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Discoidin domain receptor 1 regulates ErbB2/ErbB3 signaling in mammary epithelial cells

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Abstract

The ErbB2 receptor tyrosine kinase plays a key role in mammary gland development. It forms large clusters which serve as signaling platforms for integration of extracellular information. The discoidin domain receptor (DDR) family are collagen receptor tyrosine kinases which, together with ErbB2, are involved in many physiological and pathological processes. Here, we investigated interaction of ErbB2 and DDR1 receptors in breast cancer cells. In contrast to beta1-integrin, DDR1 colocalizes with ErbB2 in membrane clusters regardless of their expression levels. We demonstrated that this spatial coexistence is a consequence of the physical interaction between these receptors. In addition, these receptors are coexpressed in the normal mammary gland but not in breast tumor samples. Together, these results present DDR1 as a novel modulator of the ErbB2/ErbB3 signaling pathway.

Keywords

ErbB2, Cell adhesion receptors, membrane clusters, breast cancer

Abbreviations

Acyl Carrier Protein (ACP), Phosphopantetheinyl Transferase (ACPwtS), Bovine serum albumin (BSA), Breast cancer (BC), Collagen (COL), Confocal laser scanning microscopy (CLSM), Discoidin Domain Receptor (DDR), Epidermal growth factor receptor (EGFR), Extracellular matrix (ECM), Fibronectin (FN), Heregulin-beta1 (HRG), Matrix Metalloproteinases (MMP), Paraformaldehyde (PFA), Phosphate buffer solution (PBS), Poli-D-Lysine (PDL), Receptor-

Tyrosine Kinase (RTK), Room temperature (RT), Terminal end buds (TEBs), phosphoinositide 3-kinase (PI3K).

Introduction

The epidermal growth factor receptor (EGFR) family of Receptor-Tyrosine Kinase (RTK) plays a role in many physiological and pathological processes. This family consists of four members: EGFR (also named ErbB1), ErbB2, ErbB3, and ErbB4. Ligand binding stimulates homo- and heterodimerization of EGFR inducing cross-phosphorylation of tyrosines within the cytoplasmic domains that become binding sites for downstream effectors [1,2]. ErbB2 is a peculiar member of the EGFR family given its lack of identified ligand. In the absence of growth factors, ErbB2 self-associates inducing autophosphorylation and self-activation in an expression-dependent manner [3,4]. Additionally, ErbB2 dimerizes with other ErbB family members for ligand-dependent activation, being EGFR and ErbB3 its most frequent companions. The main feature of ErbB3 is its extremely low enzymatic activity [5], requiring interaction with ErbB2 for activation upon Heregulin-beta1 (HRG) binding [6].

ErbB receptors play a key role in mammary gland's development. Mammary glands of ErbB2 dominant negative mutants have shown defective lobuloalveolar structure and reduced milk protein expression [7]. Deletion of ErbB3 in murine mammary gland epithelium also resulted in reduced outgrowth, ductal penetration and smaller TEBs, although proliferation was not affected [8]. Altogether, the presented evidence places ErbB2 and ErbB3 as key regulators of the morphogenesis and proliferation of mammary epithelial cells during breast development.

ErbB2 overexpression has been described in several types of neoplastic processes including breast cancer (BC) [9]. The aggressiveness of mammary carcinoma with ErbB2 amplification is

consistent with the importance of ErbB2 in promoting invasive penetration of the mammary fat pad [10,11]. Understanding ErbB2 interactomics and its regulation is a key factor to improve therapeutic approaches and overcome trastuzumab resistance, a clinical phenomenon that occurs in 66 to 88% of ErbB2-positive metastatic breast tumors [12].

ErbB2 has been described to interact with several partners in the plasma membrane including proteins involved in cell adhesion such as focal adhesion kinase, p130Cas and Src [13–16]. In addition, extracellular matrix (ECM) receptors such as integrins have been found to interact and regulate ErbB2 signaling in BC cells [17,18]. The Discoidin Domain Receptor (DDR) family is composed by two collagen (COL) receptor tyrosine kinases: DDR1 and DDR2 [19]. Unlike ErbBs, quiescent DDRs exist as homodimers. While DDR1 is capable of binding several types of COL, DDR2 binds exclusively fibrillar COL [20]. DDR1 is generally expressed in epithelial and immune cells, while DDR2 is generally expressed in mesenchymal cells including fibroblasts and chondrocytes [21].

Ligand-binding to DDRs induces autophosphorylation and association with several proteins including scaffolds, kinases and phosphatases [19,22]. Well-differentiated epithelial cancer cells usually express DDR1 but not DDR2. In contrast, several poorly differentiated epithelial cancer cells co-express both receptors and were shown to modulate cancer cell migration [21,23].

Similarly to ErbB2 KO models, DDR1 depletion results in delayed ductal outgrowth during mouse mammary gland development [24], probably due to the fact that DDR1 promotes the expression of MMP1, 2, 7 and 9 [25]. On the contrary, TEBs are enlarged and epithelial cells have higher proliferative rates. DDR1 has also been found to be expressed in several BC cells although its relation to tumor phenotype remains unclear and contradictory. While studies reported abundant expression of DDR1 in primary invasive carcinoma and lymph node metastases [26], others

showed a moderate reduction in DDR1 mRNA levels in the majority of intermediate and high-grade human breast carcinomas compared to normal mammary tissue [27]. These discrepancies may be attributed to differences in the histological type and/or tumor grade [28,29].

In addition, DDR1 was shown to interact with other RTKs such as Insulin-like Growth Factor 1 and Insulin Receptor [30]. Given the role of ErbB2 and ErbB3 in both normal and tumorigenic mammary gland and the antagonic effect of DDR1 on breast development, we propose that this RTKs could be forming part of a signaling cluster that regulates mammary gland development and physiology.

The aim of the present work is to study the interaction of ErbB2 and DDR1 receptors in BC cells. We showed that COL negatively modulates Akt activation, while fibronectin (FN) shows no cooperativity. As COL can be sensed by both beta1 integrin and DDR1 [20], we studied the interaction of both receptors with ErbB2 in the plasma membrane of BC cells. While beta1 integrin coexists with ErbB2 in very particular situations, as previously shown by our group [31]. DDR1 interacts with ErbB2 regardless of its expression levels, as it was shown by CLSM quantitative colocalization, immunoprecipitation and pull-down assays. In this context, we propose DDR1 as a novel regulator of ErbB2. These results reveal the complexity of the ErbB2 interactome and its regulation, supporting the role of the ECM as a RTK regulator on both physiological and pathological processes.

Materials and Methods

Cell culture

MCF7, SKBR3 and COS7 cell lines were obtained from the American Type Culture Collection (Rockville). Culture and transfection was performed as previously described [31]. A previously established pool of MCF7 cells stably-expressing the fusion protein ErbB2-TagRFP was used for imaging experiments [31]. Approximately 10% of these cells shown to be positive for TagRFP. Glass or plastic surface was treated with 40μg/mL Poli-D-Lysine (PDL, Sigma) in 0.1 M Borate buffer pH = 8.5 for 1 hour prior COL or FN incubation. The surface was then washed 3 times with phosphate buffer solution (PBS), incubated 2 hours at 37°C with a 50 μg/ml of FN solution (Invitrogen) in PBS or 100 μg/ml of rat COL type I (Inamed Purecol) solution. The surface was washed 3 times with PBS prior usage.

DNA constructs

ErbB2-mYFP and ACP-ErbB2 constructs were kindly provided by Dr. Tom Jovin and Dr. Donna Arndt-Jovin (Max Planck Institute for Biophysical Chemistry, Göttingen, Germany). DDR1a-ECFP construct was kindly provided by Dr. Rafael Fridman (Wayne State University School of Medicine and Barbara Ann Karmanos Cancer Institute, Detroit, USA). ErbB2-TagRFP and beta1 integrin-ECFP were obtained as previously described [31]. All constructs were verified by sequencing.

Western blot

Whole cell protein extracts were prepared by scrapping the culture dishes on ice with RIPA buffer (50 mM Tris, pH = 8.0 containing 150 mM NaCl, 0.1% SDS, 0.5% deoxycholate and 1% NP40) containing protease and phosphatase inhibitors (40 uM phenylmethylsulfonyl fluoride, 5 μg/ml

leupeptin, 50 µg/ml aprotinin, 40 mM sodium fluoride and 100 mM beta-glycerophosphate). Protein concentration was measured by Bradford. 50 µg of each sample were then run in SDS-PAGE mini gels and transferred to PVDF membranes (Amersham Biosciences). Precision Plus Protein Dual Color Standard (BioRad) was used as the protein size marker in SDS-PAGE. Membranes were blocked 1 hour at room temperature (RT) in blocking buffer (5% bovine serum albumin (BSA) in Tris-Buffered Saline plus 0.1% Tween-20 (TBST)). Primary antibodies were prepared in blocking buffer and incubated at 4°C ON. After washing 5 times with TBST, membranes were incubated with secondary antibodies for 1 hour at RT and washed 5 times with TBST. Signal was detected by using an enhanced chemiluminescence kit (ECL, Amersham Biosciences).

Antibodies used were as follow: Akt (C67E7, Cell Signaling Technology, 1:1000), phosphorylated Akt at Ser473 (D9E, Cell Signaling Technology, 1:1000), ErbB2 (OP15, Calbiochem-Merck Biosciences, 1:1000), phosphorylated ErbB3 at Tyr1289 (21D3, Cell Signaling, 1/1000), ErbB3 (1B2E, Cell Signaling, 1/1000), DDR1 (D1G6, Cell Signaling Technology, 1:1000), beta1 integrin (D2E2, mAb9699, Cell Signaling Technology, 1:1000), GFP (Sc-8334, Santa Cruz, 1:1000), beta actin (C-4, Santa Cruz Biotechnology, 1:5000), goat anti-mouse HorseRadish Peroxidase (HRP) conjugated and goat anti-rabbit HRP conjugated (Genentech, 1:5000).

Immunofluorescence

Fifty thousand cells were seeded on 12 mm glass coverslips (Marienfeld) placed in 24-well plates. After treatment, cells were washed with PBS and fixed 10 minutes at RT with 4% paraformaldehyde (PFA) 4% sucrose in PBS. PFA was quenched by incubation with 10mM Tris-PBS 5 minutes at RT. Fixed cells were incubated in blocking buffer (0.1% Triton-X100. 5% BSA

in PBS) 1 hour at 4°C. Primary antibodies diluted in blocking buffer were incubated for 1 hour at 4°C and washed 5 times with PBS. Secondary antibodies were incubated in blocking buffer for 1 hour at 4°C and washed 5 times with PBS. Samples were mounted in a Mowiol-based mounting media.

Antibodies used were as follow: phosphorylated Akt at Ser473 (D9E, Cell Signaling Technology, 1:500), pan Akt (C67E7, Cell Signaling Technology, 1:1000), ErbB2 (Trastuzumab-Alexa Fluor 488, kindly provided by Dr. Tom Jovin and Donna Arndt-Jovin, Max Planck Institute for Biophysical Chemistry, Göttingen, Germany, 5 ng/µL), DDR1 (D1G6, Cell Signaling Technology, 1:800), Goat anti-mouse Alexa Fluor 488, goat anti-mouse Alexa Fluor 647, goat anti-rabbit Alexa Fluor 488 and goat anti-rabbit Alexa Fluor 555-conjugated antibodies (Life Technologies, 1:600).

Confocal microscopy

Confocal laser scanning microscopy (CLSM) images were acquired in an Olympus FV1000 microscope (Olympus) using an Olympus 60x / 1.42NA UPLAN SAPO oil immersion objective. Excitation and filters were as follows: Alexa Fluor 488: excitation 488 nm Argon laser line - emission 505-525 nm BP filter. TagRFP and Alexa Fluor 555: excitation 543 nm Helium/Neon laser - emission 560-620 nm BP filter. Alexa Fluor 647: excitation 633 nm diode laser - emission 650-750 nm BP filter. ECFP: excitation 440 nm pulsed laser excitation- emission 470-490 nm band pass (BP) filter. EYFP: excitation 515 nm Argon laser line - emission 525-535 nm BP filter. Images were acquired in sequential mode. Bleedthrough was checked by imaging of samples labeled with a single fluorophore. There was no registred shift between images.

Colocalization analysis

Quantification of Pearson's correlation, Manders' overlap, M1 and M2 coefficients was performed using Villalta's algorithm [32]. This algorithm runs on Matlab (MathWorks) using the image processing toolbox DIPimage (Delft University of Technology). We used the Costes thresholding method to determine the threshold values [33]. The maps for these coefficients were computed by estimating the contribution of each single pixel to the coefficient.

Akt phosphorylation assay

One hundred thousand cells were seeded on 24-well plates coated with PDL, COL or FN. Cells were starved for 16 hours and then stimulated with Recombinant Human Heregulin beta1 (Peprotech) in RAB-Tyrode buffer (135 mM NaCl, 10 mM KCl, 0.4 mM $MgCl_2$, 1 mM $CaCl_2$, 5.6 mM glucose and 10 mM HEPES, pH=7.4). For Western blot, cells were lysed in RIPA buffer and 10 µg were loaded in a 10% SDS-PAGE gel. For immunofluorescence, cell plated on 12 mm glass coverslips were stimulated, fixed and labeled as previously described.

PI3K inhibitor LY294002 (Sigma-Aldrich) was used 20 uM. ErbB kinase domain inhibitor CI1033 (Sigma-Aldrich) was used 1 uM [34]. ErbB2 blocking antibody 2C4 (Pertuzumab, Omnitarg) was used 100 nM [35]. All inhibitors were incubated 3 hours prior stimulation.

Acyl Carrier Protein (ACP) tag-based pull-down assay

COS7 cells transfected with ACP-ErbB2 plus ErbB2-mYFP, beta1 integrin-ECFP or DDR1a-ECFP were labeled with 1 uM biotin-CoA and 10 ng/µL purified recombinant Phosphopantetheinyl Transferase (ACPwtS) in Rab-Tyrode buffer 2 hours at 37°C [36]. Cells were washed

and lysed with NP40 lysis buffer (50 mM Tris pH=8, 150 mM NaCl, 1% NP40). Subsequently, cell lysates were centrifuged 5 minutes at 12000 rpm and supernatant (500 µg of proteins) was incubated while stirring with 0.2 mg of streptavidin-sepharose beads (Sigma-Aldrich) ON at 4°C. Beads were washed 3 times with NP40 lysis buffer, resuspended in 20 µL of 2X sample buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue, 150 mM Tris HCl pH=6,8) and processed as described before.

Co-immunoprecipitation

Cells were lysed in NP40 buffer containing protease and phosphatase inhibitors. Cell lysates were centrifuged 5 minutes at 12000 rpm and 500 µg of total proteins were incubated while stirring with 0.2 mg of protein A-sepharose beads (Sigma-Aldrich) 1 hour at 4°C to avoid nonspecific labeling. The soluble fraction was incubated with the antibody overnight at 4°C before the addition of 0.2 mg of protein A-sepharose beads for additional 2 hours. After five washing steps, samples were resuspended in 20 µL of sample buffer 2x and processed as described above.

Antibodies used were as follow: ErbB2 (Trastuzumab, Genetech, 10 ng/µL), DDR1 (D1G6, Cell Signaling Technology, 1/200) and unspecific antibody (used as negative control, DA1E, Cell Signaling Technology, 1/200).

Bioinformatic analysis

Coexpression analysis for normal samples was performed using the online database CoexpressDB (coxpresdb.jp) [37]. Microarray data expression was obtained from GEO DataSets [38]. mRNA

expression data published by TCGA [39] was obtained from cBioPortal Cancer Genomics Portal [40].

DDR1, ERBB2, ERBB3, and GAPDH expression data from 8 microarray data sets were obtained using GEO2R. The exclusion criteria of the data sets were that the samples come from normal or cancerous breast tissue of patients, which microarray platforms used contain at least one probe associated with each gene studied and, in the case of having more than one probe associated, that the expression of these was similar between them. The selected data sets were GSE70951, GSE78958, GSE86374, GSE10797, GDS1329, GSE3744, GSE5764, and GSE9574.

Statistical analysis

Statistical analyses were carried out using Prism 8 (GraphPad Software). Tests and sample numbers used are indicated in the figures legends.

For expression analysis, a mixed linear model was used for each gene, including the datasets ID as a random effect variable with the lmer function of the lme4 v1.1-27.1 R package [41]. Multiple comparison tests were performed with the emmeans v1.7.2 R package [https://CRAN.R-project.org/package=emmeans]. Pearson correlation coefficient (PCC, also known as Pearson's r) was calculated for each group within each study and modeled using the rma.mv function of the metafor v3.0-2 package [42]. Plots were made using the ggplot2 v3.3.5 R package ran in R version 4.1.1. Statistically differences from zero were analyzed using a mixed multivariate linear model from metafor v3.0-2 package.

Results

COL, but not FN, reduces HRG-induced Akt phosphorylation

Given the fact that ErbB2 is located in restricted regions of the cell membrane [4] and the importance of the subcellular localization of phosphorylated form of Akt in its cellular function [43], we analyzed the localization of the phosphor-Akt to such structures upon this TRK activation. We used HRG to specifically induce ErbB2 ligand-dependent activation as HRG-induced Akt activation requires ErbB2/ErbB3 interaction and phosphorylation in MCF7 cells (Fig. S1). Due to MCF7's low ErbB2 expression levels (undetectable by immunofluorescence), we used a pool of cells expressing the fusion protein ErbB2-TagRFP in order to perform imaging experiments [31]. Although these MCF7 cells overexpress the fusion protein ErbB2-TagRFP, Akt phosphorylation is not constitutive and depend on stimulation for Akt to be activated (Fig. 1A).

Consistently with our hypothesis, phospho-Akt colocalizes with ErbB2-TagRFP upon HRG stimulation (Fig. 1 A). These results support the idea that ErbB2 clusters would behave as signaling complexes where the HRG sensing machinery is placed, allowing an efficient integration of extracellular information.

As ECM can modulate the behavior of membrane clusters [44], we plated untransfected MCF7 cells on PDL, FN or COL-coated coverslips and stimulated them with HRG before Akt phosphorylation was assessed by quantitative immunofluorescence. Although no differences were observed at low HRG concentrations, 1nM of HRG induced significantly higher levels of Akt activation when cells were grown over PDL and FN than cells plated on COL (Fig. 1B and C), suggesting that this ECM protein could be negatively modulating ErbB2 signaling pathway. The effect of COL on Akt phosphorylation induced by incubation with on 1nM HRG was confirmed by Western blot (Fig. 1D).

This behavior was not observed in the ErbB2-overexpressing BC cell line SKBR3, in which a constitutive activation of ErbB3 and Akt was shown [45–49] . This feature responds to the fact that overexpression of ErbB2 induces ligand-independent phosphorylation and activation of this RTK and proteins downstream its signaling pathway.

DDR1 coexists with ErbB2 in membrane clusters

MCF7 and SKBR3 cells express two COL receptors: beta1 integrin and DDR1 [20]. As we showed that HRG-induced Akt phosphorylation occurs specifically in ErbB2 clusters, we studied the localization of both COL receptors expressed in MCF7 and SKBR3 cells by CLSM.

Beta1 integrin does not colocalize with ErbB2 (Fig. 2). These experiments recapitulate the data shown in our previous work [31], where ErbB2 was shown not to be coexisting with beta1 integrin in the membrane of MCF7 and SKBR3 cells. Our work is supported by Weinberg and collaborators [50], who demonstrated an anti-colocalization between ErbB2 and FA markers such as Talin and Vinculin in the membrane of SKBR3 cells. Together, these results provide solid evidence that ErbB2 and beta1-integrin would not be interacting in these cell models. As Akt is specifically phosphorylated in ErbB2 clusters which are not directly related to FAs, we propose that other ECM receptor, such as DDR1, could be playing a part in the modulation of Akt activation observed by COL in MCF7.

ErbB2 and DDR1 physically interacts in membrane clusters

After characterizing the coexistence of DDR1 and ErbB2 in membrane clusters, we wonder if these receptors are physically associated. In order to achieve this objective, pull down experiments were

performed by using an ACP-tagged ErbB2 fusion protein. This fusion protein consists of an ACP consensus sequence added to the N-terminal region of ErbB2. This approach allowed us to specifically biotinylate the fusion protein expressed in the plasma membrane and pull down the receptors' complexes using streptavidin-sepharose beads.

To perform pull-down experiments, we used COS7 cell line due to its high transfection efficiency (around 90%). In addition, we chose to overexpress the ErbB2 and DDR1 fusion proteins due to the low expression levels of the endogenous proteins in this cell system. Moreover, the expression of DDR1-ECFP and beta1-integrin-ECFP allow us to detect the fusion protein by using both anti-GFP and specific antibodies.

ACP-ErbB2 was cotransfected with ErbB2-mYFP, beta1-integrin-ECFP or DDR1-ECFP in COS7 cells. Precipitated proteins were detected by Western blot using anti-GFP, anti-beta1-integrin and anti-DDR1 antibodies (Fig. 3 A). Given the self-association between ErbB2 receptors, ACP-ErbB2 coprecipitates with the fusion protein ErbB2-mYFP. In addition, ErbB2/DDR1 complexes were detected by using both GFP antiserum and anti-DDR1 monoclonal antibodies after pulling down ACP-ErbB2. These results support the hypothesis of a direct or indirect physical interaction between such receptors. On the contrary, ErbB2 showed no interaction with beta1-integrin-ECFP nor the endogenous beta1-integrin (highly expressed in these cells).

In order to discard if the interaction observed in ACP-ErbB2 pull-down was forced by protein overexpression, receptors' interaction was assessed in untransfected MCF7 and SKBR3 cells. It was possible to detect DDR1 in ErbB2 immunoprecipitates of both MCF7 and SKBR3 cells (Fig. 3 B and C). ErbB2 was detected in DDR1-immunoprecipitation of SKBR3 lysates but not in MCF7 cell immunoprecipitates, probably due to its low expression levels. In both assays, beta1-integrin was bellow detection levels.

Although it remains unclear whether ErbB2 and DDR1 are directly interacting or are part of larger macromolecular complexes, the results here presented allow us to conclude that these receptors are located together in membrane structures of BC cells regardless of DDR1 and ErbB2 expression levels.

ErbB2, ErbB3 and DDR1 expression is correlated in normal mammary gland

In order to study the expression in normal cells, we used the COXPRESdb database [51] to generate a correlation tree for DDR1, ERBB2, ERBB3 and ITGB1 expression (genes coding for DDR1, ErbB2, ErbB3 and beta1-integrin, respectively) (Fig. 4 A). Such tree was constructed using the Hsa-u.c2-0 database (February 2019) and represents the correlation between the expression of the proteins here studied as the logarithm of the mutual rank (MR, a geometric average of the PCC rank from gene A to gene B and from gene B to gene A [52]). We found that ERBB2, ERBB3 and DDR1 gene expression form a cluster of correlation in human normal tissues, not discriminated by organ nor cell type. Surprisingly, DDR1-ErbB2 expression correlation is comparable to ErbB2-ErbB3, whose functional interaction has been largely described [46,53]. While ERBB2, ERBB3 and DDR1 expression is grouped, ITGB1 expression does not correlate with the RTKs here studied.

We also studied the expression of these receptors in both normal and tumoral breast samples. While DDR1 expression does not significantly correlates with ErbB2 when this analysis was performed using bulk data from BC samples, we found a strong correlation between DDR1-ERBB2 and DDR1-ERBB3 in normal mammary gland samples (Fig. 4 B-E). Although PCC value for DDR1-

ERBB3 is higher in normal samples, there is a significant correlation between such receptors' expression in both normal and BC datasets (Fig. 4 E). Together, these results endorse our hypothesis that DDR1, ErbB2 and ErbB3 would be forming a signaling cluster, with the coordinated expression of these RTKs observed in normal mammary gland tissue being necessary for such a signaling cluster to be functional.

BC is a complex and heterogeneous disease. Accumulating evidence has suggested that BCs with different histopathological and biological features exhibit distinct behaviors that lead to different treatment responses and therapeutic strategies [54]. Given such complexity, we decided to analyze the expression correlation of such RTKs using data obtained from different BC subtypes samples. Only Luminal A and B subtypes showed a positive correlation between ErbB2 and DDR1 (Fig. 5 A and C). Additionally, we found that Normal tissue presents the highest PCC between these RTKs. Consistently with bulk data analysis, ErbB3 and DDR1 expression correlate in both normal breast and BC subtypes (Fig. 4 E, Fig. 5 B and D). In addition, Normal tissue and Normal-like BC samples showed significantly higher PCC than the other BC subtypes (Fig. 5 B and D). In all cases, there was no difference in the expression of the RTKs here studied with the exception of the HER2-positive subtype, in which ErbB2 was overexpressed.

Discussion

ErbB2 plays a key role in breast development, normal and tumor mammary gland physiology [55]. ErbB2 dimerization and phosphorylation leads to Ras/MAPK and PI3K/Akt signaling pathway activation [2], which is an important feature for both physiological and physiopathological processes. In the present study we showed that COL can modulate HRG induced Akt-

phosphorylation in MCF7 cells. Such phosphorylation occurs in conspicuous membrane structures where ErbB2 and DDR1 are located. The main COL receptors in mammary epithelial cells are beta1 integrin and DDR1 [20]. As previously shown by our group, beta1 integrin is located in Focal Adhesion Complexes outside ErbB2 clusters [31,50]. In contrast, DDR1 is placed in the same structures where ErbB2 and phospho-Akt can be found. We also proved that this spatial coexistence in such clusters is a consequence of a direct or indirect physical interaction between ErbB2 and DDR1, which occurs regardless of their expression levels. All together, these results present DDR1 as a novel player in ErbB interactomics. Although the mechanism of Akt activation in this conditions has not yet been explored, we proposed that Akt phosphorylation at Ser473 can be triggered by activation of mTOR complex 2. While the canonical mechanism of such complex activation relays on the activity of phosphoinositide 3-kinase (PI3K) in the plasma membrane, both PI3K-dependent and independent activation of mTOR complex 2 was described [56]. Given the complexity of such signaling pathway the localization of mTOR complex 2, the formation of phosphatidylinositol 3,4,5-trisphosphate by PI3K and the dynamics of Akt recruitment to ErbB2/DDR1 clusters must be further studied.

During mammary gland development, ErbB2 plays part in ductal morphogenesis as well as in lobuloalveolar remodeling during lactation [57]. Together with ErbB2 and ErbB3, HRG expression and ErbB3 driven PI3K/Akt pathway activation is critical for such processes [58,59]. On the other hand, KO mice for DDR1 present a hyperproliferative mammary gland epithelium with compromised epithelial cells' differentiation and lack of milk secretion [24].

The contradictory phenotypes show an antagonic role of ErbB2/ErbB3 and DDR1 in the normal mammary gland development and physiology. These results are coherent with the negative regulation of Akt phosphorylation by COL in MCF7 cells shown in the present study. By both

biochemistry and CLSM, we demonstrated the spatial interaction between ErbB2 and DDR1, which is accompanied by a correlation between these receptors' expression in Normal tissue.

Together with Luminal A, Normal-like tumors show the highest overall survival and better prognosis among BC subtypes [60]. Moreover, MCF7 interactomics results are coherent with the bioinformatic analysis as this cell line resembles the expression and immunohistochemical characteristics of Luminal A subtype BC cells [61], which showed a significant correlation between DDR1 and ErbB2 expression. Taking these results together, we propose that interaction between these RTKs would be a feature of normal epithelial cells that remains functional in less aggressive BC subtypes, being lost during the development of the most aggressive epithelial-derived tumors. Although further studies must be performed, correlation between DDR1 and ErbB2 expression in breast samples could be associated to low grade breast tumors.

Despite the fact that DDR1 was shown to modulate cancer cell migration [21,23] and promote the expression of MMP1, 2, 7 and 9 [25], data analyzed showed no overexpression of MMPs in any of the BC subtypes analyzed (data not shown). We suggest that the DDR1 role in BC would depend on the cell context. While it could be playing part as a regulator of other RTK signaling pathways in less aggressive phenotypes (such as Normal-like tumors), a feature remnant from its physiological role, more aggressive tumors would be unaffected by COL negative regulation and DDR1 activation could become an adaptive advantage by promoting MMP expression and invasiveness. This dual role proposed for DDR1 would justify the discrepancy observed in the literature regarding its expression in tumor samples [28,29].

Although their signaling pathways' interaction must be further studied, the data presented here open a new insight to study the complex processes in which these receptors are involved. Given the role of ErbB2/ErbbB3 and DDR1 in mammary gland's development, we propose that DDR1

could be acting as a fine tuner of ErbB2/ErbB3 signaling by the regulation of Akt signaling pathway in mammary epithelial cells. Likewise, the effect observed in the ErbB2 non-overexpressed BC cell line MCF7 would be a remnant mechanism relevant in the context of the normal physiology of the mammary gland.

Data Availability

The data that supports the findings of this study are available in Fig. 1 to 5 and/or the supplementary material of this article

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Figure Legends

Figure 1. HRG-induced Akt phosphorylation occurs in ErbB2 clusters of MCF7 cells.

(A) MCF7 cells stably-expressing the fusion protein ErbB2-TagRFP were stimulated 5 minutes with 1nM HRG prior fixation and stained for phosphorylated Akt at Ser473. Colocalization map shows the preferential location of the phosphorylated form of Akt in ErbB2 clusters. Scale bar: 10μm. Images are representative of at least 20 imaged cells in three independent experiments. (B) Representative immunofluorescence images of untransfected MCF7 cells plated on PDL, FN or COL prior stimulation for 5 minutes with 1nM HRG. Scale bar: 20μm. (C) Quantification of phospho-Akt immunofluorescence of MCF7 cells plated on PDL, FN or COL. **$p<0.01$ vs. PDL and FN (Tukey's test). Three independent experiments (10 images each) were quantified. (D)

Quantification of Western blots for phospho-Akt on untransfected MCF7 cells stimulated with 1nM HRG for 5 minutes. **p<0.01 vs. PDL and FN (Tukey's test). Three independent experiments were quantified.

Figure 2. DDR1, but not beta1 integrin, coexists with ErbB2 in membrane clusters of MCF7 stably expressing ErbB2-TagRFP and SKBR3 cells.

(A) MCF7 cells stably-expressing the fusion protein ErbB2-TagRFP were plated on FN-coated coverslips and stained for beta1 integrin or DDR1. (B) MCF7 ErbB2-TagRFP cells plated on COL-coated coverslips. (C) Quantification of Pearson's correlation coefficient (PCC), Manders' overlap coefficient (MOC), M1 and M2 coefficients for MCF7 cells. (D) SKBR3 were plated on FN-coated coverslips and co-stained for ErbB2 and beta1 integrin or DDR1. (E) SKBR3 cells plated on COL-coated coverslips. (F) Quantification of ErbB2 vs. beta1 integrin and ErbB2 vs. DDR1 colocalization. Images are representative of at least 20 imaged cells in three independent experiments. Scale bar: 10μm. **p<0.01 vs. beta1 integrin, n=20 (Student's test).

Figure 3. The COL receptor DDR1 physically interacts with ErbB2 in the plasma membrane.

(A) COS7 cells were transfected with ACP-ErbB2 fusion protein together with beta1 integrin-ECFP, DDR1-ECFP or ErbB2-mYFP as control. ACP-ErbB2 protein in the plasma membrane was specifically biotinylated by incubation with ACPwtS and biotin-CoA prior pull down using streptavidin-coated sepharose beads. Precipitated proteins were detected by Western blot using anti-GFP (which binds both mYFP and ECFP), anti-beta1-integrin and anti-DDR1 antibodies (B) Co-immunoprecipitation of ErbB2 and DDR1 in MCF7 cells. (C) Co-immunoprecipitation of ErbB2 and DDR1 in SKBR3 cells. Images representative of three independent experiments.

Figure 4. ERBB2 and ERBB3 expression correlates with DDR1 in normal tissue samples.

(A) Hierarchical clustering of DDR1, ERBB2, ERBB3 and ITGB1 expression in normal tissues represented as the logarithm of the MR. ERBB2, ERBB3 and DDR1 gene expression are closer related than ITGB1. Data obtained from CoxpressDB. (B) Dot plot for ERBB2 vs. DDR1 gene expression in normal and tumoral breast samples. (C) Dot plot for ERBB3 vs. DDR1 gene expression. PCC for both ERBB2 (D) and ERBB3 (E) vs. DDR1 in normal and cancer mammary gland samples. Purple dots: normal breast samples. Blue dots: BC samples. ***$p<0.001$ vs. normal samples (Tukey's test).

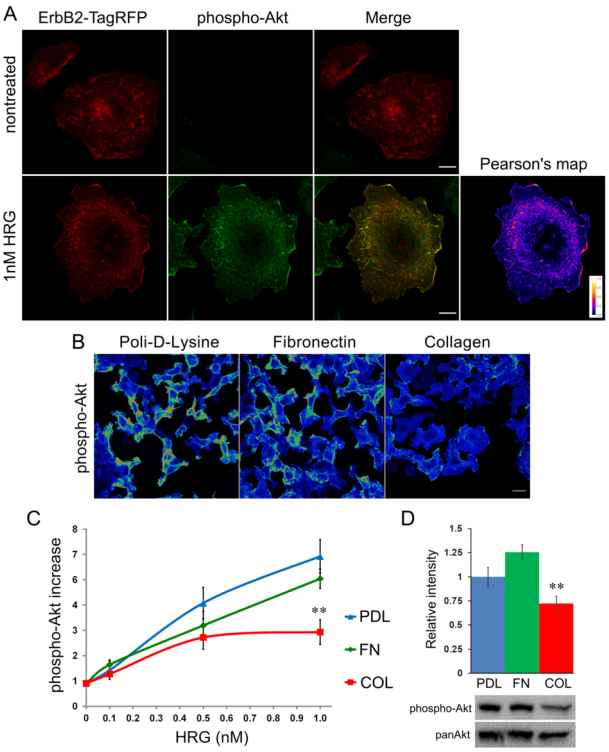
Figure 5. ERBB2 and DDR1 expression correlates in normal-like BC tumors.

Dot plots correlating expression levels of: (A) ErbB2 vs. DDR1 and (B) ErbB3 vs. DDR1. (C) PCC for ErbB2 vs. DDR1. Only Normal, Luminal A and Luminal B showed a PCC significantly different from zero ($p<0.001$). (C) PCC for ErbB3 vs. DDR1 expression. All samples showed a PCC significantly different from zero ($p<0.001$). Different letters within a BC subtype indicate statistically significant differences at a significant level of $p<0.001$ (Tukey's test).

Figure S1. HRG-induced Akt phosphorylation requires ErbB2 activity in MCF7 cells.

(A) MCF7 cells were stimulated with 1nM HRG for 5 minutes in the presence of the PI3K inhibitor LY294002, the pan-ErbB kinase domain inhibitor CI1033 or the ErbB2 blocking antibody 2C4. While PI3K inhibition completely blocks Akt phosphorylation, unaffecting ErbB3 phosphorylation, blockage of ErbB2 activity by kinase domain inhibition or the impairment of homo- and heterodimerization depletes both ErbB3 activation and Akt phosphorylation,

demonstrating that ErbB2/ErbB3 dimerization is necessary for HRG-induced Akt phosphorylation. Images are representative of three independent experiments.



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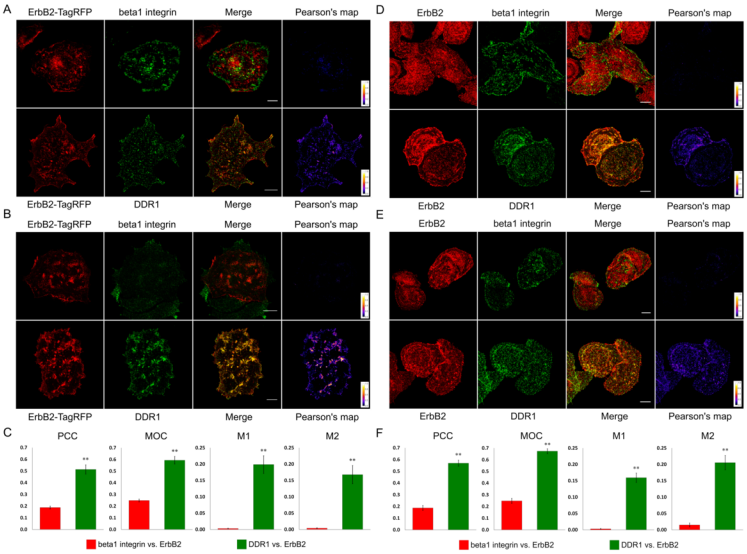

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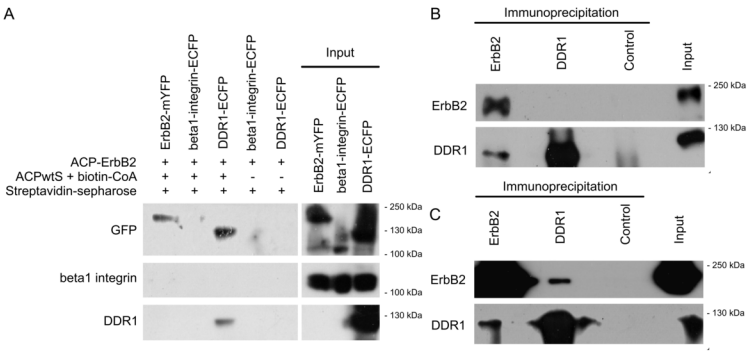

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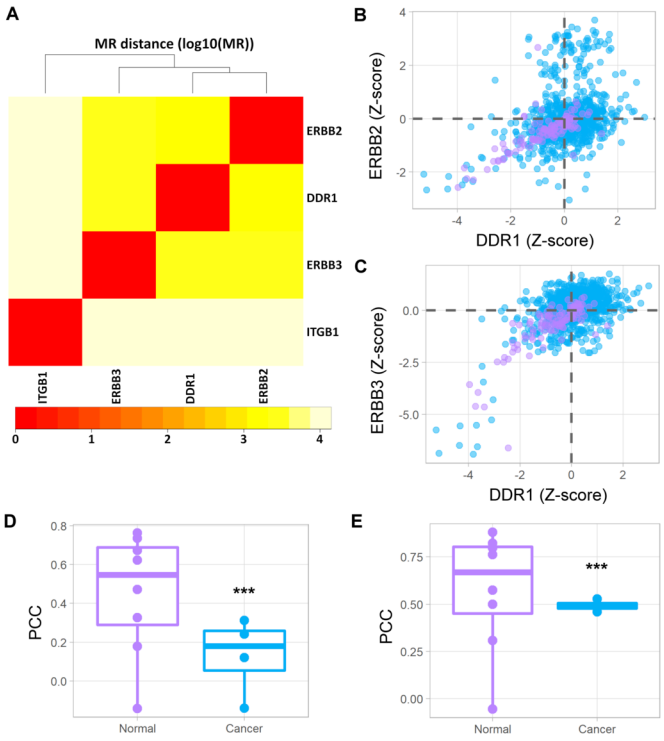

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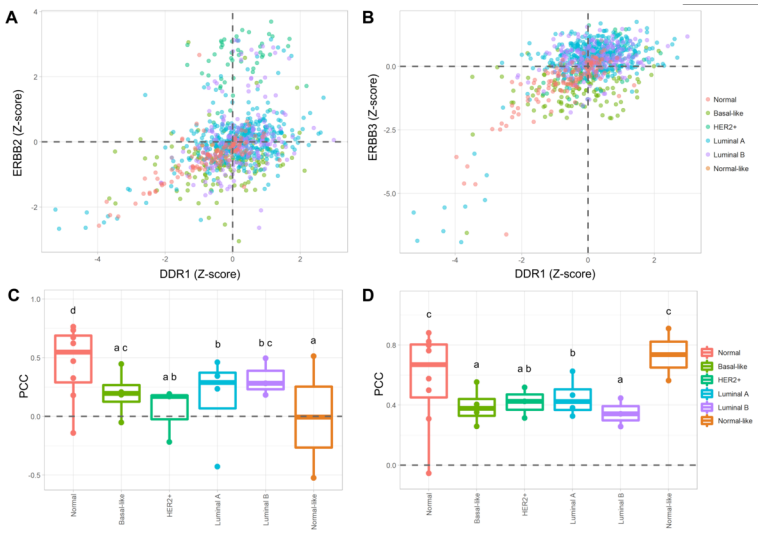

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