ErbB-2 nuclear function in breast cancer growth, metastasis and resistance to therapy

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Abstract

Approximately 15–20% of breast cancers (BC) show either membrane overexpression of ErbB-2 (MErbB-2), a member of the ErbBs family of receptor tyrosine kinases, or ERBB2 gene amplification. Until the development of MErbB-2-targeted therapies, this BC subtype, called ErbB-2-positive, was associated with increased metastatic potential and poor prognosis. Although these therapies have significantly improved overall survival and cure rates, resistance to available drugs is still a major clinical issue. In its classical mechanism, MErbB-2 activates downstream signaling cascades, which transduce its effects in BC. The fact that ErbB-2 is also present in the nucleus of BC cells was discovered over twenty years ago. Also, compelling evidence revealed a non-canonical function of nuclear ErbB-2 as a transcriptional regulator. As a deeper understanding of nuclear ErbB-2 actions would be crucial to the disclosure of its role as a biomarker and a target of therapy in BC, we will here review its function in BC, in particular, its role in growth, metastatic spreading and response to currently available MErbB-2-positive BC therapies.

Introduction

Breast cancer (BC) is the most frequently diagnosed cancer in women, with an estimated 1.5 million new cases annually. With 500,000 deaths per year worldwide, it is the prevalent cause of death by cancer in women both in the industrialized and the developing world (Maxmen 2012). BC is a complex and heterogeneous disease, comprising multiple tumor subtypes associated with distinct histological patterns and different biological features and clinical behaviors. According to BC molecular classification, the subtype overexpressing ErbB-2 in the cytoplasmic membrane (MErbB-2), a member of the ErbBs family of receptor tyrosine kinases (RTKs), or showing ErbB-2 gene (ERBB2) amplification, is called ErbB-2-positive (Slamon *et al.* 1989, Ross *et al.* 2009).

Decades of research since the discovery of MErbB-2 role as a potent inducer of BC growth *in vitro* and in preclinical models demonstrated that the ErbB-2-positive subtype is associated with high incidence of metastasis in BC patients and revealed MErbB-2 overexpression or ERBB2 gene amplification as prognostic biomarkers of the course of the disease (Slamon *et al.* 1989, Henderson & Patek 1998, Ross *et al.* 2009). Interestingly, EGF-R, another member of the ErbBs family, was detected in the cell nucleus as early as in 1984, but with no description of its role in that location (Gusterson *et al.* 1984). It was only ten years later that the pioneering work by Hung and coworkers demonstrated that NIH cells transfected with the rat variant of ErbB-2 (p185[neu]) showed strong p185[neu] nuclear presence

(Xie & Hung 1994). Remarkably intriguing were also their findings revealing that the carboxyl terminus of p185neu displays transactivation activity when tested in a GAL4-reporter assay. More than twenty years have gone by since this initial discovery, which was followed by numerous other findings identifying nuclear presence of as many as 18 distinct RTKs so far (reviewed in Carpenter & Liao 2013). We have come a long way since the days when the notion of ErbBs in the nucleus was so utterly challenging that it was regarded as either '*ridiculous or sublime*' (Waugh & Hsuan 2001). Indeed, compelling evidence has shown ErbB-2 localization in the nucleus of BC cells, where it functions as a transcriptional regulator (Tan *et al.* 2002, Wang *et al.* 2004, Kim *et al.* 2009, Beguelin *et al.* 2010, Li *et al.* 2011, 2012, Diaz Flaque *et al.* 2013*a*,*b*, Cordo Russo *et al.* 2015, Venturutti *et al.* 2015). However, the role of nuclear ErbB-2 (NErbB-2) as a biomarker and a target of therapy in BC is still unexplored. Here, we will review the function of nuclear ErbB-2 in BC, in particular, regarding tumor growth, metastatic spreading and response to currently available BC therapies.

Studies on ErbB-2 structure

ErbB-2, also called human epidermal growth factor 2 (HER2) and Neu, is a member of the ErbBs or type I receptor tyrosine kinase (RTK) family, which also includes the epidermal growth factor receptor (EGF-R/ErbB-1), ErbB-3 and ErbB-4. ERBB2 gene is localized on the long arm of chromosome 17 (Popescu *et al.* 1989). Throughout time, this 185 kDa transmembrane glycoprotein was given different names as it was being identified in different species. It was called Neu because it was initially

identified in ethylnitrosourea-induced rat neuroblastomas (Schechter *et al.* 1984, 1985, Bargmann *et al.* 1986). The human gene, on the other hand, was designated as ErbB-2/HER2 for its strong homology with ErbB (avian erythroblastosis oncogene B), the oncogene later found to encode the EGF-R/ErbB-1 (Semba *et al.* 1985). Cloning of the gene showed that, indeed, rat Neu and human ErbB-2/HER2 are encoded by orthologs (Yamamoto *et al.* 1986). Members of the ErbBs family are transmembrane RTKs with a large glycosylated extracellular portion containing four domains (I–IV), a single membrane-spanning region and a cytoplasmic tyrosine kinase domain. Extracellular domains II and IV are cysteine-rich regions, which display conserved number and spacing of cysteine residues among the members of the family. Domains I and III participate in ligand binding. The intracellular portion of ErbB receptors is composed of a juxtamembrane domain, the kinase domain and a carboxyl (C)-terminal tail, which contains several tyrosine residues that play a key role in the transduction of ErbBs biological effects (reviewed in Riese & Stern 1998, Olayioye *et al.* 2000, Yarden & Sliwkowski 2001, Burgess 2008) (Fig. 1 depicts the structure of ErbB-2).

More than twenty years have passed since the discovery that, in addition to their canonical subcellular localization in the cytoplasmic membrane, ErbB receptors are located in the cell nucleus, where they regulate multiple biological functions (reviewed in Lee *et al.* 2015). The series of compelling findings revealing nuclear ErbBs presence (reviewed in Wang & Hung 2012) stimulated a most exciting research into the mechanisms of ErbBs nuclear migration and into the identification of the ErbBs domains involved in both trafficking from the cytoplasmic
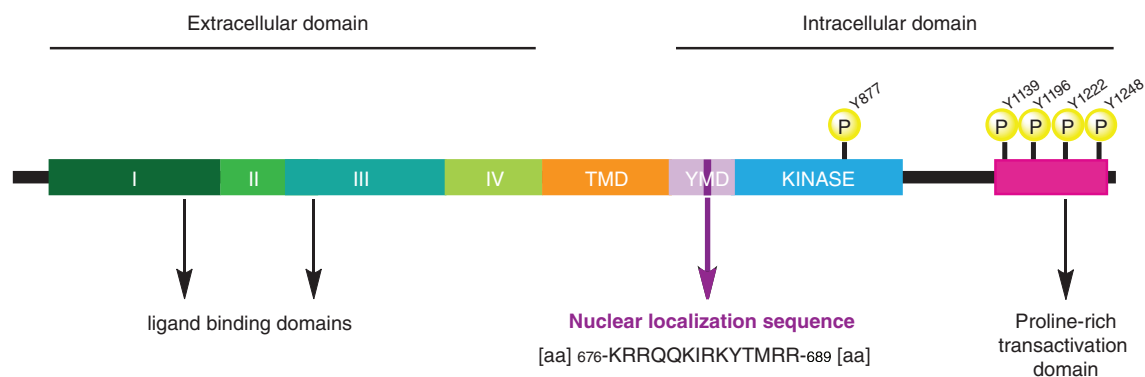
Figure 1
ErbB-2 structure. Regions that are relevant to ErbB-2 function are indicated schematically. The extracellular portion is composed by 4 domains (I–IV). ErbB-2 contains a transmembrane domain (TMD) and a juxtamembrane domain (YMD), which encloses the nuclear localization sequence (NLS, purple). The intracellular domain includes the kinase domain (blue) and a proline-rich transactivation domain near the carboxy-terminus (pink). The major phosphorylation sites are indicated (yellow circles).

membrane to the nucleus and in ErbBs nuclear function as transcriptional activators. A nuclear localization sequence (NLS) was identified in all ErbBs (Offterdinger et al. 2002, Wang et al. 2004, Williams et al. 2004, Lo et al. 2005). The NLS is located adjacent to the transmembrane domain in ErbB-2 and contains three clusters of basic amino acids (676-KRRQQKIRKYTMRR-689) (Chen et al. 2005, Giri et al. 2005, Hsu & Hung 2007) (Fig. 1). This tri-partite NLS is conserved among the members of the ErbBs family (reviewed in Lee et al. 2015). Furthermore, functional transactivation domains were mapped at the C-terminal acidic region of EGF-R, ErbB-2, ErbB-3 and ErbB-4 (Xie & Hung 1994, Lin et al. 2001, Ni et al. 2001, Wang et al. 2004, Brand et al. 2013). Figure 1 shows the proline-rich transactivation domain of ErbB-2. To date, no DNA-binding domain has been identified for the members of the ErbBs family. Therefore, it has been proposed that other transcription factors with DNA binding ability may cooperate with ErbBs to regulate gene expression.

ErbB-2 canonical mechanism of action

A large number of ligands for the ErbBs have been described. They include eight ligands for EGF-R (reviewed in Riese & Stern 1998, Burgess 2008, Schneider & Wolf 2009) and all isoforms of Neu differentiation factor/heregulins (HRG), which bind to ErbB-3 and ErbB-4 (Tzahar et al. 1996). EGF-R and ErbB-2 act as HRG co-receptors (Tzahar et al. 1996). Interestingly, ErbB-2 is an orphan receptor. The extracellular domain of ErbB-2 differs from that of the other ErbBs in that it constitutively adopts an open conformation, which exposes the dimerization loop in domain II (Cho et al. 2003, Garrett et al. 2003). This unique ErbB-2 structure helps to explain the fact that despite the absence of a known ligand that binds ErbB-2 directly, this receptor participates in an extensive network of ligand-induced formation of ErbBs dimers (Graus-Porta et al. 1997). In BC cells overexpressing MErbB-2, it also assembles homodimers and heterodimers in the absence of ligand (Landgraf 2007, Tao & Maruyama 2008). As a direct consequence of ligand binding to the extracellular domain of the ErbBs family, receptor dimers are formed and their intrinsic kinase activity is stimulated, which leads to the phosphorylation of tyrosine residues in the intracellular domain of receptors. These phosphorylated tyrosine residues link ErbBs to several signaling pathways, the classic transduction mechanism of ErbBs biological effects in BC. ErbB-3 is a unique member of this family because it displays impaired tyrosine kinase activity (Sierke et al.

1997, Jura et al. 2009). Among ErbB-2-activated cascades, the best characterized are the p42/p44 mitogen-activated protein kinases (MAPKs) and the phosphatidylinositol 3-kinase (PI3K)/AKT pathways (reviewed in Riese & Stern 1998, Olayioye et al. 2000, Yarden & Sliwkowski 2001, Burgess 2008). Accumulating evidence showed that ErbB-2 also activates the signal transducer and activator of transcription 3 (Stat3) pathway, which acts as a downstream effector of ErbB-2-induced initiation and metastatic dissemination of BC (Proietti et al. 2009, Ranger et al. 2009, Barbieri et al. 2010a,b). Src tyrosine kinase (reviewed in Ishizawar & Parsons 2004), and the serine/threonine kinase mammalian target of rapamycin (mTOR), which is activated downstream of PI3K–AKT, are also stimulated by ErbB-2 (reviewed in Mayer & Arteaga 2016).

ErbB-2 and breast cancer

Approximately 15–20% of all diagnosed breast tumors belong to the ErbB-2-positive subtype (Slamon et al. 1989, Ross et al. 2009). Until the development of anti-MErbB-2-targeted therapies, this subtype was associated with higher metastatic potential and poor prognosis in BC patients with node-positive and -negative tumors (Slamon et al. 1989, Henderson & Patek 1998, Ross et al. 2009). Notably, the overall survival (OS) of metastatic BC patients and the cure rate in the adjuvant setting improved significantly after the development of said therapies (Slamon et al. 2001, Romond et al. 2005). Current therapeutic options for patients whose tumors overexpress MErbB-2 include monoclonal antibodies (trastuzumab and pertuzumab), tyrosine kinase inhibitors (lapatinib) and trastuzumab-DM1, an antibody–drug conjugate. Trastuzumab (TZ) was the first anti-ErbB-2 therapy approved for metastatic and early-stage BC. These strategies are being used in different combinations, along also with chemotherapy in the neoadjuvant, adjuvant and metastatic settings (for review see Patel et al. 2014). Despite a significant clinical response to trastuzumab (TZ), around 40–60% of patients with ErbB-2-positive metastatic BC do not respond to TZ, showing either de novo or acquired resistance (Slamon et al. 2001, Esteva et al. 2002). Although lapatinib provides clinical benefit to a subset of patients progressing on TZ, less than 25% achieve an objective response, and the majority eventually develop acquired lapatinib resistance (Geyer et al. 2006). These data highlight that resistance to available drugs is a major clinical issue (Patel et al. 2014).

Multiple mechanisms were found to cause TZ resistance in BC cells and in preclinical models, such as expression of constitutively active truncated forms of ErbB-2, presence of ligand-induced ErbB-2/ErbB-3 dimers and ErbB-2/insulin-like growth factor receptor I (IGF1-R) dimerization (reviewed in Esteva et al. 2010). Hyperactivation of the PI3K/AKT pathway is the best acknowledged alteration in downstream signaling associated with TZ resistance. It also plays a role in the lack of response to lapatinib (Garrett et al. 2011). A plethora of clinical trials in patients with refractory ErbB-2-positive metastatic BC are assessing the efficacy of novel treatments with afatinib, an irreversible EGF-R and ErbB-2 tyrosine kinase inhibitor; neratinib, an inhibitor of ErbB-2, EGF-R and ErbB-4 activation; PI3K/AKT inhibitors; mTOR inhibitors; anti-IGF-IR antibodies; and inhibitors of heat shock protein 90 (reviewed in Stern 2012, Singh et al. 2014).

Truncated ErbB-2 variants in BC

Proteolytic cleavage of ErbB-2 in BC cells has long been reported (Lin & Clinton 1991, Pupa et al. 1993). Matrix metalloproteases (MMP), such as MMP-1 (Codony-Servat et al. 1999) and ADAM10, members of the metalloprotease-desintegrin family (Liu et al. 2006), have been found to participate in ErbB-2 cleavage within the extracellular juxtamembrane region. This results in the shedding of the ErbB-2 extracellular domain (ECD), leaving a p95 fragment that contains the transmembrane and intracellular domains, referred to as 648-CFT (carboxy-terminal fragment). Moreover, alternative initiation of translation from two methionine residues (611 and 687), which are located before and after the transmembrane domain, respectively, gives rise to other p95ErbB-2 variants, named 611-CFT and 687-CFT (Anido et al. 2006). These ErbB-2 truncated forms, collectively referred to as p95ErbB-2, have been shown to induce growth of BC xenografts in nude mice (Anido et al. 2006, Scaltriti et al. 2007). They are also frequently found in BC cell lines and in human mammary tumors, where they may even constitute the major ErbB-2 species and are associated with nodal metastasis (Molina et al. 2002). Expression of wild-type 611-CTF in the mouse mammary gland led to the development of aggressive tumors (Pedersen et al. 2009). In addition, p95ErbB-2 variants cause TZ resistance (Anido et al. 2006, Scaltriti et al. 2007). Resistance to the antiestrogen toremifene was also associated with the generation of p95ErbB-2 variants (Warri et al. 1996). The molecular mechanisms through which p95ErbB-2

variants control ErbB-2-positive BC metastatic spreading and response to anti-MErbB-2 therapies remain poorly explored. It has been demonstrated that transmembrane 611-CTF, which is able to activate p42/p44 MAPKs and AKT pathways, phosphorylates the cytoskeleton-binding protein cortactin, which is involved in 611-CTF induction of BC cells migration and metastasis (Garcia-Castillo et al. 2009). Moreover, a transcriptomic analysis revealed that 611-CTF controls the expression of genes that are associated with metastasis, including the receptor tyrosine kinases MET and EPHA2, matrix metalloproteinase 1, interleukin 11, angiopoietin-like 4 and several integrins (Pedersen et al. 2009).

Nuclear ErbB-2 action in breast cancer

Numerous findings strongly demonstrated that full-length ErbB-2 is present in the nucleus of BC cells, where it binds promoters/enhancers of target genes to regulate biological responses in BC (Tan et al. 2002, Wang et al. 2004, Kim et al. 2009, Beguelin et al. 2010, Li et al. 2011, 2012, Diaz Flaque et al. 2013a,b, Cordo Russo et al. 2015, Venturutti et al. 2015). Importantly, the development of more sophisticated techniques to study protein trafficking from the cytoplasmic membrane to different subcellular compartments, allowed further understanding of the mechanism involved in ErbB-2 nuclear translocation. It has been demonstrated that ErbB-2 is internalized from the cell membrane by endocytic internalization and endosomal sorting (Giri et al. 2005, Wang et al. 2010a). During the cytoplasmic–nuclear transport, ErbB-2 associates with importin β1, a member of the nuclear transport machinery, via ErbB-2 NLS. The ErbB-2/importin β1 complex then interacts with the nuclear pore protein Nup358, which results in ErbB-2 nuclear translocation (Giri et al. 2005). These steps are in accordance with well-acknowledged mechanisms of intracellular trafficking (Sandvig & van Deurs 2002, Giri et al. 2005, Bonifacino & Rojas 2006, Wang et al. 2010a, 2012). However, it was still necessary to explain how intact ErbB-2 could be extracted from a membrane bilayer, either at the cell surface or within an intracellular compartment like the endosome, to allow its translocation to the nucleus as a non-membrane localized molecule. That is indeed how intact transmembrane proteins are extracted from the endoplasmic reticulum (ER) to be carried into the cytoplasm. This mechanism is the ER-associated degradation (ERAD) system, which detects mal-folded proteins in the ER and carries them to the cytoplasm, where they are ubiquitinated and degraded

(Tsai *et al.* 2002). A series of studies revealed that for EGF-R and ErbB-2, the Sec61 translocon located in the ER, which is part of the ERAD pathway, mediates retrotranslocation of said receptors to the cytoplasm and their import to the nucleus. Furthermore, a membrane-bound mechanism of EGF-R and ErbB-2 trafficking from the cell surface to the nucleus, named INTERNET (integral trafficking from the ER to the nuclear envelope transport) has been identified (Wang *et al.* 2010*b*, 2012). Importin β1 and the Sec61 translocon also participate in this mechanism, where membrane-embedded full-length ErbBs are transported to the nucleus (Wang *et al.* 2010*b*, 2012). Figure 2 illustrates the proposed trafficking pathway of full-length ErbB-2 to the nucleus, induced by either HRG binding to ErbB-3,

which leads to the formation of ErbB-2/ErbB-3 dimers and ErbB-2 activation or by constitutive ErbB-2 activation induced by the formation of ErbB-2 homodimers. The mechanisms of ErbB-2 nuclear translocation has been extensively reviewed elsewhere (Giri *et al.* 2005, Wang *et al.* 2010*b*, Wang & Hung 2012, Carpenter & Liao 2013, Bertelsen & Stang 2014, Chen & Hung 2015). In spite of all these findings highlighting nuclear ErbB-2's key role in BC and providing a mechanistic explanation for its nuclear trafficking, the clinical significance of nuclear ErbB-2 remains almost unexplored. Furthermore, to the best of our knowledge and for reasons that remain unclear, there is no single drug designed to target the function of nuclear ErbB-2, which plays a major role in BC.
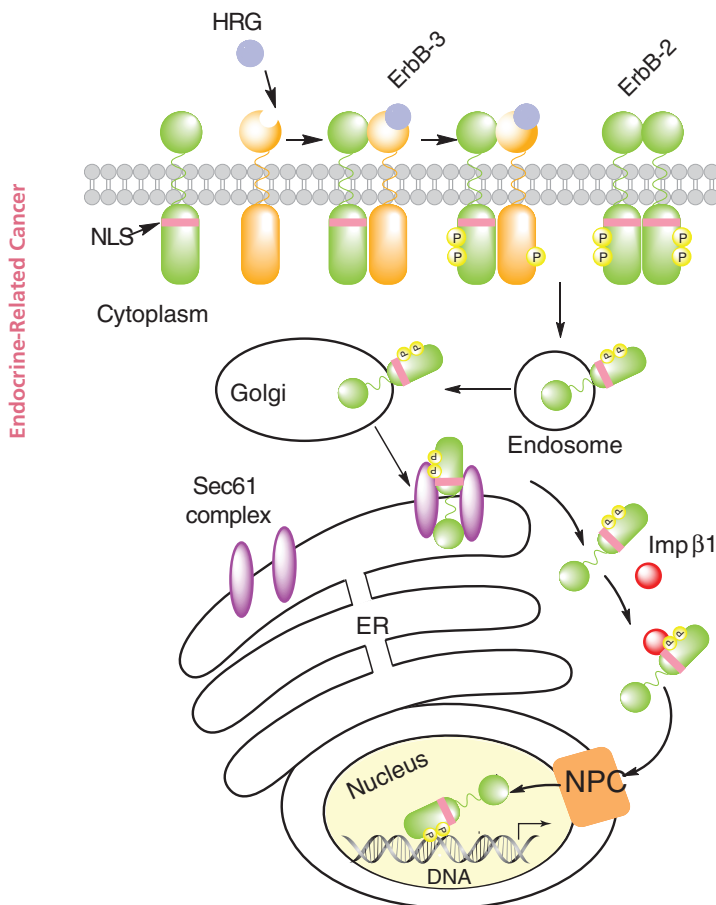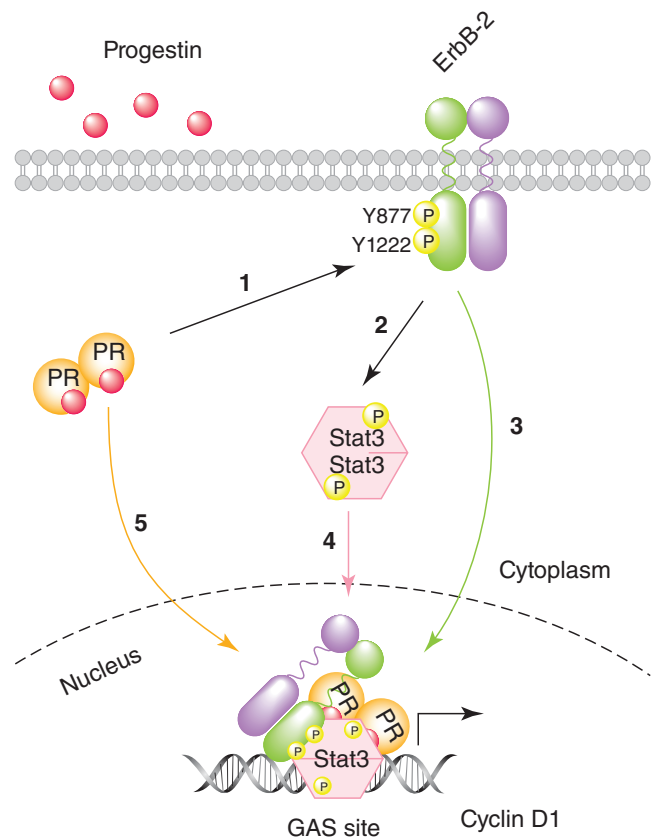

Figure 2
Proposed model of ligand (HRG)-induced and constitutive ErbB-2 nuclear transport from the cell surface to the nucleus. In this model, the Sec61 translocon, part of the ERAD pathway, associates with ErbB-2 at the endoplasmic reticulum (ER) and extracts full-length ErbB-2 from the membrane bilayer allowing its interaction with importin β1 (Imp β1). The ErbB-2/importin β1 complex then interacts with the nuclear pore protein Nup358, a member of the nuclear pore complex (NPC), which results in ErbB-2 nuclear translocation. NLS, nuclear localization sequence.

Nuclear ErbB-2 in breast cancer proliferation and metastasis

The dogma of ErbB-2 mechanism of action in BC has been challenged by Hung and coworkers who demonstrated that in MErbB-2-overexpressing BC cells, ErbB-2 migrates to the nuclear compartment where it binds to the DNA at specific sequences, which they named HER-2-associated sequences (HASs) (Wang *et al.* 2004). In this startling work, the full-length ErbB-2 protein was found in the nucleus. Furthermore, ErbB-2 tyrosine phosphorylation was required for its nuclear localization. Cyclooxygenase-2 (COX-2) was revealed in the said study as the first gene whose expression was modulated via the role of ErbB-2 as a transcription factor (TF) (Wang *et al.* 2004). The association of ErbB-2 with the COX-2 promoter at the HAS-binding site was detected in BC cell lines overexpressing ErbB-2, as well as in ErbB-2-positive human primary breast tumors (Wang *et al.* 2004). Inhibition of COX-2 activity in MCF-7 BC cells engineered to overexpress ErbB-2, and in parental MCF-7 cells, which display low endogenous levels of MErbB-2, had no effect on the proliferation of the latter, but suppressed the invasive activity of ErbB-2-overexpressing MCF-7 cells (Wang *et al.* 2004), indicating that nuclear ErbB-2's function as a TF may play a role in BC metastasis. These authors also found functional recruitment of ErbB-2 at the HAS sites of the PRPK (p53-related protein kinase) (Abe *et al.* 2001) promoter, which depends on ErbB-2-intact tyrosine kinase activity. As ErbBs lack a putative DNA-binding domain, it was proposed that they exert their nuclear function as coactivators of other TFs, which bind DNA directly. A role of nuclear ErbB-2 as a transcriptional coactivator was indeed revealed in BC models, which express estrogen

receptor (ER) and progesterone receptor (PR) – members of the steroid receptor superfamily – and also overexpress or show moderate levels of MErbB-2 (Beguelin *et al.* 2010). In these models, the presence of bidirectional interactions between PR and MErbB-2 classical signaling pathways had long been identified. On the one hand, PR was found to activate the HRG/ErbB-2 signaling pathway (Balana *et al.* 1999). On the other hand, it was reported that HRG induces PR transcriptional activation in breast tumors through a mechanism that requires functional ErbB-2 (Labriola *et al.* 2003). Stat3 was revealed as the nodal convergence point between PR and HRG/ErbB-2 signaling in BC (Proietti *et al.* 2005, 2009). Interestingly, Stat3 plays a key role in mammary cancer (reviewed in Kamran *et al.* 2013). Nuclear interaction between PR and ErbB-2 was next identified, showing that PR activated by its ligands (the steroid hormones progestins) induces ErbB-2 nuclear translocation and its colocalization and physical association with Stat3 in the nuclear compartment of BC cells. As previously shown for constitutively activated ErbB-2 (Wang *et al.* 2004), PR induced full-length ErbB-2 protein translocation to the nucleus. Nuclear ErbB-2 was also phosphorylated at tyrosine (Tyr) 1222, a major ErbB-2 autophosphorylation site, as well as at Tyr 877, a site different from the autophosphorylation ones, located at the kinase domain, in the proximity of the NLS (Guo *et al.* 2006, Xu *et al.* 2007). In the nucleus, ErbB-2 assembles a transcriptional complex in which it acts as a coactivator of Stat3 bound to its response elements (GAS sites) at the promoter of cyclin D1 (Beguelin *et al.* 2010). Importantly, this region of cyclin D1 proximal promoter lacks HAS sites and canonical PR response elements (PREs), further confirming nuclear ErbB-2's role as coactivator. Notably, PR was also loaded onto the Stat3/ErbB-2 complex. In this newly discovered class of complex, the transcription factor (Stat3) is first phosphorylated at cytoplasmic level via its coactivator (ErbB-2) function as an upstream effector (Beguelin *et al.* 2010) (Fig. 3 shows a model of ErbB-2/Stat3/PR nuclear interaction consistent with these findings). Interaction of EGF-R and Stat3 at the promoter of the inducible nitric oxide synthase (iNOS), containing both EGF-R-binding sites (AT-rich sequences (ATRSs)) and Stat3 response elements, was identified in a startling study (Lo *et al.* 2005). The presence of two clusters of ATRS- and Stat3-binding sites was essential for the regulation of the iNOS promoter by EGF-R. This highlights a major difference with respect to the nuclear function of the ErbB-2/Stat3 transcriptional complex in the cyclin D1 promoter, which requires only Stat3 binding to the GAS

Figure 3
Stat3/ErbB-2/PR transcriptional complex induced by progestins in breast cancer. PR activated by progestins induces the phosphorylation of ErbB-2 at Tyr 1222 and Tyr 877 (1). ErbB-2 activated by a nongenomic PR action stimulates Stat3 phosphorylation (2) (Beguelin *et al.* 2010). ErbB-2 (3) and Stat3 (4) then translocate to the nucleus where they assemble a transcriptional complex at the Stat3 response element (GAS sites) of the cyclin D1 promoter, in which ErbB-2 acts as a coactivator of Stat3. PR is also loaded on the Stat3/ErbB-2 complex (5).

sites and ErbB-2 recruitment to these sites, where it acts as a Stat3 coactivator. This discrepancy may indicate that EGF-R/Stat3 and ErbB-2/Stat3 complexes regulate chromatin targets by distinct mechanisms as a general rule. It may also indicate that the nature of the interaction between ErbBs and Stat3 within intact cells depends on the set of Stat3/ErbB-binding motifs available in the target gene promoter/enhancer regions, as well as on the specific sequences and unique structure of the DNA in the vicinity of the Stat3/ErbB-binding sites. Consistent with the latter, Stat3 and EGF-R do not associate at the cyclin D1 promoter, which is regulated by nuclear EGF-R (Lin *et al.* 2001) and which also contains a cluster of ATRS/ Stat3 sites (Lo *et al.* 2005).

Nuclear ErbB-2 action in the proliferation of ER-, PR- and ErbB-2-positive BC models was explored by

transfection of cells with hErbB-2ΔNLS, a human ErbB-2 nuclear localization domain mutant engineered by Hung and coworkers, unable to translocate to the nucleus and which also functions as a DN inhibitor of endogenous ErbB-2 nuclear migration (Giri *et al.* 2005, Beguelin *et al.* 2010). hErbB-2ΔNLS retains intrinsic Tyr kinase activity and the capacity to activate ErbB-2 cascades, including p42/p44 MAPKs and Stat3, and it does not affect endogenous ErbB-2 signaling (Wang *et al.* 2004, Giri *et al.* 2005, Beguelin *et al.* 2010). Transfection of BC cells expressing endogenous ErbB-2 with the mutant hErbB-2ΔNLS disrupted the assembly of the nuclear Stat3/ErbB-2/PR transcriptional complex at the cyclin D1 promoter, and consequently inhibited growth induced by progestins, both *in vitro* and in preclinical models (Beguelin *et al.* 2010). As inhibition of COX-2, a gene induced by ErbB-2 acting as a transcription factor, did not affect the proliferation but inhibited invasion (Wang *et al.* 2004), further studies are needed either to identify genes regulated by ErbB-2 through its role as a transcription factor, and which may be involved in ErbB-2 proliferative effects, or to define whether the function of ErbB-2 as a transcriptional coactivator may be the one directly involved in ErbB-2-induced stimulation of BC growth. In support of this latter possibility, it was reported that in hormone-dependent BC cells, progestins induce the expression of cell cycle regulator p21^{CIP1} via phosphorylation of c-Src, Stat3 and ErbB-2 (Diaz Flaque *et al.* 2013*b*). Notably, it was also found that ErbB-2 nuclear function plays a key role in progestin induction of p21^{CIP1} expression (Diaz Flaque *et al.* 2013*b*). Progestins were found to induce the assembly of a transcriptional complex where PR is recruited along with Stat3 and ErbB-2 to a GAS site at the p21^{CIP1} promoter. In this complex, ErbB-2 functions as a coactivator of Stat3 (Diaz Flaque *et al.* 2013*b*). The link between nuclear ErbB-2 function as a Stat3 coactivator and BC growth was provided by the findings that under progestin stimulation of BC cells, p21^{CIP1} induces cell cycle progression and that blockage of p21^{CIP1} expression strongly inhibited *in vitro* and *in vivo* progestin-induced BC proliferation (Diaz Flaque *et al.* 2013*b*). Also, providing consistent correlation between ErbB-2's role as a coactivator and its ability to promote BC growth, another study demonstrated that progestins induce the assembly of a transcriptional complex among activator protein 1 (AP-1), Stat3, PR and ErbB-2 at a region of the cyclin D1 promoter containing both AP-1 response elements (TRE) and GAS sites (Diaz Flaque *et al.* 2013*a*). This array of transcription factors (AP-1 and Stat3) whose

response elements are clustered in the DNA, plus their interacting cofactors (PR and ErbB-2) and coactivators (P300 and CBP), function cooperatively to induce cyclin D1 promoter activation as well as *in vitro* and *in vivo* BC growth driven by progestins (Diaz Flaque *et al.* 2013*a*).

The role of nuclear ErbB-2 in the growth of BC cells overexpressing MErbB-2 was also revealed by a study demonstrating the nuclear colocalization and physical association between ErbB-2, β-actin and RNA polymerase I (RNA Pol I), which coincide with active RNA Pol I transcription sites in nucleoli (Li *et al.* 2011). Knockdown of ErbB-2 reduced the synthesis of 45S pre-RNA and of nascent nuclear RNA levels. On the contrary, MCF-7 cells transfected with increasing amounts of wild-type ErbB-2 showed a dose-dependent enhancement of pre-rRNA synthesis (Li *et al.* 2011). Transfection of cells with the mutant hErbB-2ΔNLS resulted in impaired RNA Pol I binding and in significantly lower increase of pre-rRNA synthesis, highlighting the critical role of nuclear ErbB-2 in the regulation of rRNA transcription (Li *et al.* 2011). Simultaneous recruitment of ErbB-2, β-actin and RNA Pol was found at the 5′-terminal region of human rDNA, and at the 28S rRNA transcribed region, in multiple ErbB-2-expressing BC cell lines (Li *et al.* 2011). Furthermore, association of ErbB-2 with rDNA requires ongoing transcription (Li *et al.* 2011). Based on the facts that regulation of rRNA transcription by RNA Pol I is a key process for ribosome biogenesis and that the extent of rRNA gene transcription reflects the requirement of protein synthesis associated with the rate of cell growth, the authors of this study analyzed the protein content and biosynthetic rate as a way to explore the function of nuclear ErbB-2. Their findings revealed that nuclear ErbB-2 increases total protein synthesis and cell size, indicating that it may drive BC growth by enhancing rRNA synthesis and protein translation (Li *et al.* 2011). Moreover, stimulation of BC cells with the ErbBs ligand HRG promoted ErbB-2 nuclear translocation and increased 45S pre-rRNA levels. Further support of direct nuclear ErbB-2 involvement in the enhancement of RNA pol I transcription was provided by their demonstration that inhibition of MErbB-2-stimulated downstream signaling cascades, such as p42/p44 MAPKs and PI3K-AKT, did not affect HRG-induced 45S pre-rRNA synthesis or HRG enhancement of total protein synthesis (Li *et al.* 2011).

Interaction of nuclear ErbB-2 with the atypical histone macroH2A1.2 (mH2A1.2) was found in several cancer cell types, including BC, where this interaction participates in the proliferation of cancer cells (Li *et al.* 2012). The study

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of the molecular mechanism underlying ErbB-2/mH2A1.2 crosstalk and its biological effects revealed that ErbB-2 tyrosine kinase activity upregulates mH2A1.2, which in turn stimulates the proliferation and tumorigenicity of ErbB-2-positive BC cells by enhancing ErbB-2 expression at both protein and mRNA levels (Li *et al.* 2012). Increase of ErbB-2 transcription in mH2A1.2-transfected SKOV-3 ovarian cancer cells was induced by mH2A1.2 recruitment to a series of positions at the ErbB-2 proximal promoter encompassing a region up to 5 kb upstream of the translational start site (Li *et al.* 2012). It remains to be established whether similar mH2A1.2 occupancy of the ErbB-2 promoter occurs in BC cells, which will highlight ErbB-2's ability to modulate its own transcription as a common mechanism driving cancer growth.

The critical role of both Stat3 and MErbB-2 in BC metastasis has long been demonstrated (reviewed in Freudenberg *et al.* 2009, Kamran *et al.* 2013). Furthermore, a series of BC models where transgenic mice carry ErbB-2 under the control of the mouse mammary tumor virus (MMTV) promoter disclosed the involvement of Stat3 as a downstream effector of MErbB-2-induced metastatic dissemination in BC (Muller *et al.* 1988, Guy *et al.* 1992, Siegel *et al.* 1999, Finkle *et al.* 2004, Ursini-Siegel *et al.* 2008). Interestingly, a recent study revealed that an unanticipated direction of ErbB-2 and Stat3 interaction, involving nuclear ErbB-2, also plays a key role in BC metastasis (Venturutti *et al.* 2015). It was reported that Stat3, activated downstream of ErbB-2, binds to its GAS sites at the ErbB-2 promoter, which results in the upregulation of ErbB-2 transcription in metastatic, ErbB-2-positive BC cells (Venturutti *et al.* 2015). It is highly likely that the fact that Neu/ErbB-2 expression in transgenic mice models was driven by a strong viral promoter previously hampered the study of the TFs and the epigenetic mechanisms controlling endogenous ErbB-2 promoter activation in metastatic BC. ErbB-2 overexpression can occur in the absence of gene amplification, indicating the importance of the dysregulation of the transcriptional control of ErbB-2 expression in BC. Although little is known about the TFs that bind to ErbB-2 promoter/enhancers in BC (Bates & Hurst 1997, Delacroix *et al.* 2005, Zuo *et al.* 2007), the discoveries that ErbB-2 activates mH2A1.2 and Stat3 and recruits them to its promoter, raise the possibility that the general mechanisms by which ErbB-2 modulates its own overexpression in BC is through the regulation of transcriptional activators, which in turn bind to the ErbB-2 promoter and induce ErbB-2 transcription. The above-mentioned study (Venturutti *et al.* 2015) also reported

that as outcome of the feed-forward loop between Stat3 activation and ErbB-2 expression, Stat3 co-opts nuclear ErbB-2 function by recruiting ErbB-2 as its coactivator at the GAS sites in the promoter of microRNA-21 (miR-21), a miRNA whose expression is regulated by MErbB-2, and whose metastasis-promoting effects in BC are well acknowledged (Loffler *et al.* 2007, Yan *et al.* 2008, Huang *et al.* 2009, Iliopoulos *et al.* 2010, Song *et al.* 2010, Yang *et al.* 2011, Han *et al.* 2012, Niu *et al.* 2012, Marino *et al.* 2014). By means of the hErbB-2ΔNLS nuclear localization domain mutant and a constitutively activated ErbB-2 variant, it was shown that nuclear ErbB-2, in its role as a Stat3 coactivator at the miR-21 promoter, and also, notably, in its direct role as a transcription factor bound to its HAS sites, upregulates miR-21 in metastatic BC (Venturutti *et al.* 2015). This was the first demonstration that nuclear ErbB-2 regulates a microRNA in any normal or malignant cell type. Increased levels of miR-21, in turn, downregulate the expression of the metastasis-suppressor protein programmed cell death 4 (PDCD4), a validated miR-21 target (Zhu *et al.* 2008, Huang *et al.* 2009, Nieves-Alicea *et al.* 2009). Using an *in vivo* model of metastatic ErbB-2-positive BC, in which Stat3 was silenced and the expressions of ErbB-2 or of miR-21 were reconstituted, it was shown that both are downstream mediators of Stat3-driven metastasis (Venturutti *et al.* 2015). Furthermore, an inverse correlation between ErbB-2/Stat3 nuclear coexpression and PDCD4 expression was found in ErbB-2-positive primary invasive BCs (Venturutti *et al.* 2015). These findings, consistent with the previous study where inactivation of COX-2, a direct transcriptional target of nuclear ErbB-2, hampered migration of ErbB-2-positive BC cells (Wang *et al.* 2004) strongly support nuclear ErbB-2's role in BC metastasis.

Nuclear ErbB-2's role in the response to BC therapies

Full-length ErbB-2 located in the nuclear compartment was found to participate in the mechanisms of BC resistance to chemotherapeutic agents and to anti-MErbB-2 therapies.

Chemotherapy

ErbB-2 phosphorylates the cyclin-dependent kinase Cdc2 at tyrosine 15 (Tyr15) in BC cells, which leads to the inhibition of Cdc2 activation (Tan *et al.* 2002). This inhibitory phosphorylation of Cdc2 by ErbB-2 participates in taxol resistance (Tan *et al.* 2002). Notably,

it was also found that in the nuclear compartment of ErbB-2-overexpressing BC cells, ErbB-2 colocalizes with the Cdc2/cyclin B complexes, raising the exciting possibility that nuclear ErbB-2 may contribute to the inhibition of Cdc2 activation and thus to resistance to taxol (Tan *et al.* 2002). Another study demonstrated that lapatinib, a dual tyrosine kinase inhibitor of EGF-R and ErbB-2 (Medina & Goodin 2008), sensitizes ErbB-2-amplified gastric cells to the antiproliferative effects of the chemotherapeutic agents fluoropyrimidines, such as 5FU, which inhibit thymidylate synthase (TS) activity (Kim *et al.* 2008, 2009). Lapatinib treatment of said cells was found to reduce TS expression at protein and mRNA levels (Kim *et al.* 2009). The study of the molecular mechanism underlying lapatinib effects demonstrated that EGF induced the nuclear translocation of EGF-R and ErbB-2, where both bind to the TS promoter (Kim *et al.* 2009). Lapatinib prevented EGF-R and ErbB-2 nuclear migration and thus their recruitment to the TS promoter, which ultimately results in TS downregulation (Kim *et al.* 2009). Interestingly, lapatinib also blocks EGF-induced recruitment of EGF-R and ErbB-2 to the TS promoter in ErbB-2-overexpressing SKBR3 BC cells (Kim *et al.* 2009). The study provided no description of the response element to which EGF-R or ErbB-2 binds at the TS promoter (Kim *et al.* 2009).

Anti-MErbB-2 therapies

Recently, basal nuclear ErbB-2, ErbB-3 and Stat3, as well as ErbB-2/ErbB-3 and ErbB-2/Stat3 nuclear colocalization were detected in multiple TZ-responsive and -resistant ErbB-2-positive human BC lines (Cordo Russo *et al.* 2015). HRGβ1 increased nuclear migration and colocalization of all three proteins. Basal MErbB-2/MErbB-3 dimers were more numerous in TZ-sensitive cells than in -resistant cells, whereas, conversely, nuclear dimers were more abundant in resistant cells, highlighting a role of nuclear ErbB dimers in TZ response (Cordo Russo *et al.* 2015). Basal and HRGβ1-induced assembly of a Stat3/ErbB-2/ErbB-3 transcriptional complex at the GAS sites of the cyclin D1 promoter was found in both cell types, where ErbB-2 and ErbB-3 act as Stat3 cofactors (Cordo Russo *et al.* 2015). This was the first report of a nuclear function of the ErbB-2/ErbB-3 dimer, resulting in the regulation of cyclin D1 expression. Inhibition of ErbB-2 nuclear localization by transfer of hErbB-2ΔNLS blocked basal and HRGβ1-stimulated *in vitro* growth of ErbB-2-amplified human BC cells, both TZ sensitive and resistant, as well as the proliferation of a preclinical model of

TZ-resistant BC in the presence of ErbB-2/ErbB-3 dimers, of ErbB-3 phosphorylation and of PI3K/AKT pathway activation (Cordo Russo *et al.* 2015), conditions where TZ is inefficient (Yakes *et al.* 2002, Nagata *et al.* 2004, Junttila *et al.* 2009, Ghosh *et al.* 2011). Disruption of the Stat3/ErbB-2/ErbB-3 nuclear complex driving cyclin D1 expression was identified as the differential molecular signature underlying hErbB-2ΔNLS's growth inhibitory effects in TZ-resistant cells (Cordo Russo *et al.* 2015). These series of findings support a model (Fig. 4) where, in cells displaying higher levels of MErbB-2 than of nuclear ErbB-2, proliferation is driven by signaling and by nuclear ErbB-2 actions, both hierarchically equal. Inhibition of signaling with TZ or of nuclear actions with hErbB-2ΔNLS would therefore block proliferation. On the contrary, nuclear ErbB-2's role on proliferation is stronger than that of MErbB-2 in BC cells displaying higher levels of nuclear ErbB-2. Thus, only inhibition of nuclear ErbB-2 effects would inhibit proliferation (Fig. 4).

Truncated nuclear ErbB-2 variants in the response to BC therapies

The biological and clinical significance of the presence of truncated ErbB-2 forms in the nucleus of BC cells still remains to be unequivocally established. It has been demonstrated that transfection of BC cells, including T47D and MCF-7 cells displaying low-to-moderate MErbB-2 levels, and of MDA-MB-468 cells, which lack MErbB-2 expression, with p95ErbB-2 variants, results in cytoplasmic and nuclear presence of these truncated ErbB-2 forms (Anido *et al.* 2006, Scaltriti *et al.* 2007). Also, p95ErbB-2 variants have been found in primary breast tumors (Scaltriti *et al.* 2007). As reported for the full-length ErbB-2 (Wang *et al.* 2004, Cordo Russo *et al.* 2015), tyrosine kinase activity of p95ErbB-2 variants appears to be required for their nuclear translocation (Anido *et al.* 2006). The role of nuclear p95ErbB-2 variants in the response to anti-MErbB-2 therapies remains poorly explored. Lapatinib was found to increase the expression of a tyrosine phosphorylated p95ErbB-2 form (p95L) in MErbB-2-positive BC cells, which was located in the nuclear compartment (Xia *et al.* 2004). The generation of p95L appears to be mediated by the proteosome. Tyrosine phosphorylation of p95L was not inhibited by either lapatinib or GW2974, another tyrosine kinase inhibitor (Xia *et al.* 2004). Furthermore, enhanced expression of p95L was detected in BC cells and in tumor xenografts resistant to lapatinib, and metastasis
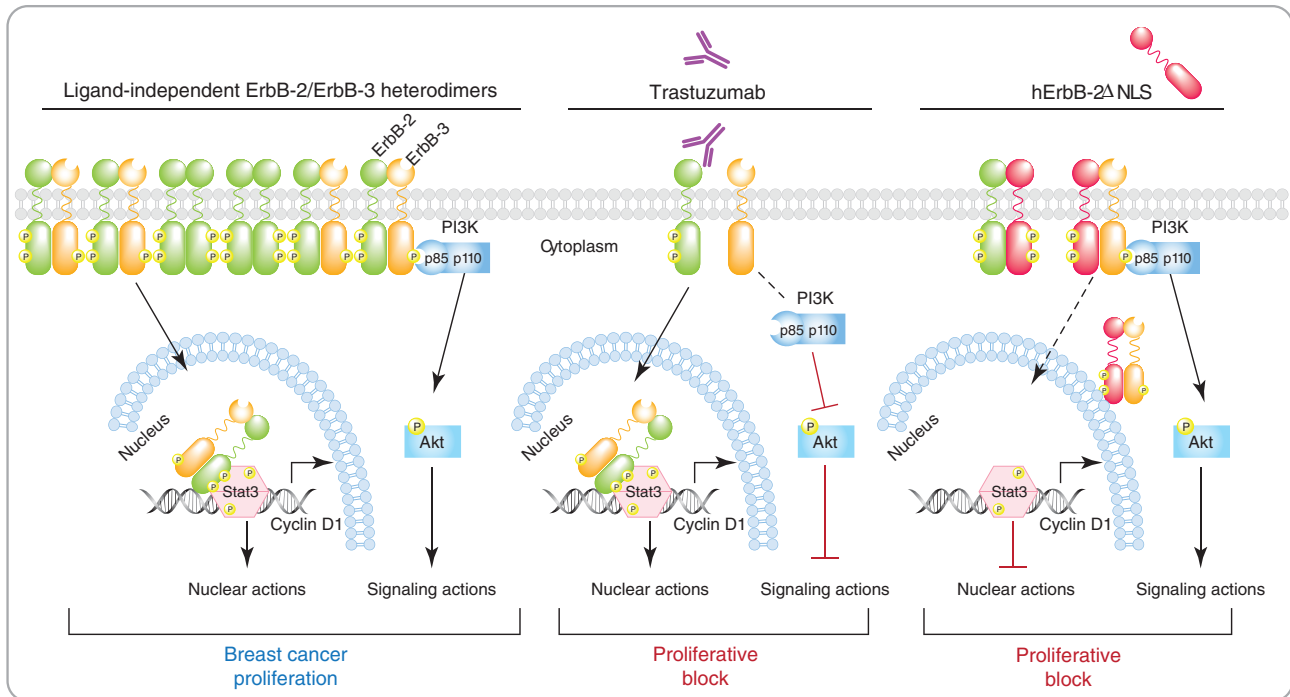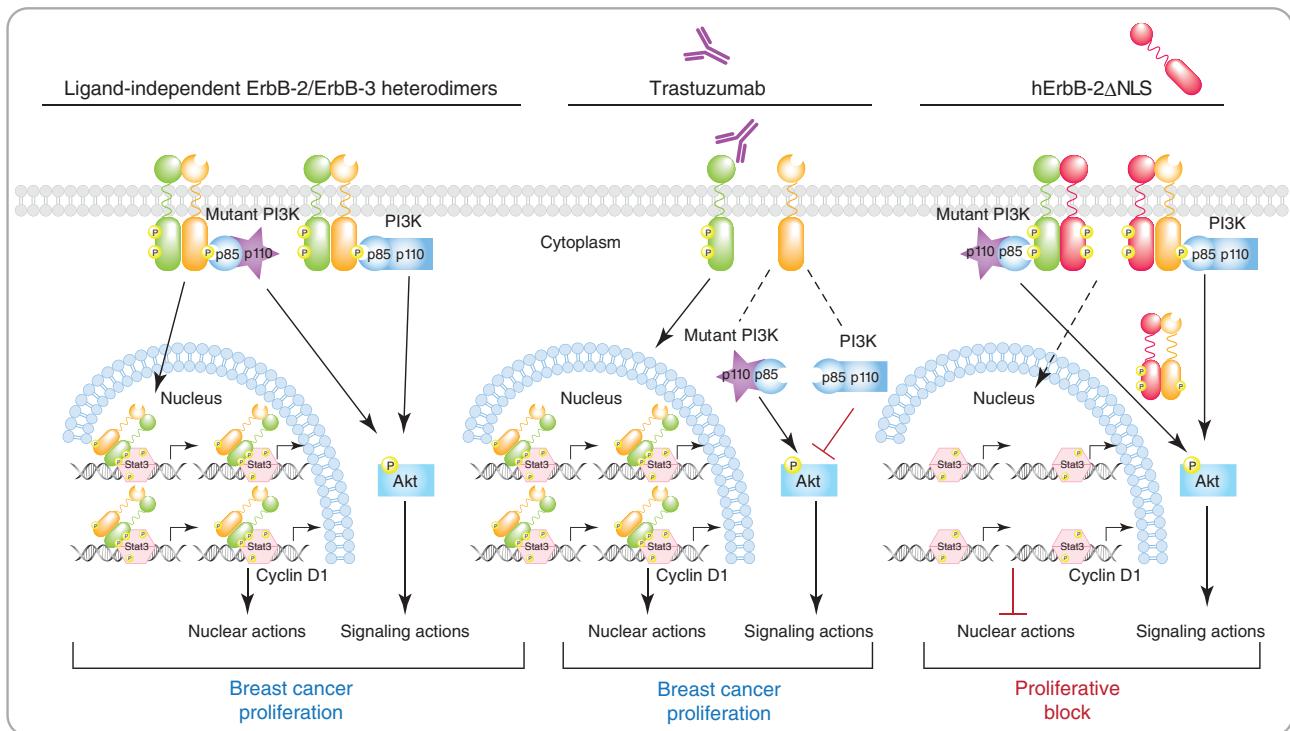
Figure 4

Nuclear ErbB-2's role in the response to trastuzumab (TZ). BC cells sensitive to TZ show higher levels of MErbB-2 than those of NErbB-2. Their proliferation is stimulated by ErbB-2 signaling (e.g. PI3K/AKT) and nuclear (e.g. Stat3/ErbB-2/ErbB-3 transcriptional complex at cyclin D1 promoter) actions, both hierarchically equal. Inhibition of signaling with TZ or of nuclear action with hErbB-2ΔNLS inhibits proliferation. TZ-resistant cells show high levels of NErbB-2, which may be higher than those of MErbB-2. Nuclear ErbB-2 effect on proliferation is stronger than that of MErbB-2. Blockade of nuclear ErbB-2 effect with hErbB-2ΔNLS prevents BC growth in spite of the activation of signaling cascades.

developed while patients were treated with lapatinib (Xia *et al.* 2004). Also, when tyrosine-phosphorylated p95ErbB-2 variants, generated by alternate initiation of translation, were expressed in BC cells, they localized at the nucleus and rendered BC cells resistant to lapatinib (Xia *et al.* 2004). These results point to a role of nuclear ErbB-2 CTFs in acquired resistance to lapatinib. However, the above findings still have to be reconciled with a series of other studies, which show that lapatinib antiproliferative effects in BC are, indeed, mediated by lapatinib's ability to prevent MErbB-2 phosphorylation and nuclear migration, and to block its ubiquitination, which results in a marked accumulation of inactive ErbB-2, ErbB-2 homodimers, and ErbB-2/ErbB-3 heterodimers at cell surface (Kim *et al.* 2009, Scaltriti *et al.* 2009, Vazquez-Martin *et al.* 2011). Also, it has been reported that along with lapatinib's ability to increase the concentration of the unphosphorylated form of ErbB-2 in the membrane of BC cells, lapatinib increases the basal shedding of ErbB-2 ECD to inhibit BC cell growth (Vazquez-Martin *et al.* 2011). Previous findings showed that transfection into BC cells of 611-CTF induces a significant downregulation of ER and causes resistance to antiestrogen therapy (Parra-Palau *et al.* 2010). Since, as described previously, it was shown that truncated ErbB-2 variants localize in the nuclear compartment when introduced into BC cells (Anido *et al.* 2006, Scaltriti *et al.* 2007), it is tempting to speculate that the nuclear function of 611-CTF may also be involved in endocrine therapy resistance.

Clinical significance of nuclear ErbB-2 as a biomarker in BC

To the best of our knowledge, there are only few works that have explored in the clinic the role of full-length nuclear ErbB-2 as a biomarker of the course of the disease and as a predictor of response to therapy. In a cohort of primary breast tumors, an inverse correlation was reported between ER expression and expressions of nuclear ErbB-2 and Cox-2 (Dillon *et al.* 2008), a gene that, as described previously, is a direct transcriptional target of nuclear ErbB-2 (Wang *et al.* 2004). Nuclear ErbB-2's presence was associated with tumor size and grade. In this study, both nuclear ErbB-2 and COX-2 were associated with reduced disease-free survival in patients receiving tamoxifen in the adjuvant setting (Dillon *et al.* 2008). More recent clinical studies in a small cohort of primary invasive BCs revealed a role for nuclear ErbB-2 as an independent prognostic

factor of poor clinical outcome in MErbB-2-positive tumors (Schillaci *et al.* 2012).

Conclusions

Compelling evidence has shown ErbB-2's localization at the nucleus of ErbB-2-positive BC cells, where it regulates multiple biological responses. Nuclear ErbB-2 participates in key features of BC, which turn this disease extremely difficult to defeat. Nuclear ErbB-2 is involved in BC proliferation, as well as in BC's ability to metastasize at distant organs. Lastly, it is also involved in BC's *de novo* resistance to systemic therapies and in BC's ability to develop compensatory growth mechanisms leading to acquired resistance to therapy. All these findings notwithstanding, and in spite of the increasing knowledge on the mechanism of ErbB-2 nuclear trafficking, the clinical significance of nuclear ErbB-2 in BC still remains to be established. Furthermore, for reasons that remain unclear, there is no single drug designed to target nuclear ErbB-2 function. Intriguingly, genome-wide studies to explore the genomic function of ErbB-2 in MErbB-2-overexpressing BC cells, or in any other cell type, have not yet been done. The specific gene signature resulting from the ErbB-2 cistrome might serve as a novel biomarker of clinical outcome. In addition, genes unraveled through a genome-wide approach might constitute novel targets of therapy. A most interesting question that also needs to be addressed is whether ErbB-2 exerts its tyrosine kinase activity also in the nuclear compartment. We hope that the findings here reviewed provide enough food for thought and help us wonder whether targeting nuclear ErbB-2 could be a mighty weapon to fight ErbB-2-positive BC.

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