glandular/tubule formation and mitotic index (Lester 2009). In contrast to DCIS, where the use of molecular markers is still debated, the utility of ER, PR and HER2/neu is well accepted for IDC and it is recommended that their status should be determined on all invasive carcinomas. Furthermore, the College of American Pathologists (CAP) acknowledges, but does not require or recommend, the use of other ancillary tests (e.g., gene array profiling or immunohistochemical staining for markers other than ER, PR and HER2/neu) as long as sufficient tissue is available (Lester 2009). The use of ER, PR and HER2/neu determination in IDC exemplifies the potential of molecular biomarkers in guiding clinical decisions (Maughan 2010). Nowadays, the status of these markers helps to determine which patients are able to respond to targeted therapies (i.e., tamoxifen or aromatase inhibitors for ER+/PR+ patients and trastuzumab or lapatinib for HER2/neu patients) (Rakha 2010 Payne 2008).

Molecular Breast Cancer Classification

Breast cancer classification schemes have evolved over many decades into a tool that is used to aid in treatment and prognosis. However, with recent advances in cancer research and an increased molecular understanding of breast cancer heterogeneity, the current clinical model for breast cancer classification may benefit from the addition of several factors. **(**Malhotra 2010) The use of array-based technologies has revolutionized research into genomic markers of prognosis or response to treatment, with the ability to test a large number of potential markers in a high-throughput, cost-effective manner (Sanjay 2006). Using gene-expression array analysis the rapid analysis of multiple markers has become possible, thus allowing the identification of several intrinsic molecular subtypes of breast cancer that were later confirmed and classified as: basal-like, ErbB2+, normal breast like, luminal subtype A and luminal subtype B (Fig. 2) (Perou 2000, Sortie 2001, Sortie 2003). Recently, a new subtype classified as "claudinlow" has also been identified (Herschkowitz 2007, Prat 2010).

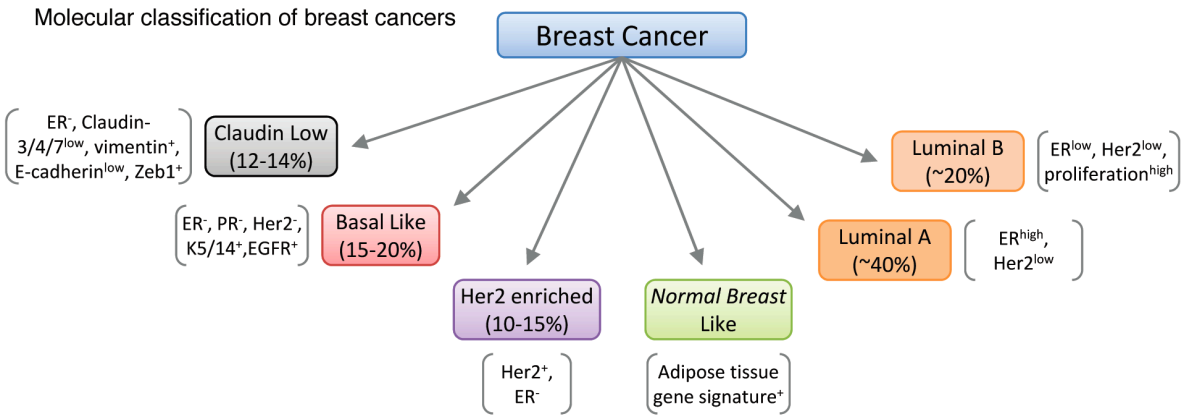

Figure 2 Molecular classification of breast cancer. This classification is based on the intrinsic molecular subtypes of breast cancer identi!ed by microarray analysis of patient tumor specimens.1(Malhotra 2010)

These molecular subtypes of cancer were identified by microarray-based gene expression analysis and unbiased hierarchical clustering. Notably, the molecular subtypes display highly significant differences in prediction of overall survival, as well as disease-free survival with the basal-like/ triple-negative (ER-/PR-/ErbB2-) subtype having the shortest survival. Furthermore, this molecular classification was able to stratify the ER^+ population into several subtypes that, again, demonstrated a difference in patient survival. This is significant because even though clinical assessment of IDC utilizes ER, PR and ErbB2 status, these markers did not allow separation of the two distinct ER^+ subtypes (i.e., Luminal A and Luminal B) that have very different clinical outcomes (Sortie 2001, Sortie 2003). It is unknown how the subtypes relate to the cell of origin, how to classify the many samples (about 10-15%) that

could not be assigned any subtype, how homogeneous are the different subtypes), and what are the molecular alterations specific to each subtype. Furthermore, it has been suggested that breast tumors subtypes are part of a continuum (Finetti 2008). A recent study has shown that genes associated with susceptibility variants are differentially expressed in the major subtypes (Nordgard 2007) thus opening up interesting perspectives. (Fig. 3)

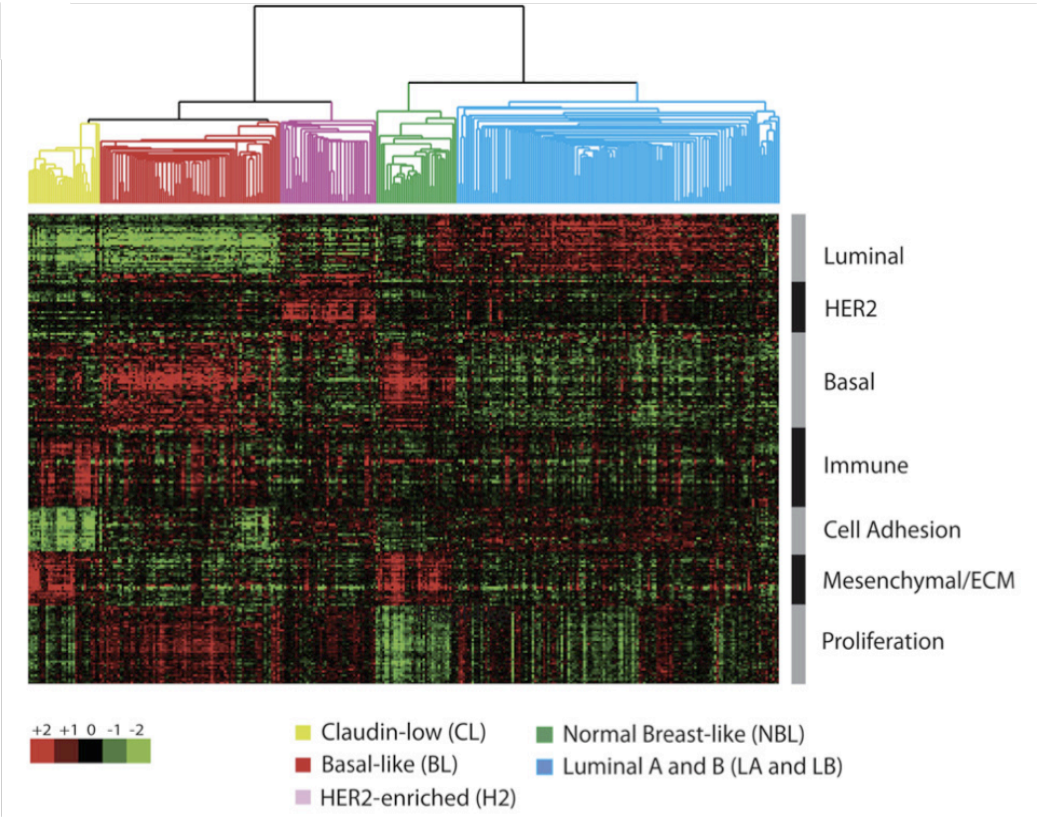

Figure 3. Intrinsic hierarchical clustering. Characteristic expression patterns are highlighted including the Luminal, HER2, Basal, Immune, Cell adhesion, Mesenchymal/Extracellular matrix (ECM) and Proliferation gene clusters. Each colored square represents the relative transcript abundance (in log2 space) with highest expression being red, average expression being black, and lowest expression being green.(Prat 2010)

The utility of this new molecular classification to predict outcomes has raised hopes of its adaptation in clinical practice; however, routine use of microarray analysis or genome sequencing is still cost prohibitive. To overcome this obstacle, investigators narrowed down a 50-gene signature that can effectively differentiate the molecular subtypes using quantitative real time PCR (qRTPCR). This 50-gene signature, termed PAM50, has been shown to be an effective replacement for full microarray analysis with an ability to classify tumors into one of the intrinsic subtypes.(Parker 2009) Furthermore, it has been demonstrated that a model using the PAM50 gene set for molecular classification had a significantly improved ability to predict risk of relapse as compared to a model utilizing only clinical variables (tumor size, node status and histologic grade) when tested on ER+/node-negative patients. However, it is important to mention that utilizing both clinical variables and molecular subtyping resulted in

significantly better predictive value than either one alone. In light of the tremendous variability in response to therapy, it is perhaps most notable that using the molecular subtypes generated a model with 94% sensitivity and 97% negative predictive value for predicting pathological complete response (Parker 2009). Thus, it is reasonable to expect that the application of molecular subtyping in clinical practice will provide useful information regarding patient-specific prognosis, risk of relapse and probability for pathological complete response. A major benefit of improved risk stratification will be the identification of patients for whom the benefits of neoadjuvant therapy outweigh the risks. Alternatively, patients with increased risk of relapse may benefit from a more aggressive treatment strategy or increased surveillance. It is important to note that the PAM50 is not the only multi-gene predictor for breast cancer; there are many others that are useful for cancer classification, grading, prognosis and response to therapy (Ross 2008).

Molecular subtyping has not yet matured sufficiently for stable stratification of luminal and HER2-enriched breast carcinomas. Furthermore, it has limited clinical relevance for subtyping of basal-like breast tumors because of the large overlap between triple-negative and basal-like cancers (de Ronde 2010, Kreike 2007). Till now, other means ie, immunohistochemistry for HER2 and oestrogen receptor status, and array comparative genomic hybridisation to test for *BRCA* gene status and homologous recombination deficiency are more predictive in clinical decision-making for neoadjuvant and adjuvant systemic therapy. Therefore, molecular subtyping should not be used instead of morphology and immunohistochemistry but rather in addition to these classic approaches, in order to increase clinical relevance and robustness.

Breast Cancer Genetics

Breast cancer is a genetic disease. At the moment breast cancer is diagnosed clinically; mutations can be demonstrated in at least four to six major regulatory genes, located on various chromosomes present in the nucleus of the breast cancer cell. These genes play a role in maintaining the physiological balance between proliferation, apoptosis and differentiation. Other genes regulate expression of steroid receptors, cell adhesion molecules and angiogenic factors, and of various other proteins important for invasion and the establishment of metastases. It has been proposed that the process of breast cancer tumorigenesis is best described by a multi-step progression model (Beckmann 1997) in which the normal breast epithelium evolves via hyperplasia and carcinoma in situ into an invasive cancer, which eventually can disseminate via lymph and blood vascular systems to form metastases. Each of these steps is thought to correlate with one or more distinct mutations in regulatory genes.

The major high penetrance genes in which mutations increase susceptibility to breast cancer are the breast cancer susceptibility gene 1 (BRCA1) and the breast cancer susceptibility gene 2 (BRCA2). Mutations in these genes account together for 2–3% of all breast cancers and around 30–40% of all familial breast cancers (Wooster 2003). In addition to BRCA1 and 2, several other genes are mutated in familial breast cancer. Mutations in the cell cycle checkpoint kinase gene (CHEK 2) account for about 5% of familial cancer cases. Mutations in TP53 (causing the Li-Fraumeni Syndrome) and those in PTEN (Cowden's disease) are responsible for no more than 1% of all familial breast cancer cases. (Wooster 2003). Predisposition to breast cancer has also been related to a variety of genetic polymorphisms in genes involved in metabolism of steroid hormones (e.g. CYP17 and CYP19) and carcinogens (e.g. CYP1A1, NAT1 and NAT2) (Dunning 1999). So far, the majority of breast cancers are so-called sporadic cancers that result from the accumulation of acquired and uncorrected genetic alterations in somatic genes, without any involvement of germline mutation . Risk factors for sporadic breast cancer are often hormonal in nature (Clemons 2001)**.**

A long list of genes has been implicated in breast cancer Tumorigenesis. (Table 2) Many oncogenes, with different functionality and cellular localization, have been reported to play a role in human breast carcinogenesis. In sporadic breast cancer oncogenes amplification is frequently found, but only a few of these amplified genes are crucial in the development of breast cancer, e.g. MYC, Int2, EMS1, CCND1 and ERBB2 (Nass 1997. Ormandy 2003, Barnes1998, Miles 1999 Fioravanti 1997. An 1997). Factors like EGF, TGFβ and IGF-1 could be also involved in proliferation and growth of breast cancer Growth Tumor suppressor genes.

Gene	Locus	Role in hereditary breast cancer	Role in sporadic breast cancer	Reference(s)
BRCA1	17q12-21	Germline mutation (hereditary breast ovarian cancer syndrome)	Inactivation by hypermethylation of the BRCA1 promotor region	[2,23–30]
BRCA2	13q12-13	Germline mutation (hereditary breast ovarian cancer syndrome)	Silenced by overexpressed EMSY	[28,29,75]
TP53 (p53)	17p13.1	Germline mutation (Li-Fraumeni syndrome) TP53 mutations frequent in BRCA1 and BRCA2 mutant breast cancers	Late event	[31–36]
Rb1	13q14.1	No specific role	Late event	[30,37,38]
PTEN (MMAC1)	10q.23-24	Germline mutation (Cowden disease syndrome)	Rare	[39]
MYC	8q24	No specific role	Overexpressed in 25–30%	[11,30]
ERBB2/Her2/neu	17q21	Frequently underexpressed in BRCA1 mutant breast cancers	Overexpressed in 25–30%	[16]
CDH1 (E-Cadherin)	16q22.1	No specific role	Early event in lobular breast cancer	[44]
CCND1 (Cyclin D1)	11q13	Frequently underexpressed in BRCA1 mutant breast cancers	Overexpressed in 30–40%	[14,15,63]
ERα	6q25.1	Frequently underexpressed in BRCA1 mutant breast cancers	Underexpressed in 25%	[47,48,50,51]
ERβ	14q22-24	Not known	Not known	[49–51]

Table 2 Most important genes implied in breast cancer tumorigenesis. (Kenemans 2004)

The BRCA1 gene, located on chromosome 17q12-21, was cloned in 1994 (Miki 1994). BRCA1 is involved in many transcriptional processes. It has been associated with more than 15 different proteins involved in transcription, either in transcriptional activation or transcriptional repression (Cable 2003). It also plays a role in apoptosis. As a tumor suppressor, *BRCA*1 has got a role in maintaining genomic stability. It interacts with various proteins, and the complexes formed are involved in DNA recognition and repair (Jhanwar-Uniyal 2002, Venkitaraman 2002). Germline mutations in *BRCA1* confer susceptibility to breast and ovarian cancer. Mutations of *BRCA1* are scattered throughout the gene and consist of insertions, deletions, frame-shifts, base substitutions and inferred regulatory mutations. In sporadic breast cancer the gene is rarely mutated, but frequently functionally impaired (Lambie 2003 Vidarsson 2002). The *BRCA2* gene is located on chromosome 13q12-13. The gene codes for proteins involved in DNA repair, cell cycle control and transcription (Kerr 2001).and may have a function in terminal differentiation of breast epithelial cells (Vidarsson 2002). In sporadic breast cancer, mutational inactivation of BRCA2 is rare as inactivation requires both gene copies to be mutated or totally lost (Venkitaraman 2002 Kerr 2001 Lerebours 2002).The *PTEN* tumor suppressor gene is located on chromosome 10q23. Germline mutations play a role in breast cancer (within the Cowden disease syndrome). Somatic mutations in sporadic breast cancer are rare (Ueda 1998). The cell cycle checkpoint kinase *CHEK2* gene (on chromosome 22) is a key mediator in DNA damage-response (Rouse

2002, Myung 2001). The 1100delC variant of the *CHEK2* gene was thought to cause low-penetrance susceptibility to familial breast cancer (Meijers-Heijboer 2002 Vahteristo 2002). The *CDH1* gene (on 16q22.1) encodes for the adhesion molecule E-Cadherin. In sporadic lobular breast cancer, CDH1 is claimed to behave as a tumor suppressor gene (Berx 1995). The estrogen receptor (ER) a gene located on chromosome 6q25.1, is the most important growth factor receptor involved in hormone-dependent breast carcinogenesis. The ER-β gene is located on 14q22-24. Estrogens can act as tumor initiator, by causing direct DNA damage (Liehr 1997). By induction of incessant mitosis, estrogens can promote accumulation of DNA replication damage ultimately leading to a malignant phenotype (Kenemans 2003). The two receptor isoforms are encoded by two different mRNAs, but share the same structural and functional domain composition (Kuiper 1996). Estrogen receptors regulate gene expression by both estrogen-dependent and estrogen-independent mechanisms leading to activation of gene transcription, e.g. of cell cycle control proteins. These processes may result in cell proliferation. Overexpression of ER-a is frequently observed in early stages of breast cancer(Hayashi 2003). The significance of ER-β in breast cancer is less clear than that of ER-a. The presence of ER-β mRNA has been demonstrated both in normal as well as in malignant mammary gland tissue (Cullen 2001).

c-Myc

Ever since Bishop and his co-workers discovered the c-*myc* gene in the late 1970s. (Bishop 1982) Most if not all, types of human malignancy have been reported to have amplification and/or overexpression of this gene, although the frequency of these alterations varies greatly among different reports (Nesbit *et al.* 1999). In 1992, researchers started to realize that aberrant expression of c-*myc* could cause apoptosis (Evan *et al.* 1992, Shi *et al.* 1992), although the phenomenon had actually been observed much earlier (Wurm *et al.* 1986).

The c-*myc* gene is transcribed to three major transcripts that start from different initiating sites , yielding three major proteins named c-Myc1, c-Myc2, and c-MycS (Fig.4) (Henriksson & Luscher 1996, Xiao *et al.* 1998). c-Myc2 is an approximately 62-kDa protein that is the major form of the three c-Myc proteins and the one referred to as 'c-Myc' in most studies. c-Myc1 arises from an alternative initiation site at an in-frame, non-AUG codon, yielding a protein 2–4 kDa larger than c-Myc2. c-MycS arises from a leaky scanning mechanism, and initiates at two closely spaced downstream AUG codons, resulting in a protein lacking about 100 amino acids at the N-terminus of c-Myc2 (Claassen & Hann 1999).

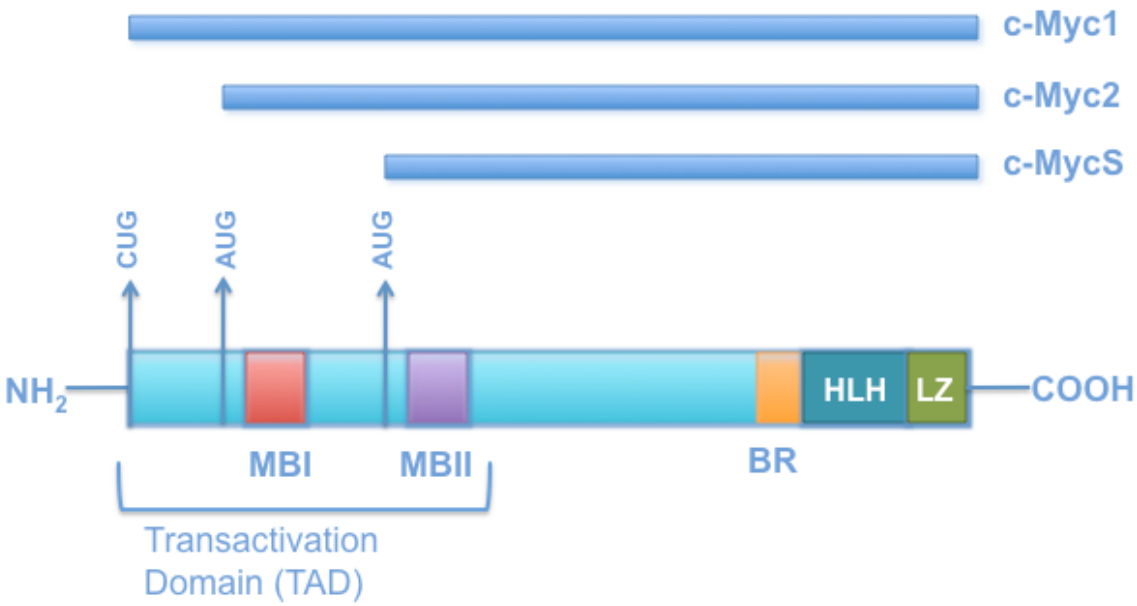

Figure 4 Schematic diagram of the Myc family proteins. Within the TAD at the N-terminus there are two *myc* homologyboxes (MBI and MBII), which are conserved among Myc family proteins. At the C-terminus, the HLH/LZ domain links to the basic region (BR) of the c-Myc protein. The initiation sites of the three c-Myc proteins are indicated.

An unusual property of the c-*myc* gene that is often neglected by investigators is that the antisense strand of the gene also yields transcripts (Spicer & Sonenshein 1992).The amino terminus of each full-length c-Myc protein (c-Myc1 and c-Myc2) harbors a transactivation

domain (TAD), within which are two regions that are highly conserved among members of the Myc family; these regions are termed Myc homology boxes I and II (MBI and MBII) (Fig. 4). The carboxyl terminus of the c-Myc proteins contains a basic region and a helix-loop-helix/leucine zipper (HLH/LZ) domain. Through the HLH/LZ domain, a c-Myc protein heterodimerizes with another transcription factor, Max. The c-Myc/Max complex then binds to a specific DNA recognition sequence, the so-called E-box element that contains a central CAC(G/A)TG motif (Amati *et al.* 1998, Dang 1999).

Genes containing this Myc E-box element in their regulatory regions may be c-Myc targets, and thus subjected to transactivation or transrepression by the c-Myc/ Max complex (Cole & McMahon 1999). The c-Myc RNA and protein have short half lives (30 min and 20 min, respectively) as compared to those of Max (3 hrs and >24 hrs, respectively), and in most systems Myc appears to be the limiting, regulated component of the heterodimer. Within the TAD, the MBI has been shown to be required for the transactivation activities of c-Myc, whereas the MBII is needed for the trans-suppression activities (Sakamuro & Prendergast 1999). c-MycS lacks the MBI but still retains the MBII in its TAD; this may be the reason why c-MycS is deficient for transactivation but retains the activity of trans-suppression. Thus, c-Myc1 and c-Myc2 can both activate and repress transcription of specific target genes, whereas c-MycS can only repress transcription and can thus function as a dominant-negative inhibitor of certain (but not all) activities of the full-length c-Myc proteins (Xiao *et al.* 1998, Sakamuro & Prendergast 1999). Under normal growth conditions, expression of c-Myc1 and c-Myc2 proteins is differentially regulated (Batsche & Cremisi 1999). Regulation of c-MycS is much less known, relative to c-Myc1 and c-Myc2. It has been shown that its expression is increased to the levels comparable to those ofc-Myc2 during rapid cell growth, and constitutively high levels ofc-MycS have been found in some tumor cell lines as well (Spotts *et al.* 1997).

Although it could also act as a a transcriptional activator, exspecially for genes involved in differentiantion. c-Myc also acts as transcription repressor by interacting with histone deacetylases (Kurland2008) . In the past decades, various approaches have been used to identify c-Myc target genes. So far, as many as 15%-20% of human genes can be regulated directly or indirectly by c-Myc. These genes are related to cell cycle control, protein synthesis, cytoskeleton and cell motility, cell metabolism, and microRNA- the small regulatory molecules that regulate the stability and translation of target mRNA (Gao 2009).How these genes interact with each other to modulate growth, differentiation, apoptosis, and survival is largely unknown (Lin 2010).

c-Myc was found overexpressed, through different mechanisms, in a lot of different human cancers. Some work has tried to established a role for c-*myc* in cell cycle progression, metabolism, apoptosis and genomic instability. One model proposes that c-*myc* promotes cell proliferation and genomic instability by accelerating cells through G1 and S phases of the cell cycle, abrogating cell cycle checkpoints, and increasing cell metabolism. In many settings these alterations will lead to apoptosis, or cell death. But in the background of additional mutations that activate anti-apoptotic signals, c-*myc* can lead to full blown neoplastic transformation.) Most studies focused on c-Myc's effects on regulatory proteins of the G1-S phase transition of the cell cycle. This transition is promoted when cyclin dependent kinases (CDKs) are activated by association with specific cyclins. Cyclin dependent kinase inhibitors (CDKIs) inhibit this activation. c-Myc has been implicated in inducing cyclin D1 and D2, cyclin E, CDK4, and cdc25A, a phosphatase, which activates CDK2 and CDK4. c-Myc has also been shown to reduce the amounts or inhibit the function of the CDK inhibitor, p27, potentially by increasing cyclin D levels which can then sequester p27. c-Myc also induces Cul1, which mediates the degradation of p27 (Gardner 2002).

A highly regulated cell cycle allows cells to repair DNA damage before replicating, thus promoting genomic fidelity. Inappropriate cell cycle proliferation can lead to genomic instability, resulting in new mutations and abnormal chromosome number and structure. c-Myc overexpression, even transiently, can induce genomic instability that is characterized by gene amplification, aneuploidy and polyploidy. Other studies suggest that c-Myc induces the production of reactive oxygen species (ROS) by mitochondria, leading to DNA damage and genomic instability. (Gardner 2002)

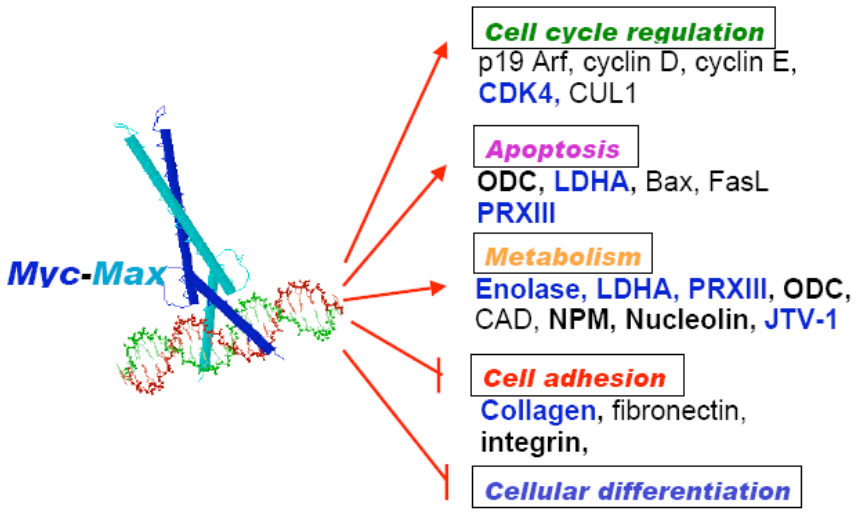

Figure 5 The Myc-Max basic-helix-loop-helix leucine zipper heterodimer binds a target DNA site, termed E-box. c-Myc regulates downstream target genes resulting in activation cell cycle regulation, apoptosis, or inhibition of cell adhesion. Examples of c-Myc target genes associated with different cellular functions are listed.

The EGFR family

The EGFR family consists of four closely related tyrosine kinase receptors: ErbB-1 (also termed epidermal growth factor receptor [EGFR] or HER-1), ErbB-2 (also termed HER-2 or HER-2/neu), ErbB-3 (HER-3), and ErbB-4 (HER-4) (Lin 2004).

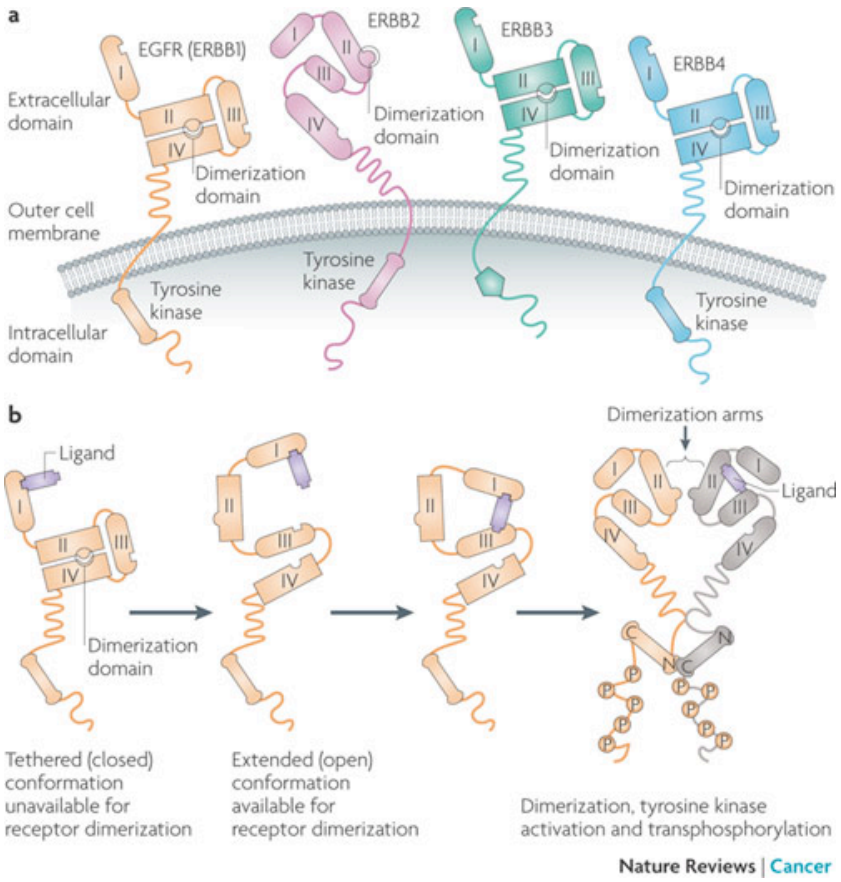

Figure 6 EGFR family. A. The ErbB family includes four members. Each receptor is composed of three functional domains: an extracellular domain responsible for ligand binding, a segment α-helix transmembrane, and an intracellular domain with tyrosine kinases activity. The Epidermal Growth Factor Receptor (EGFR), ErbB3 and ErbB4 may exist in a bound conformation (closed), in absence of ligand, in which the dimerization domain is not available to interact with another receptor. There is no known ligand for ErbB2; this receptor exists in an extended conformation (open) and permanently available for dimerization. **B**. change of conformation in response to ligand binding. The ligand that binds to ErbB receptors seems to to induce a conformational change in folded structure of the molecule that exposes the dimerization domain, this step is necessary for the formation of omo or hetero-dimers and the functional activation of EGFR, erbB3 and erbB4. Baselga 2009

Under normal physiological conditions, ErbB receptors activation is controlled by spatial and temporal expression of their ligands, members of the EGF-related peptide growth factor family. These peptides are produced as transmembrane precursors, and the ectodomains are processed by proteolysis leading to shedding of soluble growth factors. The peptides of EGF-family are divided into three groups, based upon their receptor specificity. The first group includes EGF, transforming growth factor (TGF)-α, amphiregulin (AR) and epigen (EPG),

which bind specifically to EGFR; the second group includes betacellulin (BTC), heparin-binding EGF (HB-EGF) and epiregulin (EPR), which exhibit dual specificity, binding both EGFR and ErbB4. The third group, composed of the neuregulins (NRGs), forms two subgroups based upon their capacity to bind ErbB3 and ErbB4 (NRG-1 and NRG-2) or only ErbB4 (NRG-3 and NRG-4) (Riese 1998, Kochupurakkal 2005 Hynes 2008)

Each ErbB receptor has an extracellular domain involved in ligand binding, a helical transmembrane segment, and an intracellular protein tyrosine kinase domain (Fig. 6). After ligand binding, the extracellular domains of the receptors undergo conformational changes, which allow them to form homodimers or heterodimers of the EGFR family (Lin 2004, Atalay 2003, Rosen 2010) ErbB3 has impaired kinase activity because of substitutions in critical residues in its kinase domain. It acquires signaling potential only when dimerized with another receptor. Hence, ErbB3 is phosphorylated and functions as a signaling entity only when dimerized with another ErbB receptor (Kim 1998). Furthermore, none of the EGF family ligands directly binds ErbB2, however, ErbB2 is activated via heterodimerization with another activated ErbB receptor and generally is the preferred heterodimerization partner (FIG CC)(Graus-Porta 1997).

Putative ligands of ErbB 2 have been characterized, but no specific ligand has yet been identified. This has clinical implications in terms of there is not an alternative approach to block this pathway, and this may be related to the development of resistance to ErbB 2 blockade. The specific receptors involved in each dimer affect the type and number of downstream effectors activated, and also influence the downregulation mechanism for the ligand-bound receptors Dimerization of ErbB receptors induces phosphorylation of their intracellular tyrosine kinase domains, which provide docking sites for adaptor proteins and signaling enzymes. These molecules act as a link between membrane receptor kinases and “downstream” intracellular protein kinases, which results in the activation of multiple signaling pathways, of which the MAPK and PI3K pathways are probably the best understood (Atalay 2003). Given the absence of known ligands for ErbB 2, and lack of tyrosine kinase activity of ErbB3, it is assumed that these receptors must form heterodimers with another member of the EGFR family in order to activate signaling. ErbB 2 is the preferred dimerization and signaling partner for all other members of the EGFR family, and it appears to function mainly as a coreceptor, increasing the affinity of ligand binding to dimerized receptor complexes (Atalay 2003, Graus-Porta 1997). With their multiple ligands, many dimerization combinations, and large number of downstream effectors, the EGFR family mediates an extensive range of signals, controlling a variety of cellular processes, including

cellular proliferation, apoptosis, and angiogenesis (Prenzel 2001). Through their interconnected cellular signaling network, the EGFR family regulates different biological processes, including cell proliferation, differentiation, and survival, and plays a key role in the development and progression of many types of human cancer including carcinoma of the breast, lung, ovaries and stomach (Holbro 2004, Hynes 2005, Hines 1994). Cancer patients,with alterations in ErbB1 or ErbB2 genes, generally develop a more aggressive disease, associated with parameters predicting a poor clinical outcome (Slamon 1987, Hynes 1994, Nicholson 2001). Expression of EGFR family members in breast tumors has a significant impact on tumor aggressiveness and patient survival. ErbB-1 and ErbB -2 are expressed in approximately 16%–48% and 25%–30% of breast tumors, respectively, and their expression correlates with a more aggressive disease course, shorter survival time, and higher risk for resistance to endocrine therapies. ErbB3 expression, observed in approximately 18% of breast tumors, also correlates with shorter overall survival. Interestingly, expression of ErbB-4 (found in approximately 12% of breast tumors) has been associated with more favorable tumor characteristics and longer survival (Vogt 1998, Witton 2003)

Based upon these clinical findings, ErbB receptors start appealing as candidate therapeutic targets. Nowadays numerous agents targeting individual members of the EGFR family have been developed for using in the treatment of breast cancer. Existing therapeutic approaches have largely focused on two classes of agents. The first comprises monoclonal antibodies that bind to extracellular regions of ErbBs to interfere with receptor function (e.g., trastuzumab, pertuzumab, and a number of pan-HER inhibitors). Trastuzumab binds to the juxtamembrane region of ErbB-2 with high specificity, but it is not currently known how it specifically interferes with ErbB2 function (Cho 2003). Pertuzumab is the first in a class of ErbB2 dimerization inhibitors; its binding to ErbB2 inhibits the dimerization with other ErbB receptors and this is thought to result in slowed tumor growth) (Gelmon 2008). The second class of ErbB-targeted agents comprises the small molecule tyrosine kinase inhibitors (TKIs) that inhibit enzyme function of EGFR family members intracellular.

ErbB2 and cancer

The proto-oncogene *ERBB2* (HER-2 or *neu)* is located on chromosome 17q and encodes a 185-kd tyrosine kinase receptor of the epidermal growth factor receptor family.ErbB2 has the same structure of other members of the family ErbB but ErbB2 remains an orphan receptor, with no diffusible ErbB2-specific ligand identified.

However, it has been shown that EGF-like ligands are bivalent in nature, that is, they bind to their receptors at two different sites: high- and low-affinity binding sites (Olayioye 2000, Yarden 2001). Although ErbB2 is not a high-affinity receptor for any of the ligands shown to bind ErbBs, it is the favorite low-affinity co-receptor for all EGF-like ligands, and therefore it emerges as the preferred dimer-mate for the three other ErbBs once these primary (high-affinity) receptors are occupied by their ligands (Fig 7)(Klapper 1999, Brennan 2000). Thus, it is assumed that at least 30 ErbB-binding growth factors can utilize HER- 2-related signaling pathways, although none directly binds ErbB2. ErbB2 is crucial in the induction of growth signal by the ligand-occupied ErbBs, because in the presence of ErbB2 (Pauletti 1996) it is the preferred heterodimerization partner for all ligand-binding ErbB RTKs (Press 1997).

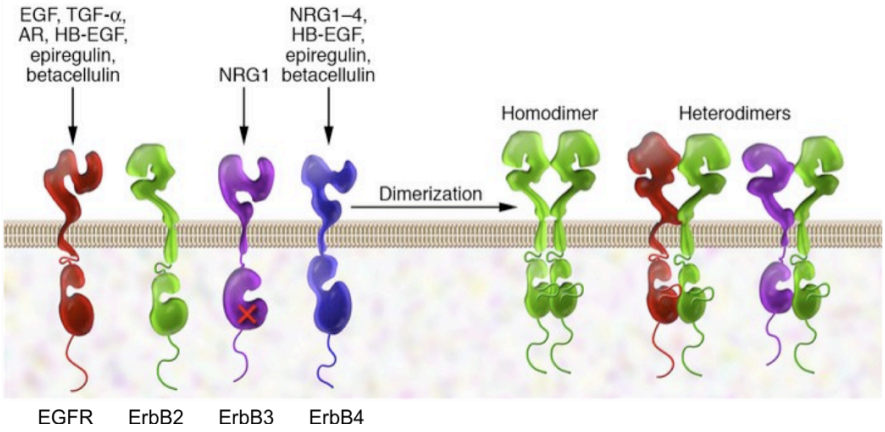

Figure 7. ErbB2 form heterodimers with other EGFR family members. There is no known ligand of ErbB2,however ErbB2 can form dimers with another ErbB2 receptor activated by ligand binding

ErbB2-containing heterodimers have also been characterized by an extremely high growth factor-induced signaling potency and mitogenesis. ErbB2-containing heterodimers potently induce the major mitogenic signaling cascades, by the activation of the mitogen-activated protein (MAP) kinase and phosphatidylinositol 3-kinase (PI3-kinase) pathways (Fig.8) (Daly 1999, Lane 2000).

The high signaling potency of ErbB2 containing heterodimers, in turn, results from several specific features: capacity of ErbB2 to (1) reduce the rate of ligand dissociation from its high-affinity receptor, (2) capacity to induce lateral signaling by recruiting and activating other (unoccupied) ErbB receptors (Brennan 2000), and (3) from efficient signaling through protein

kinases such as MAP and Jun N-terminal that are expecially potent activators of mitosis. In addition, (4) ErbB2-containing receptor dimers are recycled from endosomes back to cell surface instead of being degraded by lysosomes, thus resulting in their overexpression at the cell surface (Waterman 2001). The oncogenic potential of the ErbB2 receptor may be activated through multiple genetic mechanisms including point mutations, truncation of the protein, or amplification of the non-mutated proto-oncogene. Of these, gene amplification is so far the most common mechanism in breast cancer and leads to ErB2 overexpression (Bange 2001). Thus, the amplification of *ERBB2* gene increases dramatically the likelihood of ErbB2 forming heterodimeric complexes with the other ErbBs on the cell surface of the cancer cell. This, in turn, indicates that several dozen potent ligands that bind to other members of the ErbB family can take advantage of ErbB2-dependent signaling pathways leading to the oncogenic activation of cells.

As previously said, ErbB2 requires dimerization with another ligand-activated ErbB receptor to mediate signaling. Considering ErbB2-overexpressing cancers, it is interesting to discuss whether signaling originating from ErbB2 alone is enough to drive transformation, or whether there still might be a requirement for other ErbB receptors in the transformation process. Targeting overexpressed ErbB2 in cancer cells has an anti-proliferative effect, which is accompanied by a strong decrease in PI-3K/PKB signaling activity (Munster 2002, Neve 2000) and can be prevented by expression of a constitutive active form of PKB (Yakes 2002). Surprisingly, while ErbB2 couples very well to the MAPK pathway through several Shc and Grb2 binding sites, it appears unable to recruit the p85 adaptor subunit of PI-3K . Thus, in order to activate this pathway, ErbB2 should either heterodimerize with another ErbB receptor containing a p85 binding site, or activate the pathway indirectly, for example through Ras signaling (Rodriguez-Viciana 1994). ErbB3 contains six docking sites for the p85 adaptor subunit of PI-3K and efficiently couples to this pathway (Fig.8) (Fedi 1994, Hellyer 2001) . Furthermore, inactivation of ErbB2 leads to loss of ErbB3 phosphorylation (Motoyama 2002). These observations suggest that ErbB3's role in cancer cells might be to act as a partner of overexpressed ErbB2, by promoting the activation of the PI-3K/ PKB pathway. Some studies suggest that the ErbB2/ErbB3 heterodimer works as an oncogenic unit to drive proliferation of breast cancer cells. A suggestion that ErbB2/ErbB3 heterodimers might have a role in an in vivo setting comes from results with a transgenic mammary tumor model induced by expression of activated ErbB2. In these tumors it was observed that expression of ErbB3 was increased and, importantly, its phosphotyrosine content was also elevated (Siegel 1999).

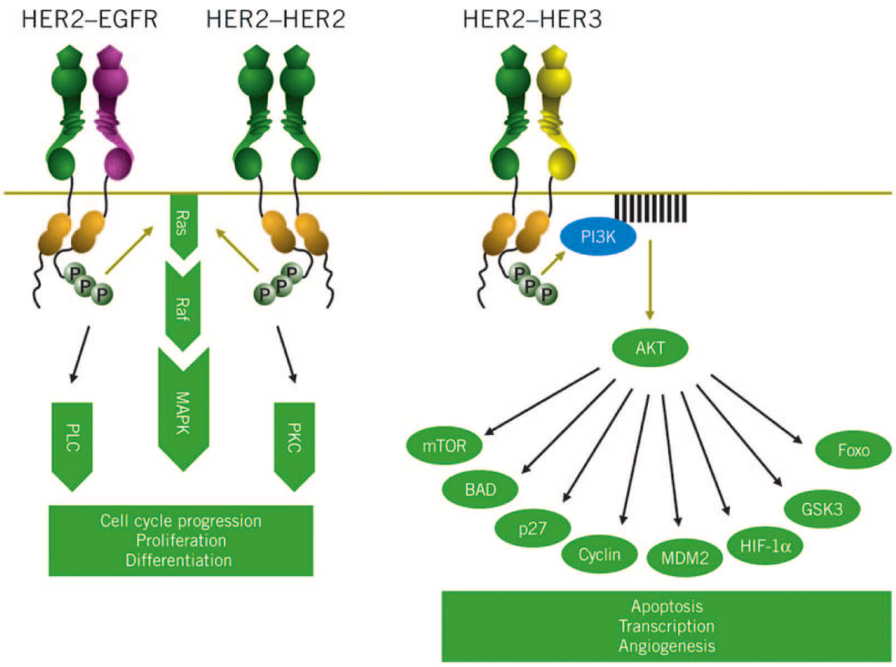

Figure 8. ErbB2 activates numerous cellular signalling pathways. EGFR-ErbB2 and ErbB2-ErbB2 dimers activate the Ras-Raf-MAPK, the PKC and the PLC pathways. ErbB2-ErbB3 dimers activate PI4K and the downstream AKT pathway. However there is cross-talk between downstream pathways that connets them to each other, adding considerable complexity to the signaling network. ErbB2 overespression and overactivity results in increased signaling throught all these pathways, leading to malignant trasformation.

Amplification or overexpression of *ERBB2* has been shown to be a prognostic factor associated with resistance to some forms of adjuvant chemotherapy and sensitivity to others, short disease-free interval, and short survival time in both node-negative (Andrulis 1998) and node-positive breast cancer patients (Slamon 1987). It is also an objective indicator of high histological grade, nuclear atypia, biological aggressiveness and high metastatic potential of breast cancers (Farabegoli 1999 Wright 1989). Approximately 25–30% of invasive female breast cancers overexpress Erbb2. In 90–95% of these cases, overexpression is a direct result of gene amplification (Pauletti 1996). The high prevalence of ErbB2 overexpression in breast cancer and the apparent clinical significance led to the development of the recombinant humanized monoclonal antibody, the Trastuzumab (Herceptin®), against ErbB2 (Bange 2001, Carter 2001). The Trastuzumab works against the tumor cells by binding to the extracellular domain of the ErbB2 receptor and thus reduces the cell-surface expression of ErbB2 . In addition, in order to down regulating cell-surface ErbB2 expression, Trastuzumab induces the cyclin-dependent kinase inhibitor p27Kip1 and the retinoblastoma protein (Rb)-related protein p130, which reduces the number of carcinoma cells in the S phase of the cell cycle (Yarden 2001). The recruitment and activation of immune effector cells to the ErbB2-overexpressing tumor may also contribute to Herceptin's mechanisms of action (Klapper 2000). In the USA,

Trastuzumab was approved by the Food and Drug Administration (FDA) for treating breast cancer patients with HER-2- overexpressing tumor since 1998. Alternative approaches to the use of naked monoclonal antibodies, such as Herceptin, have been used to target ErbB2 receptor directly or against its signaling pathways. Murine antibody 2C4 (Pertuzumab) was also isolated by Ullrich and colleagues and showed to bind ErbB2 with high affinity on an epitope distinct from trastuzumab (Hudziak 1989). Moreover, unlike Trastuzumab, it did not have strong anti-proliferative activity in ErbB2 over-expressing breast tumor cell lines. Nevertheless, 2C4 has the ability to interfere with HRG induced ErbB2/ErbB3 heterodimerization (Agus 2002), while Trastuzumab is unable to do it (Motoyama 2002). Detailed knowledges on the crystal structure of each ErbB receptor ectodomain provided insight into ligand binding and could explain why the two antibodies have different biological activites. Trastuzumab infact binds domain IV of ErbB2, close to the membrane (Cho 2003), while Pertuzumab binds domain II, which is involved in ligand induced receptor dimerization (Franklin 2004). Thus, Pertuzumab, which is currently in clinical trial, represents a novel class of targeted therapeutics, namely ErbB2 dimerization inhibitors (Attard 2007). Other immunological approaches include the use of ErbB2 targeting antibodies able to induce toxicity to cancer cells by combining them with radionuclides, toxins or prodrugs.(reference), DNA vaccines against ErbB2, and a combination of vaccines with cytokine therapy. Different approaches have also been developed to block transcription, translation, or maturation of HER-2 transcripts or proteins with different types of gene therapy. These therapies include use of anti-ErbB2 intracellular single-chain antibodies, transcriptional regression of ErbB2 expression by adenovirus type 5 *E1A* gene, suicide gene therapy directed at the ErbB2, different antisense-approaches as well as adenovirus-mediated hammerhead ribozymes specific for ErbB2 (Yarden 2001, Yu 2000). Other alternatives to the immunological and gene therapy approaches that target ErbB2 include a wide variety of drugs that target the tyrosine kinase activity of ErbB2. These compounds represent natural and synthetic inhibitors of tyrosine kinases (so-called low molecular weight inhibitors), farnesyl transferase inhibitors, tyrphostins, MAPK inhibitors, Akt inhibitors as well as antibiotics whose mechanism of action is related to the inhibition of tyrosine kinase activity. In addition, certain prostaglandins that bind to peroxisome proliferator-activated receptor δ (PPARδ) have an antiproliferative effect on breast cancer cells lines, and this is related to inhibitory action on the ErbB2 (and other ErbBs) tyrosine kinase activity (Järvinen 2002). On the other hand, increased signaling through insulin-like growth factor receptors takes place in the ErbB2- overexpressing tumors and may also induce resistance to Herceptin (Albanell 2001).

ERBB2 promoter

In primary breast tumours strong *ERBB2* gene overexpression is associated with gene amplification and increased transcription. In many primary breast cancers (Slamon 1989) and mammary adenocarcinoma cell lines (Kraus 1987), the mRNA overexpression is much more elevated and not proportional than the gene copy number, thus indicating that mechanisms other than gene amplification must be involved. Even though the ERBB2 overexpression is accepted today as a factor of poor prognosis, the reasons for this overexpression remain unknown. The protein and DNA elements controlling the breast-specific expression of the human *ERBB2* gene are not well known. Most studies investigating the transcriptional mechanisms responsible for ErbB-2 accumulation in breast cancers have focused on the *ERBB2* proximal promoter region. Indeed, the 500-bp fragment located upstream of the transcription start site, whose activity is increased in cells overexpressing *ERBB2* gene, has been well characterized. **(**Delacroix 2005). Major genomic features including the five different positive-acting control elements reported for the *ERBB-2* proximal promoter are shown in Figure 9

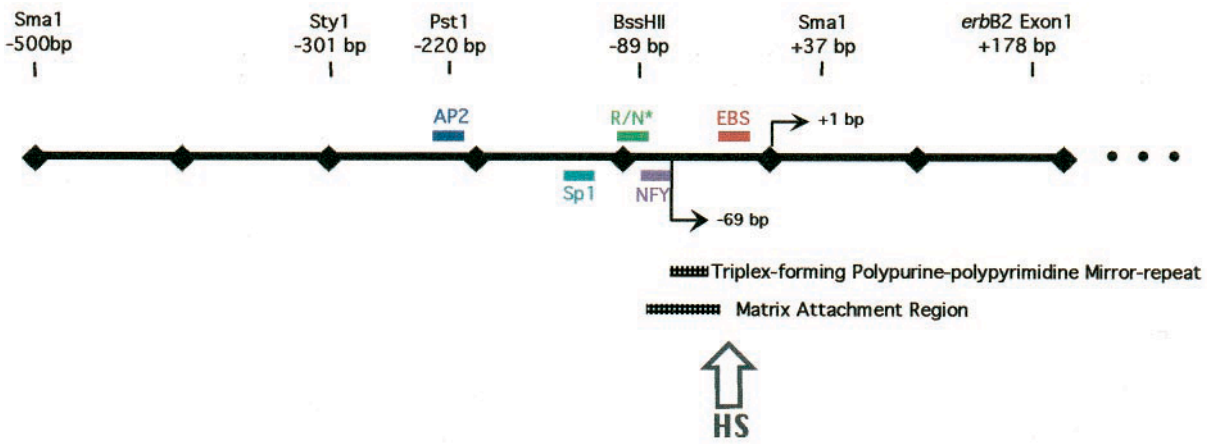

Figure 9. Proximal promoter features regulating erbB2 transcription. Genomic landmarks and known positive-acting regulatory elements (EBS, NFY, R/N*, Sp1, AP2) localized in relationship to the primary site of transcript initiation at +1 bp and a secondary site at 769 bp preferentially upregulated during promoter-driven erbB2 overexpression. Transactivator proteins thought to bind these regulatory elements include Notch-activated RBPJk (R/N*), and members of the Ets (EBS), Sp1 (Sp1), AP2 (AP2) and CCAAT box binding protein (NFY) families. Other known regulatory features include the matrix attachment region (MAR) containing the 28 bp triplex-forming polypurine(GGA)-polypyrimidine(TCC) mirror-repeat and an open-chromatin region of DNase-I hypersensitivity (HS) centered over the Ets binding site (EBS) and mirror-repeat element

A conserved CCAAT box (from 777 to 773 bp, shown binding NFY) and non-conserved TATAA box (from 728 to 724 bp, not shown in Figure 9) present in the human promoter flank a conserved Ets binding site (EBS, the GAGGAA element from 735 to 730 bp) (Scott 2000)**.** It has been shown that several ets factors, which bind a sequence lying immediately upstream of the TATA box, enhance promoter activity (Scott 1994, Benz *et al*., 1997). These

factors may act by inhibiting the formation of a DNA triple helix, which inhibits the transcription of the gene (Scott 2000). AP-2 transcription factors also stimulate the promoter activity in breast cancer cell lines by binding two sequences in the *ERBB2* proximal promoter, located respectively 213 bp (Bosher 1996) and 500 bp (Vernimmen 2003) upstream of the transcription start site. Besides this proximal promoter fragment, some experiments revealed that distal regulatory elements were implicated in *ERBB2* gene transcription regulation (Grooteclaes *et al*., 1994). The sequence of a 1-kb1 DNA fragment, located upstream to the proximal 500-bp *neu* gene promoter, was shown to possess such repressor activity. (Suen 1990, Suen 2000). The 3-kb fragment upstream of the proximal promoter inhibited the transcriptional activity in several breast cancer cell lines, where they expressed low or high levels of the *ERBB2* transcript. This indicated that the fragment contains repressing elements able to overcome the positive effects of the proximal enhancers. Interestingly, the 2.2-kb fragment further upstream is able to restore high transcriptional activity only in BT-474 cells, which strongly overexpress the gene. It was showed that the distal *ERBB2* promoter region enhances the transcriptional activity in several *ERBB2* overexpressing breast cancer cells line, and thus being required for the overexpression. This specific transcriptional activity is accompanied by a specific nuclear binding activity, underlying that *ERBB2* expression level is governed dominantly by the transcription factors population or activity in the cells.(Delacroix 2005). It has been identified a novel transcripts of *ERBB-2* produced by a novel promoter at about 12 kb upstream of the previously described promoter of c-*ERBB-2*. In theory, downregulating *ERBB-2* may be accomplished more efficiently and permanently by blocking the 2 ± 10-fold ampliflied copies of this oncogene's promoter rather than targeting a geometrically greater number of erbB2 transcripts or the 105 to 106 steady-state receptor molecules present on each overexpressing cancer cell. Several anti-transcriptional erbB2 therapeutic strategies are currently under development and at least one such therapeutic agent has entered clinical trials based on its empirically observed repression of erbB2 promoter activity (Chen et al., 1997a; Hung and Wang, 2000).

Myc and ErbB2

Amplification of *ERBB2* leading to receptor overexpression is found in 20 to 25% of primary breast tumors. In these tumors, constitutive ErbB2 activation stimulates numerous intracellular signaling pathways including Ras/Erk and PI3K/Akt, both of which impact on Myc transcription and protein stability. The role of Myc has been examined in the ErbB2-overexpressing SKBr3 and BT-474 breast tumor cell lines. Treatment of both with the ErbB2-specific antibody trastuzumab caused a cell cycle block that was accompanied by a decrease in PI3K/Akt pathway activity, and by downregulation of Myc and D-type cyclins (Lane 2000) Interestingly, ectopic expression of Myc in SKBr3 cells partially rescued the cells from functional ErbB2 inactivation (Neve2000), focusing to the importance of Myc as an ErbB2 effector.

The Enolase gene family

Enolases (EC 4.2.1.11) are a group of enzymes which main catalytic function is the dehydration of 2-phospho-D-glycerate (PGA) to phosphoenolpyruvate (PEP) in the glycolytic pathway (Rider 1974). In these last years, anyway, Enolases genes and proteins have been found to serve multiple non catalytic functions in cells: playing roles in hypoxia tolerance (Semenza 1996), tumor suppression (He 2007, Lo Presti 2010;) and cell surface plasminogen binding (Wygrecka 2009) or acting as a lens tau-crystallin (Kim 1993), a DNA-binding protein (Wang 2005, Feo 2000), a beta-actin associated protein (Perconti 2007) and a tubulin/microtubule binding protein during myogenesis (Keller 2007).

Three major ENO genes have been described on the human genome, ENO1, ENO2 and ENO3, which encode the alpha, gamma and beta isoforms, respectively (Giallongo 1990). Two other human ENO-like genes have also been reported, ENO4 (Deloukas 2004) and ENO5 (also called ENOF1 or ENOSF1), originally identified as encoding an antisense transcript to the thymidylate synthase gene (Dolnick 1993) and more recently shown to play a role in regulating the TS locus (Liang 2005). Biochemical studies of vertebrate enolases have characterized several dimeric isozymes containing α-, β- and γ-subunits which are differentially but widely distributed in tissues of the body. ENO3 encodes the β-subunit and is predominantly expressed in muscle whereas ENO2 is more restricted to neural tissues (also called neuron-specific enolase or NSE) while ENO1 is expressed in virtually all tissues of the body including embryonic tissues and encodes the α-subunit.

During vertebrate development, major changes occur in the expression of these genes with a switch from ENO1→ENO3 and a change from αα to ββ enolase isozymes in skeletal muscle and a similar switch from ENO1→ENO2 in nervous tissues with an associated change from αα to γγ enolase isozymes.

Evolutionary studies have shown that DNA sequences encoding the enolase gene family are highly conserved from yeast to mammalian organisms and that the gene duplication events generating the ENO1, ENO2 and ENO3 genes may have predated the appearance of vertebrates (Tracy 2000, Piast 2005).

Myc Promoter Binding-Protein 1 (MBP1)

The role of c-myc promoter binding protein-1 (MBP-1) in tumor suppression has been demonstrated, in certain types of cancer, to be that of a general transcriptional repressor. MBP-1 was originally identified from a human cervical carcinoma Hela cells. It has been reported to bind to the TATA box of the human c-myc P2 promoter and to negatively regulate transcription c-Myc gene transcription by competing for the binding of the TATA-Binding protein (TBP) and preventing the formation of a transcription initiation complex (Ray 1991). Exogenously expressed MBP-1 reportedly suppresses cell growth, and induces apoptosis and necrosis in breast (Ray 1995), neuroblastoma (Ejeskar 2005) , or non-small-cell lung cancer cells (Ghosh 2006) via the transcriptional repression of c-myc or through physical interplay with its cellular partners (Ghosh 2005 Perconti 2007). More recently, the results of an in vitro experiment suggested that the physiological level of MBP-1 is modulated by the concentration of glucose and that a change in the expression of MBP-1 leads to an alteration in cell proliferation (Sedoris 2007). The antitumor activity of MBP-1 has also been demonstrated in human tumor xenografted mice (Ray 1995, Ghosh 2005). Thus, the expression level of MBP-1 appears to be a determining factor for cell growth, and alterations in its level by tumor microenvironmental factors may affect cancer development. MBP-1 has been reported to be a short form of the 48 kDa ENO1 protein. Sequence analysis of MBP-1 revealed 98% sequence identity with ENO1 cDNA in both the coding region and the 3'-UTR (Giallongo 1986). Coincidentally, both genes map to the 1p36 region on chromosome 1 (Onyango 1998, White 1997). Ghosh et al. in 1999 reported that the C-terminal MBP-1 protein, which is highly homologus to ENO1, exhibited transcriptional repression activity, and its activity was sufficient to stimulate regression of prostate tumor growth in nude mice (Ghosh 2005). Additionally, has been shown that ectopic expression of the short form of ENO1, lacking the first 96 amino acids, functions in a manner similar to MBP-1 (Feo 2000). They also showed that in vitro transcription and translation of the coding sequence of ENO1 can yield two polypeptides with apparent molecular masses of 48 and 37 kDa. In an RNase protection assay, hybridization of the total RNA of HeLa cells with a cRNA antisense probe corresponding to ENO1 gave a single transcript, suggesting that the same transcript may encode both ENO1 and MBP-1. Moreover, site-directed mutagenesis of Met94 and Met97 on the ENO1 cDNA further supports this single-transcript hypothesis and suggests that MBP-1 is a product of alternative translation initiation of the ENO1 transcript (Subramanian 2000).

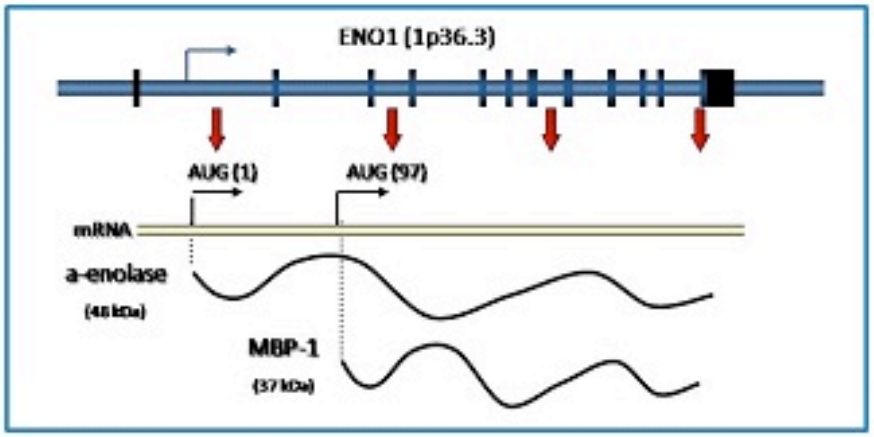

Figure 10 ENO1 gene is traslated into two proteins: α-enolase and MBP-1. From the ENO1 mRNA are traslated both α-enolase and MBP-1. The α-enolase begins from the AUG in position 1, MBP-1 begins from AUG in position 382, corresponding to a methionine in position 97 of α-enolase protein.

Aim of the study

We have previously shown that nuclear MBP-1 is expressed in almost all normal breast epithelial cells, but loss of expression has been found in 65% of primary ductal infiltrating carcinoma (IDC). In these breast tumors MBP-1 expression is associated with good survival and is inversely correlated with ErbB2 and Ki67 protein expression (Lo Presti et al., 2010).

Based on our recent results, that show that ErbB2 expression is inversely correlated with MBP1 expession, we hypothesized that the MBP1 regulate *ERBB2* gene transcription. To verify this hypotesis:

- We induced MBP1 overexpression in a cell line overexpressing ErbB2, to see the direct effect of MBP1 on the ErbB2 protein expression.
- We constructed deletion mutants of *ERBB2* promoter in order to verify the direct effect of MBP1 on that promoter and to eventually identify the region where molecular binding takes place
- We performed a chip assay in order to verify the direct binding of MBP1 protein on *ERBB2* promoter DNA sequence.

In order to understand the role of tumor protector of MBP-1, we tried to identify genes that are differentially expressed (DE) between MBP-1+ve and MBP-1-ve breast cancer patients, we performed a global gene expression analysis using Agilent whole genome 44k microarray in a two-color arrays assay.

RESULTS AND DISCUSSION

Preliminary results

We have previously shown (Lo Presti 2010) that nuclear MBP-1 is expressed in almost all normal breast epithelial cells, but we have found loss of expression in 65% of primary ductal infiltrating carcinoma (IDC). All the 177 tissue samples included in that study were scored positively cytoplasmic α-enolase expression. With respect to the number of enolase labelled cells, at least 50% cytoplasmic labeling was observed in all cases. In addition, α-enolase expression was predominantly detected in the cytosol of tumor cells, whereas a weak staining was observed in stromal and myoepithelial cells. All normal breast tissues showed moderate expression of cells labelled α-enolase, whereas stronger expression was observed in the paired tumor samples, however, no significant correlation between expression of the protein and clinicopathological characteristics of tumors or patients outcome was observed. To grade tumors relatively to MBP-1, a cut-off value for nuclear MBP-1 expression was chosen as 20% of stained nuclei, and it was used to define tumors as MBP-1- negative (<20%, absent/low expression) or MBP-1-positive (>20%, medium/high expression). Statistical analyses were done to examine the correlation between nuclear MBP-1 expression, as detected by immunohistochemical staining, and the clinicopathological characteristics of breast cancer.

As shown in Figure 11.A, in patients with IDC no correlation was found between the expression levels of nuclear MBP-1 and patient age, tumor size, death by disease or estrogen and progesterone receptors expression levels. . In contrast, MBP-1 expression was strongly correlated with the node status (P = 0.0002), tumor grade (P,0.0001) and inversely correlated with expression levels of ErbB2 (P = 0.0001) and Ki67 (P = 0.0096) proteins.

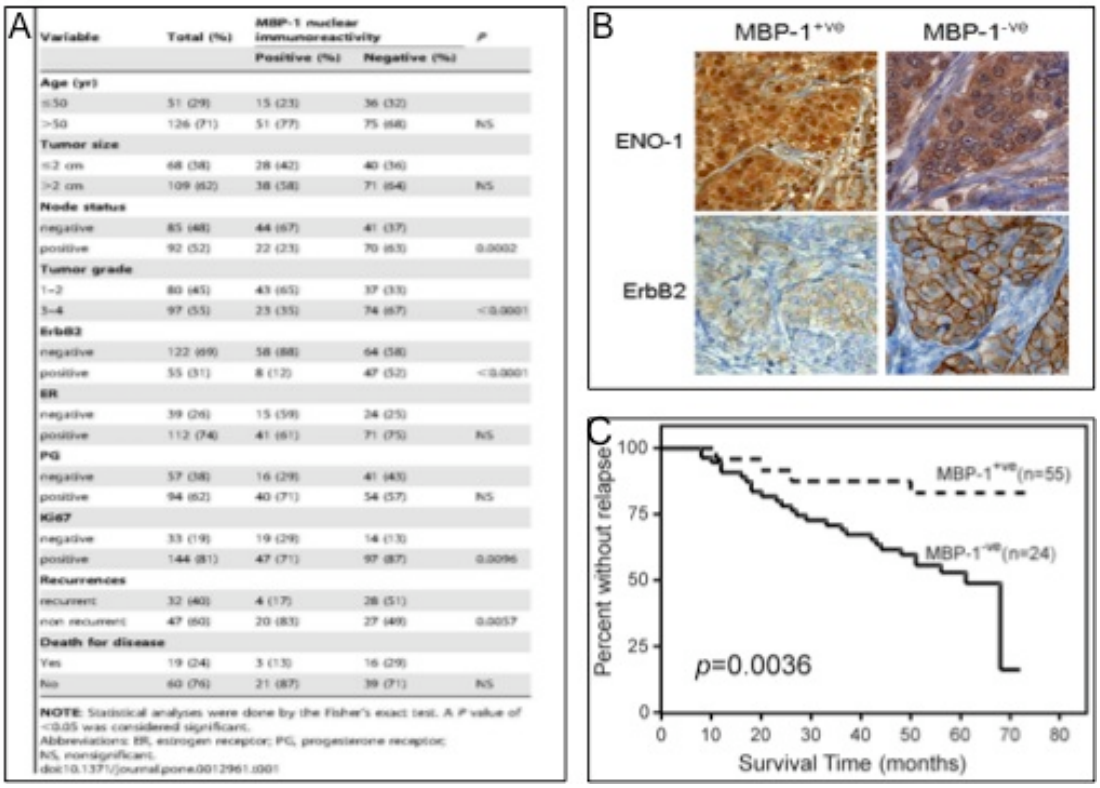

Figure 11 Preliminary results (Lo Presti 2010): A Correlation between MBP-1 expression and clinicopathological characteristics of breast cancer patients. **B** MBP-1 nuclear staining correlated with ErbB2 expression. Immunohistochemical staining for α-enolase and ErbB2 in MBP-1-positive (S1) and MBP-1-negative (S2) tumors. Magnification: 500x. **C** Survival analysis of IDC patients according to MBP-1 expression.

MBP-1-positive expression also significantly correlated with a 92% local recurrence-free survival. In contrast, for IDC samples that showed the loss of MBP-1 expression, recurrence-free survival fell to 54% at five year after surgery (p= 0.0036) (Figure 11).

A multivariate analysis was performed, according to Cox regression model, for disease-free survival, including as covariates MBP-1, ErbB2 expression and lymph node status. These last two factors were chosen based on the results of the univariate analysis and because they are known to influence survival of breast cancer patients. Nuclear MBP-1 expression was found to be an independent favourable prognostic indicator for disease-free survival .

MBP-1 regulates ERBB2 expression in SKBR3 breast cancer cells

Based on our observation that nuclear MBP-1 expression is mainly retained in ErbB2-negative tumors, we hypotized the involvement of MBP-1 in the negative regulation of the gene *ERBB2*, like already reported for the c-Myc gene. To investigate the influence of MBP-1 on *ERBB2* transciption levels we chose the SKBR3 cell line. This breast cancer derived line is characterized by high expression levels of both *ERBB2* and *MYC* onocogens. SKBR3 breast cancer cells were transfected with a vector expressing high levels of MBP-1 (pFLAG-MBP-1). In pFLAG-MBP-1 plasmid, the coding sequence of MBP-1 is placed in frame with the Flag epitope sequence (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) under the control of CMV promoter (a strong promoter). (FIG. 12)

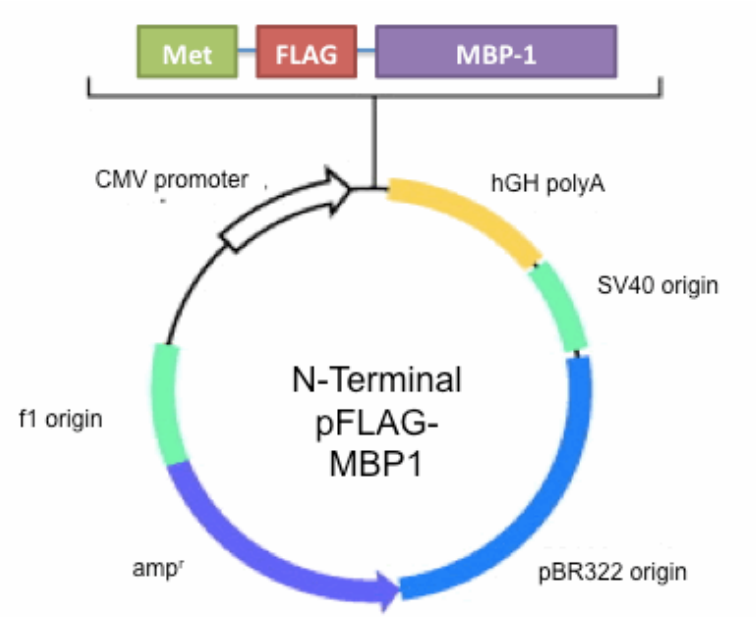

Figure 12 Plasmid pFLAG-MBP1. This plasmid contains the MBP-1 coding sequence placed in frame with the Flag epitope sequence, and placed under the control of cytomegalovirus promoter. The plasmid also contain the pBR322, a low number replication origin, the SV40 origin of replication, which allows transient episomal replication in cells expressing the SV40 large T antigen, f1 ori region which allows for the production of single-stranded phagemid DNA.

SKBR3 were trasfected with pFLAG-MBP-1 and, as control, with an empty vector (mock). After 48 hours since transfection cells were harvested for RNA extraction and the RNA was retrotrascripted in cDNA. In order to measure the abundance of the endogenous *ERBB2* and *MYC* trascripts, we performed a quantitative real-time PCR experiment using SYBR® Green dye. For the quantitation of *ERBB2* and *MYC* transcripts we used the comparative Ct method. This method allows us to compare the Ct values of the samples with a control such as a non-

treated sample or a sample treated with the mock plasmid. The Ct values of both the control and the samples of interest are normalized to an endogenous housekeeping gene (TBP *TATA Binding Protein)*. The comparative Ct method is also known as the 2-[delta][delta]Ct method where [delta][delta]Ct = [delta]Ct_{sample} - [delta]$Ct_{reference}$. Here, [delta]Ct_{sample} is the Ct value for any sample normalized to the endogenous housekeeping gene and [delta]$Ct_{reference}$ is the Ct value for the calibrator also normalized to the endogenous housekeeping gene. For the [delta][delta]Ct calculation to be valid, the amplification efficiencies of the target and the endogenous reference must be approximately equal. As shown in figure 13, endogenous *ERBB2* mRNA significantly decreases (~60%) in presence of MBP-1 overexpression. In transfected cells we can also see, as expected, the decrease of *MYC* mRNA.

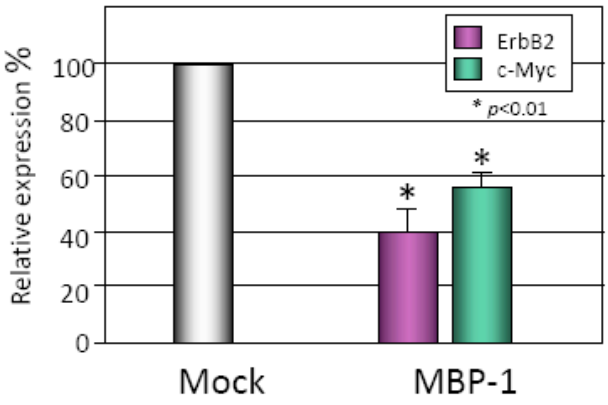

Figure 13 Real Time PCR on RNA extracted from trasfected cells and controls. SKBR3 cells were transfected either with a vector expressing MBP-1 (pFlag-MBP-1) or empty vector (mock). After transfection cells were harvested for RNA extraction and abundance of the endogenous ERBB2 and c-Myc transcripts was measured by quantitative RT-PCR. MBP-1 overexpression negatively regulates endogenous ERBB2 mRNA, as well c-Myc mRNA.

In order to verify that the decreased levels of ErbB2 transcript are due to MBP1 overexpression, ie that in cells that are overexpressing MBP1, ErbB2 decreases. SKBR3 were trasfected with pFlag-MBP-1 and, as control, with a vector expressing the green fluorescent protein GFP (pEGFP-N1). Trasfected cells were analyzed by immunofluorencence, we used specific antibodies against Flag epitope and cellular ErbB2. As shown in figure 14, trasfected cells that overexpressed MBP-1 (green) showed a strong decrease of ErbB2 (red) mainly in the membrane; control cells, overexpressing the GFP protein (green) had normal levels of ErbB2 protein (red). Spatial distribution of the proteins was visualized by confocal miscroscopy.(Fig 15)

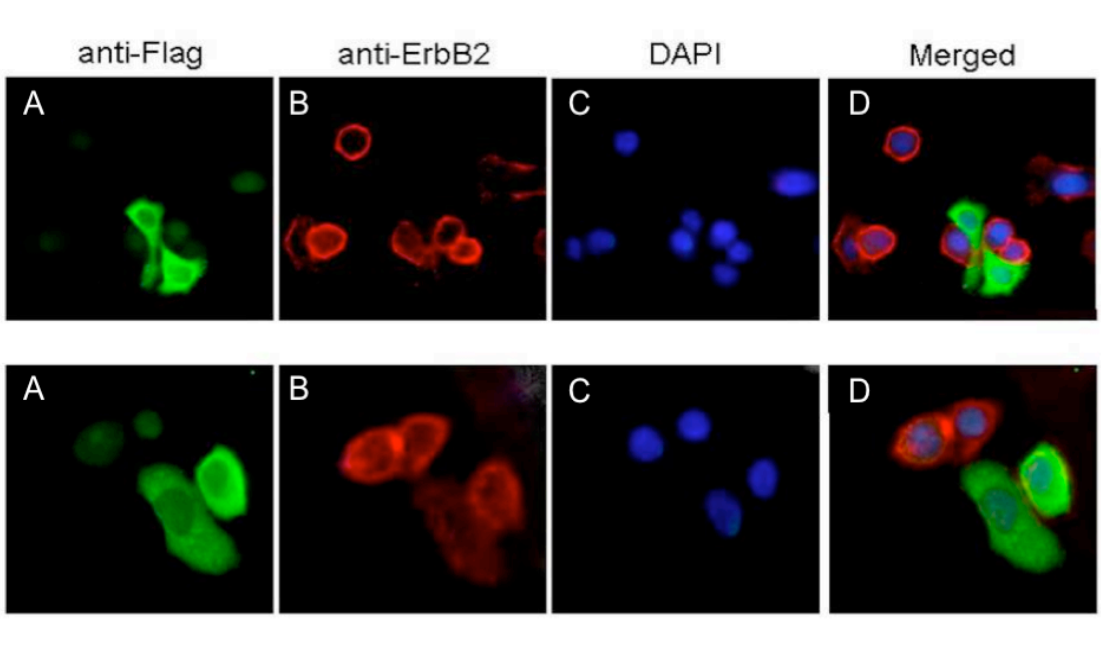

Figure 14 Immunofluorescence images made with epifluorescence microscope. The fusion protein FLAG-MBP-1, shown in green, shows a predominantly nuclear localization. This index of plasmid transfection and expression in cells SKBr3. The product of the ErbB2 gene is shown in red. Nuclei are visible in blue (DAPI). Transfected cells show a decrease of ErbB2 protein levels, as shown in the image with the fluorescence in red and the "Merged".

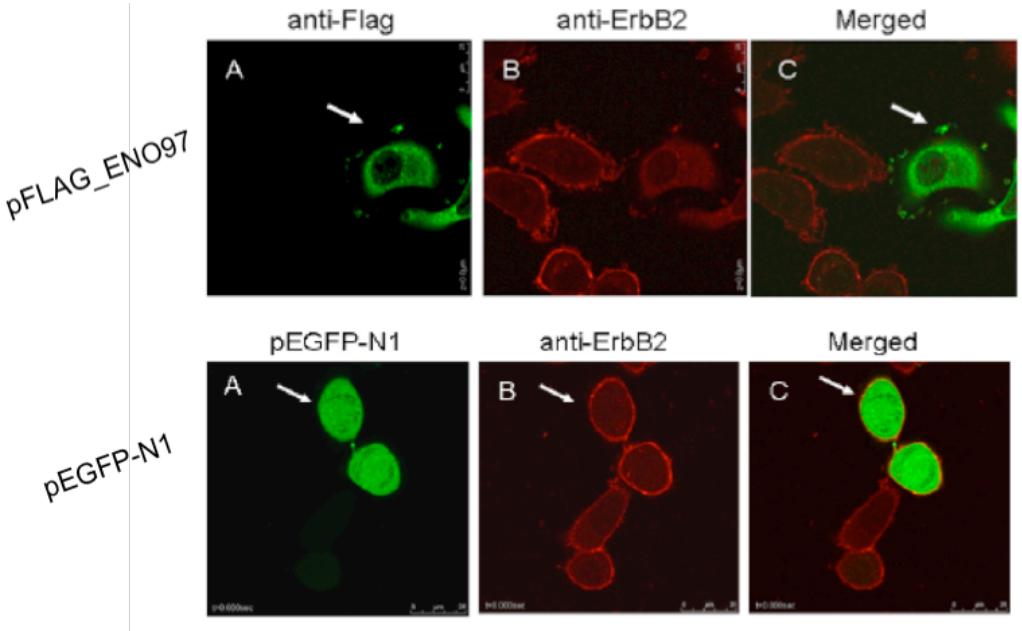

Figure 15. Images captured with confocal software and microscope (Leica). In the first lane pFLAG-ENO97 transfected SKBR3 cells, expressing MBP-1 (green), show a decrease of ErbB2 protein levels on cellular membrane (B-C). In the second lane pEGFPN1 trasfected cells, expressing GFP (green), have normal levels of ErbB2 protein on cellular membrane (B-C).

Exogenous MBP-1 regulates ERBB2 promoter

Reporter Vector costruction

To determine if MBP-1 repression activity is excerted directly to the *ERBB2* promoter and where is the binding site, we trasfercted in breast cancer cells, firefly luciferase reporter vectors, containing three ERBB2 promoter fragments of increasing sizes. This reporter vectors were constructed using the *pGL3 Luciferase Reporter Vector Promega basic* (promega). This plasmid is 4818bp long, it contains the coding sequence of Firefly (*Photinus pyralis*) luciferase but it doesn't contain promoter sequence or enhancers. We made three reporter vectors with ERBB2 promoter fragments of increasing size: 300, 560 and 780 bp. The 300 bp fragment contains the proximal promoter, the 560bp fragment contains binding sites of some regulatory proteins (Grooteclaces 1994), the 760 bp fragment contains a region that was shown to have strong trascriptional activity (Delacroix 2005). The DNA fragments were obtained by PCR amplification, starting from a genomic DNA extracted from a human-mouse somatic hybrid containing only human chromosome 17. Oligonucleotides (for sequences see Materials and Methods) used for amplification were synthesized in order to put a NheI site the 5 'end and a BglII site at the 3 'end of all the fragments amplified. (Fig.16)

300 bp and 560 bp fragments were cut with NheI e BglII and cloned into pGL3-basic, cut with same enzymes. Insertion of 780 bp fragment in PGL3-basic plasmid failed, probably due to poor efficiency of PCR. To overcome this problem, we inserted this fragment into plasmid pGEM-T-easy (that needs 3' A-tailed) and it was subsequently excised by cutting with BglII and NheI. After elution from the agarose gel, fragment was inserted into plasmid pGL3-basic. Using NheI and Bgl II we made a oriented cloning, ie we put the fragments of the promoter upstream of the luciferase gene. We checked the proper insertion of the fragments into pGL-3 basic plasmid using direct automatic sequencing. The plasmids were named pGL3-ER300 pGL3-ER560 and pGL3-ER780

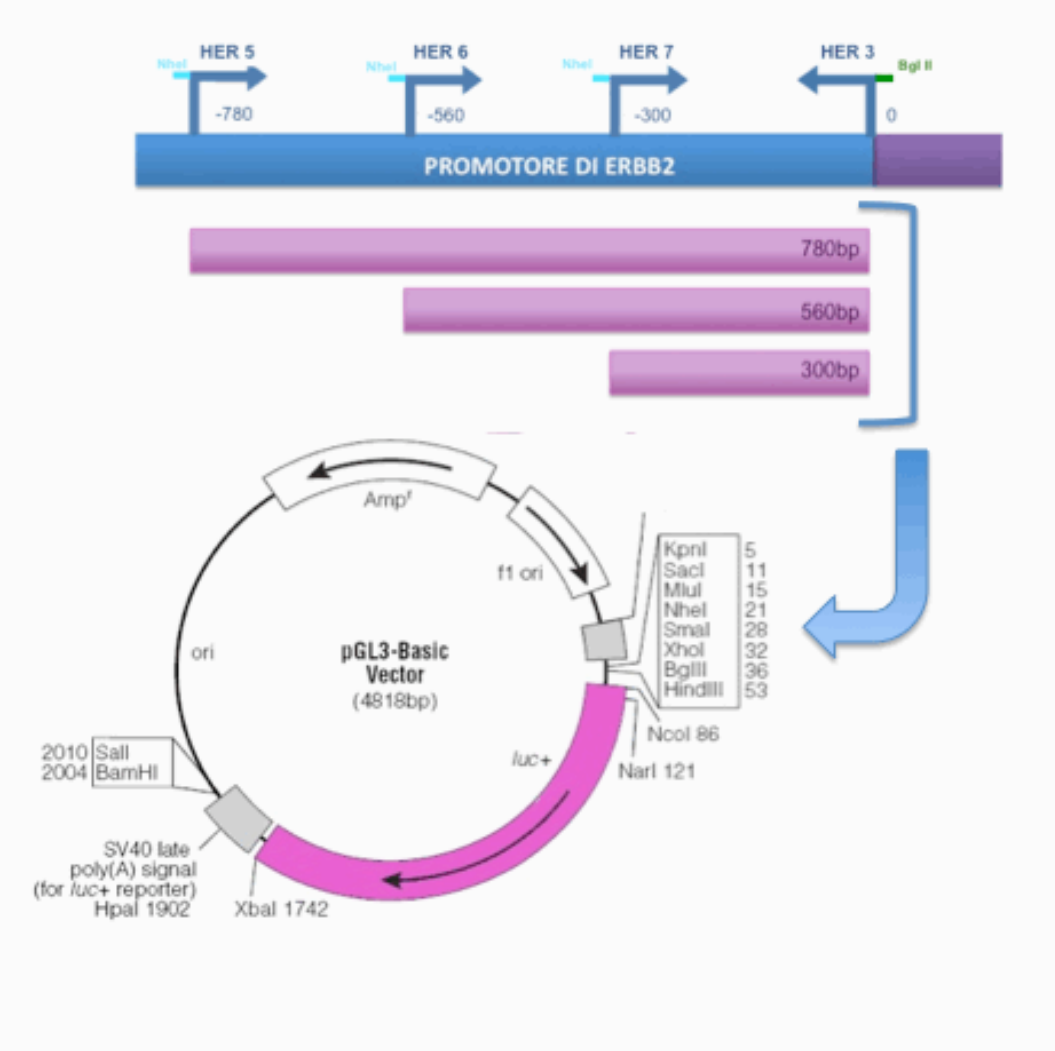

Figure 16 Construction of reporter plasmids. *ERBB2* promoter fragments of increasing size were amplified with primers that create NheI-BglII restriction sites at the ends. After enzymatic cleavage of the fragments and the plasmid, the fragment was inserted upstream of the luciferase gene (pink).

Reporter essays

In order to see if MBP1 directly influences the activity of *ERBB2* promoter, we trasfected SKBR3 cells with the three *ERBB2* promoter reporter construcs (pGL3-ER300 pGL3-ER560 and pGL3-ER780). Each trasfection was done using one of three constructs in parallel (Fig XX) with an effector plasmid, (pFlag-MBP-1 or pUC18) and with a plasmid that codify for β-galattosidase enzyme (pSV β-gal). Empty pGL3-basic and pGL3-control, containing a luciferase gene driven by the SV40-promoter, were used as negative and positive controls, respectively. pFlag-MBP-1 allows production of our effector MBP-1, pUC18B is a silent vector. pSVβ-gal allow us to normalize results for transfection efficiency (for plan of transfection see materials and methods).

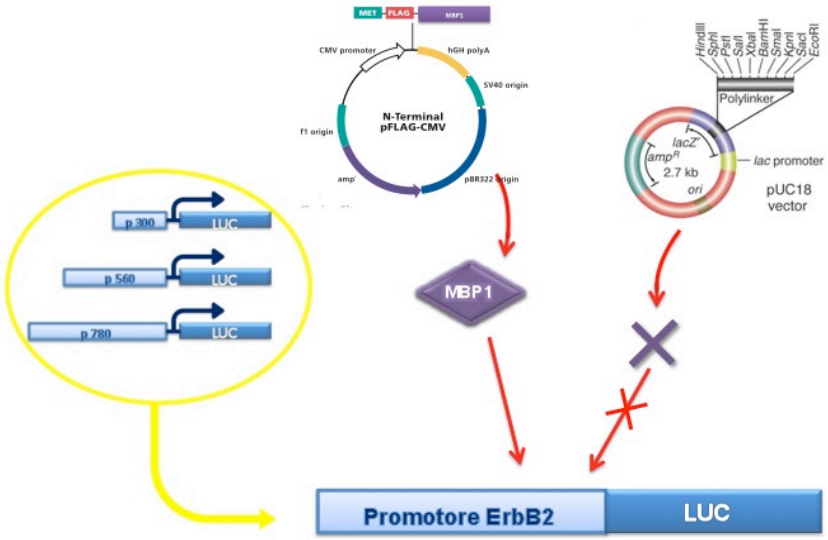

Figure 17 Schematic representation of transfection strategy into SKBr3 cells. MBP-1 was produced plasmid p-Flag-ENO97. We tested MBP-1 activity on reporter plasmids containing promoter *ERBB2* fragments of gene. We compared transcription levels of luciferase gene in presence of MBP-1 and in presence of the silent plasmid pUC18B

Cells were harvested and lised after 24 hours since trasfection, lysates were assayed for the activity of β-galactosidase and luciferase through a luminescence system. We did five series of transfection experiments and the data obtained were used as follows: the values of luminescence were normalized to the values of β-galactosidase to correct for transfection efficiency. We averaged the luminescence corrected data across experiments and the averages of each set of experiments was related to the values obtained by transfection with plasmid control pGL3basic, which was arbitrarily assigned a value of 1. The results, shown in figure 18 B, indicate that in presence of MBP-1 expression there is strong reduction (from 78% to 85%) in the reporters pGL3-ER560 and pGL3-ER780 bp.

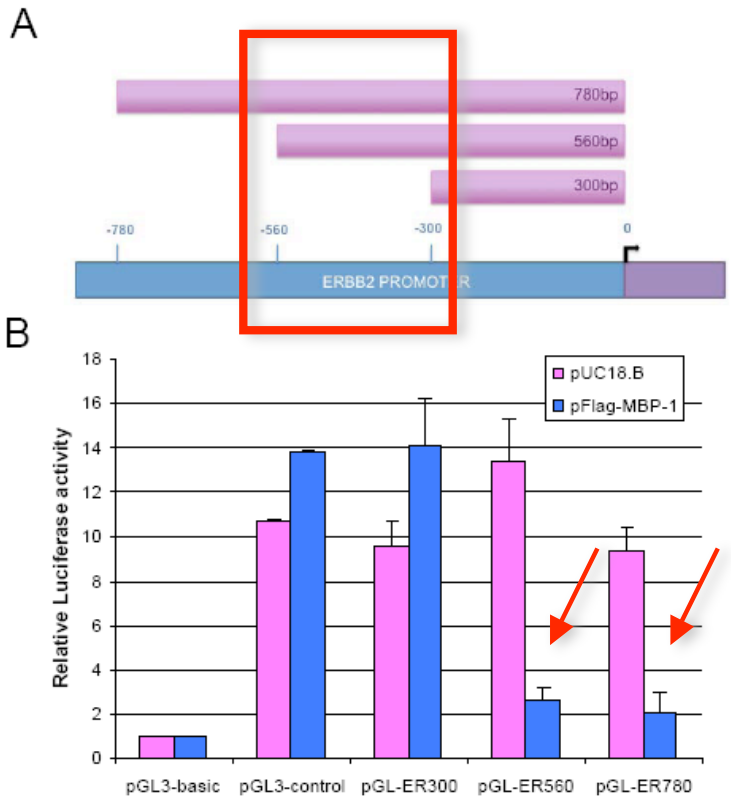

Figure 18. (A) Graphical representation of the ERBB2 promoter and the derived luciferase reporter vectors. **(B)**. Luciferase activity of ERBB2 promoter vectors in the presence (blue bars) or absence (pink bars) of effector plasmid expressing MBP-1 in SKbr3. The results are presented as fold induction above the luciferase activity obtained with the pGL3-basic vector, which was arbitrarily assigned the value of 1.

These results suggest the existence of a promoter region of the *ERBB2* gene, between nucleotides -560 and -300, which could be involved in the connection with MBP-1. This region, interacting with MBP-1, could be, in fact, responsible for trascriptional repression observed in the reporter plasmids and in the endogenous gene, as previously shown. The analysis of the nucleotidic sequence of this region suggests the existence of several consensus regions for binding factor MBP-1 (indicated in red in figure19), which was reported link a/t rich regions (Chaudhary et al., 1995).

-561ACTTCAAAGATTCCAGAAGATATGCCCCGGGGGTCCTGGAAGMCACAAGGTAAA
CACAACACATCCCCCTCCTTGACTRTCAATTTTACTAGAGGATGTGGTGGGAAAACCAT
TATTTGATATTAAAACAAATAGGCTTGGGATGGAGTAGGATGCAAGCTCCCCAGGAAA
GTTTAAGATAAAACCTGAGACTTAAAAGGGTGTTAAGAGTGGCAGCCTAGGGAATTT
ATCCCGGACTCCGGGGGAGGGGGCARAGTCAC -300

Figure 19. ERBB2 promoter sequence -561/-300 , target candidate for MBP-1 binding

MBP-1 binds ERBB2 promoter *in vivo*

Putative interactions between the *ERBB2* promoter region -560/-300 and MBP-1 protein were investigated in vivo by chromatin-immunoprecipitation (ChIP).

Because it doesn't exist an antibody that can discriminate MBP-1 from alpha enolase. In order to immunoprecipitated chromatin bound to MBP-1, we have chosen to express MBP-1 tagged with Flag epitope recognized by a specific antibody. We trasfected SKBR3 \cells with pFlag-ENO97 pasmid, transfections were performed following the protocol described in materials and methods. 3 plates 100 mm were treated simultaneously in order to have transfected cells on which to perform western blot experiments, and cells on which perform immunoprecipitation experiments. In fact, western blot analysis was performed to confirm the successful transfection and subsequent expression of recombinant protein MBP1-Flag.

Western Blot

In order to check the expression of the protein MBP1- Flag, we extracted proteins from one plate of transfected cells. We performed a western blot analyzing protein lysates of transfected cells and in parallel SKBR3 cell lysates as a negative control The results (Fig. 20 showed that only proteins extracted from cells transfected had reactivity of anti-Flag after immunodetection. This confirm that cells were transfected and that MBP-1-Flag was successfully synthesized. On the same filter we used an antibody against β-actin, to normalize MBP-1-Flag expression and and to control amount of lysate loaded on gel.

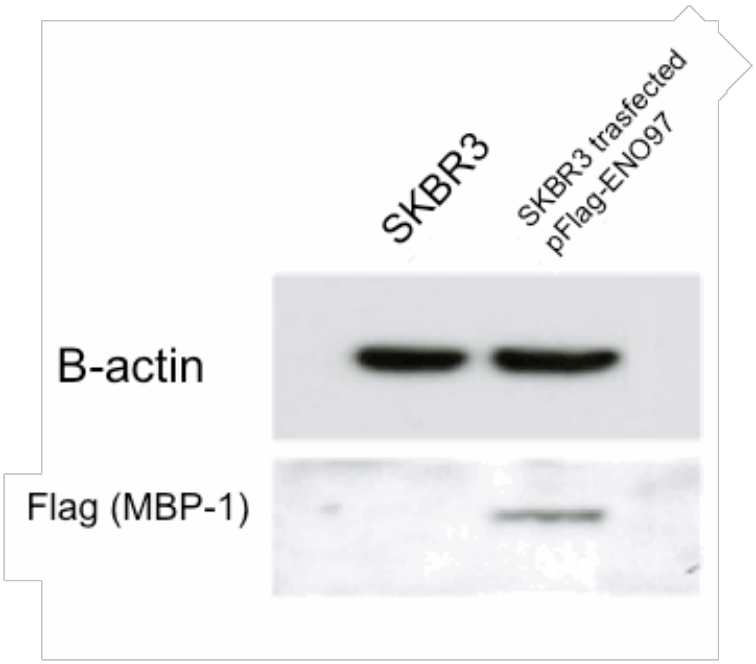

Figure 20 Western blot results. Flag-MBP-1 is expressed only in SKBR3 transfected with pFlag-ENO97. β-actin was used as control**.**

Chromatin immunoprecipitation

ChIP analysis was performed with chromatin isolated from lysates from pFlag-MBP-1 trasfected SKBR3 cells. As described in materials and methods, we cross-linked DNA and proteins with formaldehyde, then genomic DNA was fragmented by sonication in fragments from 200bp to 600bp. Correct chromatin sonication was verified by electrophoresis on agarose gel (Fig 21). DNA-protein complexes were immunoprecipitated with and without (as negative control) anti-Flag (SIGMA).

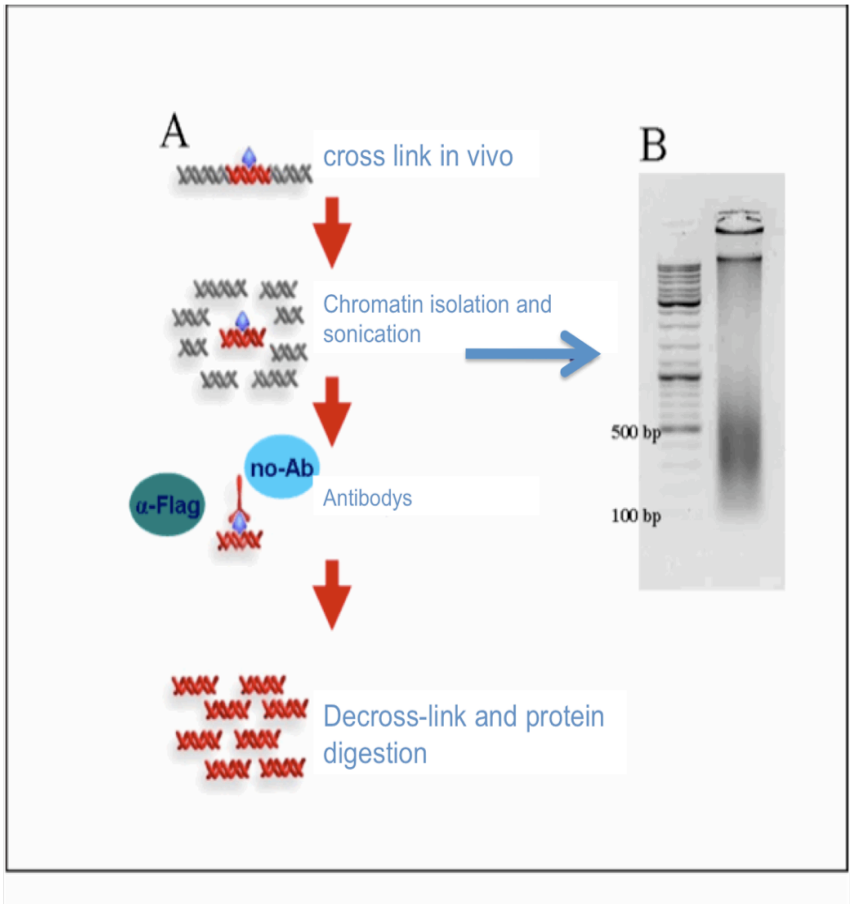

Figure 21 A. schematic representation of the various stages of chromatin immunoprecipitation **B.** Sonicated chromatin on agarose gel, average size fragments is of 200-600 bp

After de-cross-link and protein digestion, the samples obtained were analyzed by Real-time PCR. . Two sets of primers were used to amplify the ERBB2 -560/-300 promoter region: a set (ERP1-2) detects the binding to -561/-401 region and the other one (ERP2-3) detects the binding to -503/-275 (Figure 3A). As negative control we used a primer set (MB48) amplifying an 210 bp genomic located between MYC and PVT-1 human genes. As shown in figure 3B and 3C anti-FLAG abs immunoprecipitates yielded a significant enrichment of ERBB2 promoter-specific PCR products. No such enrichment was observed in the absence of

specific immunoprecipitating abs nor when MD48 primers were used. Taken together, these results indicate that MBP-1 directly binds to -560/-300 region of the ERBB2 gene promoter.

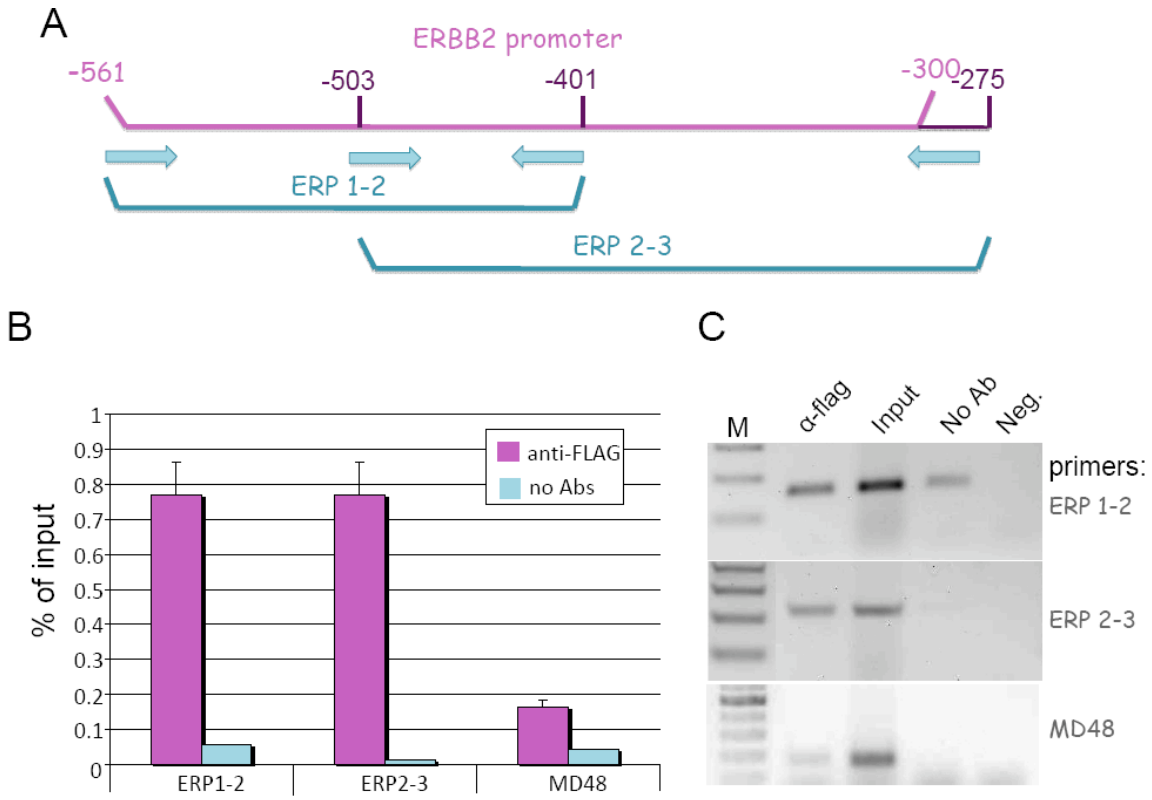

Figure 22. SKBr3 cells were transfected either with a vector expressing MBP-1 (pFlag-MBP-1) or empty vector (mock) and ChIP analysis was performed after immunoprecipitation with anti-Flag abs, as described in materials and methods. **(A)**. Graphical representation of ERBB2 promoter fragment -560/-300 and position of primers used in ChIp analysis. **(B)** Real time PCR on input, immunoprecipitated and negative control. The amount of immunoprecipitated DNA was calculated relative to the present in total input chromatin (%input). **(C)** End point PCR analysis of immunoprecipitated chromatin.

Differentially expressed gene in MBP-1^{+ve} versus MBP-1^{-ve} ErbB2^{-ve} IDC of the breast

In order to identify genes that are differentially expressed (DE) between MBP-1^{+ve} and MBP-1^{-ve} breast cancer patients, MBP-1^{+ve} and MBP-1^{-ve} non metastatic infiltrating ErbB2^{-ve} breast IDCs and their normal tissue counterparts were selected from archived material (Lo Presti et al., 2010).

The analyzed data set consists of 15 samples, including 9 tumors and 6 matched adjacent normal samples. Microarray experiments were run on double channel microarray platforms (Whole Human Genome 44K probes: Agilent G4112F). A pool of normal was used as a common baseline (see Matherials and Methods). For each sample, gene expression ratio values were calculated by comparing the individual sample (tumor or normal) with the respective common baseline.

To characterize the overall diversity between individual samples, we performed an unsupervised hierarchical clustering of all 15 samples, arbitrarily assigned to three major classes: Normal (N), MBP-1^{+ve} (MP) and MBP-1^{-ve} (MN). As hown in figure 23the first bifurcation of the hierarchical clustering dendrogram identifies two clusters of samples, which represent non-random distributions of normal and malignant samples from the complete population.

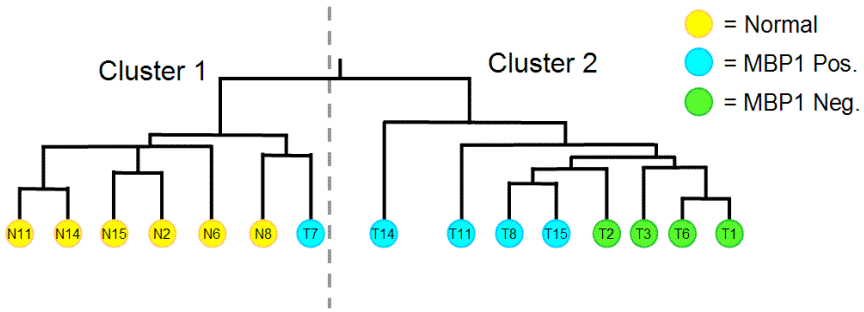

Figure 23. Unsupervised hierarchical clustering of 15 samples based on a subset of 23 most variable genes selected using a filter of Two-fold change or more on at least two samples. Samples are colored according to classification and numbered according to the individual origin.

Normal samples are over-represented in cluster 1 (Fisher's exact test; *P* =2e-4), segregating into the same sub-cluster, while tumors are over-represented in cluster 2 (Fisher's exact test; *P* =2e-4). Interestingly, samples from the MBP-1^{+ve} class were mainly distributed across the two clusters, as expected by their intermediate phenotype falling between Normal and MBP-1^{+ve} samples.

Genes differentially expressed in the three sample groups were then identified by ANOVA analysis. Using a conservative q-value threshold of 0.001 a list of 138 genes was selected. According to their patterns of expression, two groups of genes could be identified (Figure 24). Each group was analyzed separately by DAVID for gene set enrichment analysis (see Matherials and Methods).

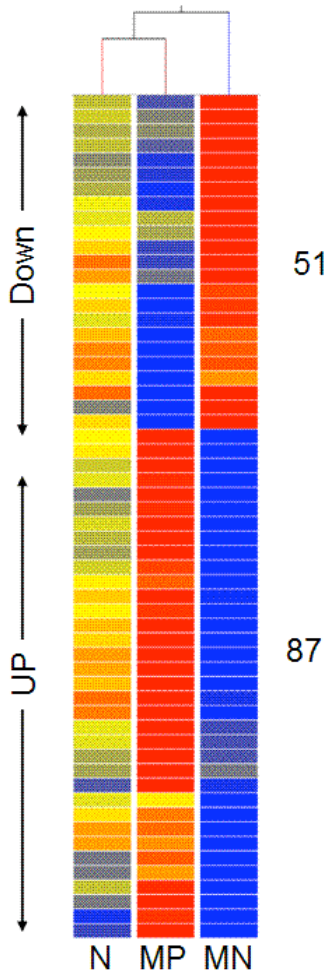

Figure 24. Identification of gene DE in mammary samples. Hierarchical clustering of samples based on a subset of 138 genes selected by one-way ANOVA (q-value < 0.001). For display purposes, samples in each class (Normal, MBP-1^{-ve}, MBP-1^{-ve}) were clustered togheter and arranged from Normal (left) to MBP-1^{-ve} (right). Genes were grouped in two groups (Down and Up) according to their pattern of expression. The gene size of each group is indicated to the right of each cluster.

Genes differentially expressed in the three sample groups were then identified by ANOVA analysis. Using a conservative q-value threshold of 0.001 a list of 138 genes was selected. According to their patterns of expression, two DE groups of genes could be identified (Figure XX). Each group was analyzed separately by DAVID for gene set enrichment analysis (see Matherials and Methods).

Gene set enrichment	P-Value	
	Down regulated	Up regulated
GO:0031982~vesicle	0.04821	
GO:0031410~cytoplasmic vesicle	0.04332	
GO:0031988~membrane-bounded vesicle	0.03173	
GO:0007049~cell cycle	0.02945	
GO:0016023~cytoplasmic membrane-bounded vesicle	0.02921	
GO:0016044~membrane organization	0.02023	
GO:0007050~cell cycle arrest	0.01097	
GO:0005576~extracellular region		0.00041
GO:0006952~defense response		0.00147
GO:0005615~extracellular space		0.00178
GO:0044421~extracellular region part		0.00225
GO:0016620~oxidoreductase activity		0.00601
GO:0006954~inflammatory response		0.00759
GO:0006081~cellular aldehyde metabolic process		0.00966
GO:0060627~regulation of vesicle-mediated transport		0.01451
GO:0005125~cytokine activity		0.01725
GO:0009611~response to wounding		0.02234
GO:0009986~cell surface		0.02821
GO:0006955~immune response		0.02971
GO:0004030~aldehyde dehydrogenase [NAD(P)+] activity		0.03012
GO:0032103~positive regulation of response to external stimulus		0.04557
GO:0002262~myeloid cell homeostasis		0.04691
GO:0008083~growth factor activity		0.04858

Figure 25 Graphical representation of the significance of gene set enrichment for the two gene groups described in (a). Enrichment scores are computed by one-sided Fisher's exact test.

Microarray data validation

We performed quantitative Real Time PCR analysis to validate expression profiles of six genes selected from the 138 differentially expressed genes in MBP1^{+ve} vs MBP1^{-ve}. (Bax, CNTNAP2 (Contactin Associated Protein-Like 2), MammaglobinA, LipofillinB, TAZ and GDF15).

Total mRNA was isolated from an independent group of MBP-1^{+ve} (n=12) and MBP-1^{-ve} (n=12) ERBB2^{-ve} IDCs. TATA-binding protein (TBP) expression levels were used to normalize qRT-PCR data. Normalized results are relative to the expression level detected for each gene in normal breast tissue (n=6). Real-time PCR result for MammaglobinA, LipofillinB, TAZ and GDF15 genes statistically confirmed the differential expression according to MBP-1 status (Fig 26). Real-time PCR result for Bax and CNTNAP2 showed no differences between MBP-1^{+ve} and MBP-1^{-ve} tumors.

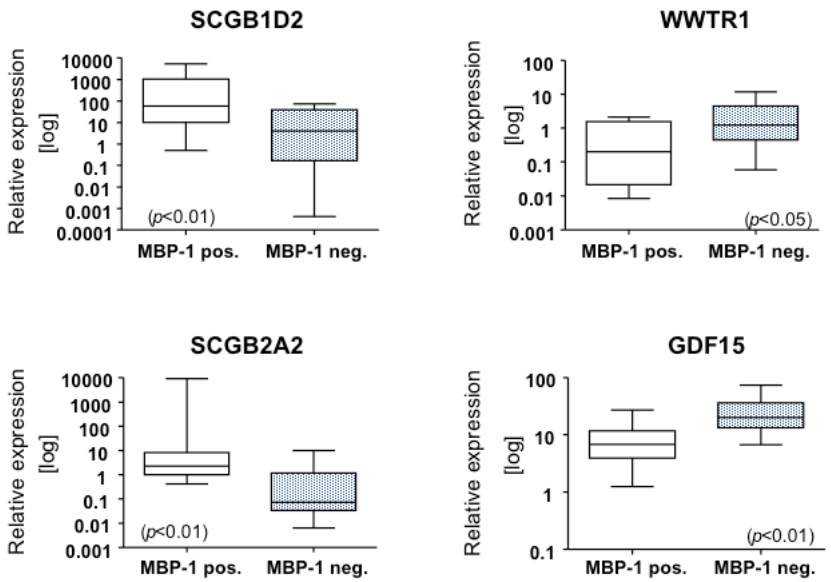

Figure 26. qRT-PCR validation of DE expressed gene in MBP-1^{+ve} vs MBP-1^{-ve} IDC. Quantitative RT-PCR validation of four genes (SCGB1D2, MammaglobinA; SCGB2A2, LipofillinB, WWTR1, TAZ and GDF15) in an independent group of MBP-1^{+ve} (n=12) and MBP-1^{-}ve (n=12) ERBB2^{-ve} IDCs. TATA-binding protein (TBP) expression levels were used to normalize qRT-PCR data. Normalized results are relative to the expression level detected for each gene in normal breast tissue (n=6). Statistic significance was computed as described in materials and methods.

MATERIALS AND METHODS

Cell culture

The human breast cancer cell line SKBR3, was purchased from American Type Culture Collection (Rockville, MD) and cultured in DMEM medium supplemented with 10% fetal bovine serum, 2 mM glutamine and 100 μg/ml penicillin/streptomycin (Invitrogen).

Transfection experiments

SKBR3 cells were transfected with Lipofectamine LTX reagent in OptiMem medium as instructed by the manufacturer (Invitrogen). For immunofluorescence assays $1.5x10^5$ SKBR3 cells, grown in multiwell culture plates (12 well), with 1.5 μg of pFLAG-MBP1 or pEGFPN1 plasmids were used. For RT-PCR, western blot and Chip analyses 1.5x105 cells, in cell culture dishes (10mm), with 7.5μg of pFLAG-MBP1 were used. Luciferase report assay were performed with $5x10^4$ cells in multiwell culture plates (12 well) with 0.5 μg of the appropriate luciferase reporter construct and 1 μg of pFLAG-MBP1 or pUC18B as effector plasmids. To correct for variability in transfection efficiency 0.5 μg of the β- galactosidase expression vector pSV-β-gal (Promega) was included in each transfection experiment.

Immunofluorescence

Trasfected SKBR3 cells were grown on glass cover slip in multiwell culture plates (12 well) for 72 hrs and fixed for 10 min in 3.7% paraformaldehyde/PBS buffer. After incubation with Triton X-100 (0.1%), the cells were blocked with 3% of BSA in T-TBS for 45 min at room temperature. The cells were incubated O.N. at 4°C with the primary anti-Flag (Sigma) and anti ERBB2 antibodies (Ventana). After washing, fixed cells cells were incubated with antirabbit *IgG* AlexaFluor488 and anti-mouse IgG AlexaFluor594. Finally the cover slips was mounted with anitifade reagent with DAPI (Invitrogen). The slide was observed with a confocal microscope Leica-DMRXA.

RNA extraction

RNA from tissues was extracted by homogenization (Polytron; Kinematica, Lucerne, Switzerland) in 1ml of TRIzol reagent (Invitogen) (added to cell pellet) at maximum speed for 90–120 s. For RNA extraction, cell were harvested, pelleted in PBS, and resuspended in TRIzol reagent. The homogenate was allowed to incubate for 5 min at room temperature, a 1:5 volume of chloroform was added, and the tube was vortexed and, finally, subjected to centrifugation at 12,000 *g* for 15 min. The aqueous phase was isolated, and a one-half volume of isopropanol was added to precipitate the RNA. After this initial isolation, a secondary purification was performed with the RNeasy Total RNA isolation kit according to manufacturer's specifications (Qiagen). The purified total RNA was finally eluted in diethyl pyrocarbonate-treated H_2O, and quantity and integrity were characterized using a Nanodrop UV spectrophotometer and Agilent Bioanalyzer 2100.

Quantitative real-time PCR analysis

Changes in gene expression were analyzed by real-time PCR, performed on an Prism 7300 sequence detection system (Applied Biosystems). cDNA was synthesized from total RNA (1 μg per sample) in a reverse transcriptase (RT) reaction in 20 μl of 5x first-strand synthesis buffer (Invitrogen) containing 1 pg of oligo (oligo dT Pharmacia, USA), 0.2 mM dNTPs, 10mM DTT, and 200 IU of Superscript II RT (Invitrogen). The reaction mixture was incubated at 42°C for 60 min and stopped by incubation at 75°C for 15 min. Amplification of cDNA (1/20) was performed using Power SYBER Green PCR ready-mix (Applied Biosystem Foster City, CA) and 0.1 μM primers (see Table 3 A-B). The thermal cycling parameters were 95°C for 10 min, followed by 40 cycles at 95°C for 15s and 60°C for 60s. Before the samples were analyzed, standard curves of purified, target-specific amplicons were created. For microarray validation: gene-specific oligonucleotides were used to PCR amplify the gene product from a pooled sample of prepared cDNA, the concentration of the amplicons was determined by UV spectrophotometry, and a standard curve was created. The mRNA expression for each gene was determined by comparing it with its respective standard curve. This measurement was controlled for RNA quality, quantity, and RT efficiency by normalizing it to the expression level of the TATA-Binding Protein (TBP) gene. TBP was used as a control gene because it was shown to be a good gene for normalization of real-time RT-PCR data in breast carcinoma (Lyng 2008). Each primer set produced a single product, as

determined by melt-curve analysis, and amplicons were of correct size, as analyzed by agarose gel electrophoresis. Statistical significance was determined by use of normalized fold changes and ANOVA using the Relative Expression Software Tool REST version 1.9.12 (Pfaffl 2002). Primers were designed using the web-based application Primer3 (http://frodo.wi.mit.edu/primer3/), biasing toward the 3'-end of the transcript and placing the two oligos in different exons, to maximize the likelihood of giving a gene-specific product. The settings used in Primer3 were 125-bp amplicon, 20mer, 60°C melting temperatures, and all others as defaults. Primer sequences were analyzed by BLAST. Gene names, forward and reverse primer sequences are listed in Table 3. E

Reporter and expression vectors

DNA fragments of the human ERBB2 gene promoter were inserted into the pGL3-Basic vector (Promega, Madison, WI), upstream of the firefly luciferase gene. Each vector has been named according to the fragment length. Promoter fragments were amplified from a human-mouse hybridoma cell lines containing only human chromosome 17, using PCR primers designed in order to insert restriction sites at the end of each fragment (Table XX C). The generated fragments of 300, 560 and 780 nucleotides were digested, respectively, with restriction enzyme pairs BglII-PvuII, BglII-Sma I, BglII-Pst I, purified from agarose gel and inserted into pGL3-basic vector. All constructs were sequenced in order to confirm the nucleotide sequence and the correct orientation of the cloned fragments. For sequencing were used primers for the pGL3basic sequence flanking the insertion site Table 3 D

Luciferase and β-galactosidase assay

Cell extracts were prepared 48 h after transfection and Luciferase activity was measured in duplicate for all samples in a Turner 20/20 luminometer (Turner Designs, Inc., Sunnyvale, CA) using the Bright-Glo™ Luciferase Assay System (Promega). Betagalactosidase activity was measured in duplicate for all samples in a Turner 20/20 luminometer (Turner Designs, Inc., Sunnyvale, CA) using the Beta-Glo® Assay System (Promega). The ratio of luciferase activity to β-galactosidase activity in each sample served as a measure of the normalized luciferase activity. All data shown were generated from at least three independent experiments. *Western Blot analysis* For Western blot analysis, total cell lysates were prepared

by directly harvesting cells in 500 µl of ice-cold RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 1 mM EDTA, 0.5 mM DTT) with freshly added protease and phosphatase inhibitors (Sigma Chemical Company, St Louis, MO). After 30 min of incubation on ice, samples were spun at 12.000 rpm for 20 min at 4°C and supernatants were collected. Protein concentrations from cell lysates were determined by the Bradford protein assay (BioRad, Hercules, CA). Aliquots corresponding to 40 µg of samples were separated on 4-12% polyacrylamide gradient gels (Invitrogen, Carlsbad, CA), and electrotransferred to PVDF membrane, according to manufacturer's instructions, (Amersham Biosciences, Sweden). Membranes were probed with primary antibodies rabbit anti-Flag antibody (Sigma Chemical Company, St Louis, MO) and horseradish peroxidase-conjugated secondary antibodies (Amersham Bioscience, Sweden). To ensure equal loading of protein among samples, membranes were additionally probed with β-actin antibody (Sigma Chemical Company, St Louis, MO). Detection was performed with chemiluminescent substrates (Pierce Biotechnology, Rockford, IL) and densitometric analysis was used to quantify signals.

Chromatin immunoprecipitation (ChIP) assay

Molecular interaction between MBP-1 and ERBB2 promoter was investigated in *vivo* by using a ChIP assay kit (Upstate Biotech, Billerica, MA). Briefly, 1,5 106 transfected SKBR3 cells were treated with 1% formaldehyde for 10 min at 37°C to cross-link proteins to chromatin. After rinsing with 125 mM glycine in PBS cells were washed with cold PBS and lysed in SDS lysis buffer (1% SDS, 10mM EDTA, 50mMTris-HCl, pH 8.1). The lysate was sonicated to shear DNA to a length between 200 and 600 bp. The sonicated supernatant was diluted 10-fold with ChiP dilution buffer (0.01% SDS, 1% Triton X-100, 20mMTris-HCl, pH 8.1, 150mMNaCl) and incubated, after a preclearing step with salmon sperm DNA/ proteinA-agarose (Upstate Biotechnology) with and without rabbit anti-Flag antibody overnight at 4°C. To collect DNA-antibodies complexes a salmon sperm DNA/proteinAagarose slurry was added to the mixture, incubated for 1 h at 4°C with rotation and DNA/proteinA-agarose complexes were recovered by centrifugation. After extensive washing the pellet was dissolved in 0.25 ml of elution buffer (0.2% SDS, 0.1 M NaHCO3), and the suspension was spun to remove agarose. Supernatant was made 0.2 M with NaCl and incubated a 65°C for 4 h to reverse cross-linking. After proteinase K treatment DNA was extracted with phenol/ chloroform and precipitated with ethanol. For PCR one-tenth of the recovered DNA was

amplified using specific primers directed to region -560/-300 of the ERBB2 promoter and primers MD48, targeted to an intergenic region between MYC and PVT1, as a negative control (table I). To verify that an equivalent amount of chromatin was used in the immunoprecipitations, DNA samples representing to 10% of the total input 7 chromatin was included in the PCR reactions.

Microarray experiments

Starting from RNA extracted from tissue was generated cRNA, by in vitro transcription using T7 RNA polymerase on 5 μg of total RNA and labelled with Cy5 or Cy3 (Amersham Pharmacia Biotech). Labelled-RNA (5 μg) of from each tumor sample were co-hybridized with 5 μg of a normal reference, consisting of an equal amount of cRNA extracted from mammary healthy samples of the corresponding tumor.

Labelled cRNAs were fragmented to an average size of 50–100 nucleotides by heating the samples to 60°C with 10 mM of zinc chloride and then adding an hybridization buffer containing 1 M NaCl, 0.5% sodium sarcosine, 50 mM MES, pH6.5, and formamide to a final concentration of 30%. The final volume was 3 ml at 40°C. The quality of cRNA, labelling efficiency and size distribution of fragmented cRNA (average size of 50–100 nucleotides) were examined by UV spectrophotometer and Agilent Bioanalyzer 2100.

The samples were hybridized on a Human 44k array containing 39,558 unique probes corresponding to ~30,000 human genes, manufactured by Agilent (Agilent Technologies Inc., Santa Clara, CA, USA). Each sample was hybridized in duplicate with fluor reversal (dye-swap) to systematically correct for dye bias. After hybridization, slides were washed and scanned using a confocal laser scanner and the Feature Extraction software (Agilent Technologies). The intensities obtained after scanning were quantified, background-corrected and normalized. Expression changes between each sample (tumours and normals) were quantified as the $\log_{10}$ of the expression ratio. All analyses were performed with Genespring GX ver. 10.0 (Agilent Technologies).

Clustering of mammary samples

Prior to clustering the human mammary dataset was filtered for non variable genes by removing those genes that showed significant changes in expression (fold-change > 2 or < -2) in less than 2 samples. For each gene, fold change was calculated as the ratio between each sample and the corresponding normal tissues reference. Therefore the fold change was the ratio against a pool of mammary normal samples.
Using these criteria, 138 genes were selected for an average linkage hierarchical clustering based on Pearson correlation coefficients. The overrepresentation of normal MBP-1 Positive and MBP-1 Negative samples in each cluster was assessed by Fisher-exact test.

Identification of group-specific genes mammary samples

Starting from the whole set of 20000 reliable probes, we selected the genes differentially expressed among the three groups of samples (MP, MBP-1 Positive; MN, MBP-1 Negative; N, normal) by applying one-way ANOVA test on gene expression ratio values, calculated against the reference pool of normal samples. The resulting p-values were corrected for multiple testing with the Benjamini and Hochberg test by converting them into the corresponding q-values which corresponds to the false discovery rate (FDR). Therefore a q-value threshold of 0.001 implies that 0.01% of the genes identified as differentially expressed are false positive. Using the conservative q-value Benjamini, Yoav; Hochberg cutoff of 0.001 we selected 8.124 genes. The selected genes have then been submitted to t-test, in which genes were individually tested for up- or down-regulation in each class of samples against the others. In this way it was determined in which sample class each selected gene was found to be differentially expressed. Genes could therefore be classified in two groups, according to their pattern of expression. For display purposes, the groups of samples (N, MP and MN) and genes up-and down-regulated were clustered separately using an average linkage hierarchical clustering and the Pearson correlation as similarity measure.

Gene set enrichment analysis

Groups of genes identified in previous steps were compared to annotated gene sets in order to identify the functional classes that were significantly over-represented. Enrichment p-values were computed according to the Fisher's exact test. All analyses were performed using The **D**atabase for **A**nnotation, **V**isualization and **I**ntegrated **D**iscovery (**DAVID**) ver. 6.7.

Primers sequence

A

c-Myc	Superarray
ErbB2	Superarray
TBP	Superarray

B

ERP1-F	ACTTCAAAGATTCCAGAAGATATGC
ERP2-R	GCTTGCATCCTACTCCATCC
ERP2-F	ACACATCCCCCTCCTTGACT
ERP3-R	CGGAGAATCCCTAAATGCAG
MD-F	ATT GTC CCC TCT CCT CCT GT
MD-R	CTT CGT CTC CCC TAC TGC TG

C

HER-3	**GAAGATCT**GGGCTCCCCTGGTTTCTC
HER-5	**GCTAGC**GCTGGTCATGGTGGCACA
HER-6	**GCTAGC**ACTTCAAAGATTCCAGAAGATATGC
HER-7	**GCTAGC**CACCAGCCTCTGCATTTAGG

D

pGL3-F	CTAGCAAAATAGGCTGTCCC
pGL3-R	GGAAGACGCCAAAAACATAAAG

E **GENE NAME**

TAZ-F	**TAZ**	CAGCAATGTGGATGAGATGG
TAZ-R		TCATTGAAGAGGGGGATCAG
LIPB-F	**LipofillinB**	GTTCAAGTTAAGTCTTGCCAAATTTGATGCC
LIPB-R		CACACTACATTTCTTCAATATTTTCACCAGGAC
GDF-F	**GDF15**	CCCGGGACCCTCAGAGTT
GDF-R		CCGCAGCCTGGTTAGCA
CAP2-F	**CNTNAP2**	TCCCGGCTATGCCAAGATAAA
CAP2-R		TTCCGATTGCCAAAGTCAACC

BAXF	**BAX**	GATGCGTCCACCAAGAAGCT
BAXR		CGGCCCCAGTTGAAGTTG
MG39F	**MammoglobinA**	CACCGACAGCAGCAGCCT
MG352		AGTTCTGTGAGCCAAAGGTCT

Table 3 Primers sequence. **A** Primers used for Real time PCR. **B.** Primers used for realtime PCR on immunoprecipitated chromatine. **C.** Primers used for PGL3 reporter plasmids costruction (in blue BglII restriction site, in red NheI restriction site) **D.** Primers used for PGL3 reporter plasmids sequencing. **E.** Primers used for Real time PCR for microarray validation data.

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