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Early Response to ErbB2 Over-Expression in Polarized Caco-2 Cells Involves Partial Segregation From ErbB3 by Relocalization to the Apical Surface and Initiation of Survival Signaling

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ABSTRACT

In several human cancers, ErbB2 over-expression facilitates the formation of constitutively active homodimers resistant to internalization which results in progressive signal amplification from the receptor, conducive to cell survival, proliferation, or metastasis. Here we report on studies of the influence of ErbB2 over-expression on localization and signaling in polarized Caco-2 and MDCK cells, two established models to study molecular trafficking. In these cells, ErbB2 is not over-expressed and shares basolateral localization with ErbB3. Over-expression of ErbB2 by transient transfection resulted in partial separation of the receptors by relocalization of ErbB2, but not ErbB3, to the apical surface, as shown by biotinylation of the apical or basolateral surfaces. These results were confirmed by immunofluorescence and confocal microscopy. Polarity controls indicated that the relocalization of ErbB2 is not the result of depolarization of the cells. Biotinylation and confocal microscopy also showed that apical, but not basolateral ErbB2 is activated at tyrosine 1139. This phosphotyrosine binds adaptor protein Grb2, as confirmed by immunoprecipitation. However, we found that it does not initiate the canonical Grb2-Ras-Raf-Erk pathway. Instead, our data supports the activation of a survival pathway via Bcl-2. The effects of ErbB2 over-expression were abrogated by the humanized anti-ErbB2 monoclonal antibody Herceptin added only from the apical side. The ability of apical ErbB2 to initiate an altered downstream cascade suggests that subcellular localization of the receptor plays an important role in regulating ErbB2 signaling in polarized epithelia. J. Cell. Biochem. 111: 643–652, 2010. © 2010 Wiley-Liss, Inc.

KEY WORDS: POLARITY; EPITHELIA; CARCINOMAS; COLON; ErbB; RAS; Bcl-2

ErbB2 is a class I receptor tyrosine kinase with exceptional features that distinguishes it from the other members of the family of ErbB epidermal growth factor receptors. No soluble ligand has been identified for ErbB2, thus the mechanism of activation differs from other ErbB family members, where the interaction with high-affinity soluble ligands induces the formation of homo- or heterodimers and phosphorylation [Yarden and Sliwkowski, 2001; Carraway and Kozloski, 2009]. Though dimer formation is necessary for signaling, it is not sufficient to initiate it. Instead, it is the adoption of a specific conformation that triggers signaling in receptor tyrosine kinases [Burke et al., 1997; Garrett et al., 2002; Ogiso et al., 2002]. Crystallographic data has revealed that ErbB2 presents a constitutively active structure that prevents canonical ligand binding, but is rather suitable for the formation of dimers [Cho et al., 2003; Garrett et al., 2003; Franklin et al., 2004]. This structure, which does not require different binding surfaces for each ErbB receptor, offers an explanation for the status of ErbB2 as the preferred heterodimer partner. ErbB2 also confers strength of signaling to its partner in two ways, via a very potent tyrosine kinase domain capable of activating both the MAPK and PI3 kinase

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pathways, and by hindering dimer internalization [Wang et al., 1999]. These interactions have been implicated in numerous developmental processes in normal tissues as well as in cancers of the breast, uterus, urinary bladder, lung, gall bladder, stomach, pancreas, and head and neck, where ErbB2 is aberrantly over-expressed. ErbB2 over-expression is regarded as a major contributor to cancer progression and an indicator of poor prognosis [Slamon et al., 1987; Alroy and Yarden, 1997; Klapper et al., 2000]. While not as commonly tested for as in cancers mentioned above, ErbB2 over-expression also plays a substantial role in patients with colon cancer. Different publications report ErbB2 over-expression in human colon cancer ranging 5–47% of the cases [Schuell et al., 2006; Park et al., 2007].

Two key effects of ErbB2 over-expression are the ligand independent constitutive activation of ErbB2 homodimers [Di Fiore et al., 1987; Lonardo et al., 1990; Worthylake et al., 1999] and the decrease in the turnover rate of its dimers by a deficiency in internalization and lysosomal degradation [Lenferink et al., 1998], which leads to progressive signal amplification. The ErbB2 carboxy-terminal region contains five tyrosine residues that, upon phosphorylation, provide potential binding sites for cytoplasmic signaling molecules [Riese et al., 1995; van der Geer et al., 1995; Dankort et al., 1997; Dankort and Muller, 2000] containing Src homology 2 (SH2) [Schlessinger, 1994] and/or protein tyrosine binding (PTB) domains [Kavanaugh et al., 1995; Dankort et al., 2001]. These cytoplasmic proteins interact in a sequence-specific manner, thereby initiating precise signaling cascades. The functions of ErbB2 are determined by the sites in the cytoplasmic domain that become phosphorylated and the effectors that dock at those sites [Hynes and Lane, 2005]. Thus, ultimately, the ability to initiate a signaling response depends on the localization of the receptor, and the binding proteins available in that specific microenvironment. Cell polarity contributes to define these biochemical and functional microenvironments within the cell, and the loss of this organization is a hallmark in the oncogenic process. Thus, potential effects of polarization, or loss of it on oncogenic receptors such as ErbB2 may be important to further understand this process in various carcinomas.

Studies in polarized human intestinal epithelial (Caco-2) cells have shown that ErbB2 localization is restricted to the basolateral surface via its interaction with Erbin [Borg et al., 2000]. We recently demonstrated that in polarized Caco-2 cells, ErbB2 is relocalized to the apical domain by over-expression of mucin4. This change of localization of ErbB2 does not involve over-expression of the receptor. In the apical localization, ErbB2 is activated at tyrosines 1139 and 1248 [Ramsauer et al., 2003, 2006], which are considered to be involved in processes of migration and proliferation [Dankort et al., 2001]. These phosphorylations, initiate a non-canonical survival pathway via p38 that includes the activation of AKT on serine 473. ErbB2 causes malignant transformation by over-expression resulting from gene reduplication [Birnbaum et al., 2009]. Thus, it is relevant to understand the initial effects of an increased level of expression of ErbB2 in a polarized epithelial cell. In this study, we present evidence that over-expression of ErbB2 leads to its apical redistribution, away from ErbB3, and phosphorylation on tyrosine 1139, which is not phosphorylated in basolateral ErbB2. Previous studies on polarized cells have shown that the phosphorylation of tyrosine 1139 on ErbB2 confers transformation potential to the cell, not by inducing proliferation, but rather by causing morphological changes such as cell spreading, and favoring survival [Dankort et al., 1997; Khoury et al., 2001; Ramsauer et al., 2006].

Because over-expressed ErbB2 is expected to saturate endogenous binding partners, and isolated ErbB2 molecules are known to partition in lipid rafts [Nagy et al., 2002], we hypothesized that over-expressed ErbB2 may relocalize to the apical domain by itself, even in the absence of over-expressed mucin4. The rationale for this idea is that partition in lipid rafts is a major sorting and transport pathway for apically bound membrane traffic in polarized epithelial cells [Fullekrug and Simons, 2004].

MATERIALS AND METHODS

CELL LINES AND CULTURE CONDITIONS

Caco-2 and MDCK cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained as described before [Rodriguez et al., 1994; Ramsauer et al., 2003]. For immunofluorescence experiments, the cells were seeded on 12 mm round cover slips (Fisher Scientific) or 6-mm Transwell-Clear™ filters (Corning Costar) at high density ($\sim 5 \times 10^4$ cells/cm^2) to obtain confluency in 2–3 days. For biotinylation experiments the cells were seeded on 24 mm Transwell-Clear™ filters (Corning Costar). Seventy-two hours prior to immunofluorescence or biotinylation experiments, Caco-2 cells (75% confluent) were transiently transfected with human full length ErbB2 cDNA clone commercially available (Origene, MD) or empty vector tagged with GFP as a control, using magnetic – assisted transfection (IBA GmbH, Germany) according to the manufacturer's instructions.

ANTIBODIES AND REAGENTS

To study ErbB2, we used two monoclonal antibodies from Lab Vision (Freemont, CA), namely NeoMarkers 2 (clone 9G6.10), which react against the extra cellular domain (ECD) of ErbB2, and NeoMarkers 8 (clone E2-4001), the polyclonal antibody against ErbB2 from Dako Cytomation (Carpinteria, CA) and from Calbiochem (anti-c-neu 5 and anti-c-neu 3). Additional antibodies against phosphorylated tyrosines included: anti-pY1248 from Millipore and Cell Signaling, and anti-pY1139 from Sigma, Upstate (Charlottesville, VA). ErbB3 was studied with the following antibodies from Lab Vision: NeoMarkers 7 and 10, and antibodies 4 and 5 from Calbiochem. p38, phospho-p38, and Bcl-2 were analyzed using antibodies from Cell Signaling and Millipore. Grb2 was detected using an antibody from BD Biosciences (San Jose, CA), and actin with a monoclonal antibody from Sigma (St. Louis, MO). p21-ras and ras-GTP were assayed using and antibody and kit from Pierce. Cell polarity was assessed using an antibody against intestinal alkaline phosphatase from BD Biosciences. All secondary antibodies were affinity purified with no cross-reactivity with other species. Peroxidase-conjugated secondary antibodies were obtained from Pierce and Promega. Alexa Fluor 488 and Texas Red-conjugated secondary antibodies (Molecular Probes, Eugene, OR), as well as FITC- and CY3-conjugated secondary antibodies (Jackson Immunological) were

used as specified by the manufacturers. Herceptin™, a humanized anti-ErbB2 monoclonal antibody, is manufactured by Genentech, San Francisco, CA, and extensively used as functional ErbB2 blocking therapy in humans. Immunoprecitation and selective cell separation was carried out with antibody-coated magnetic beads from Dynal (Invitrogen) according to the manufacturer's instructions. Methyl-β-cyclodextrin was from Sigma.

IMMUNOFLUORESCENCE

Caco-2 cells grown to confluence were fixed with 3% *p*-formaldehyde for 20 min at room temperature. After rinsing, the cells were permeabilized with 0.2% Triton X-100 for 5 min or 0.1% saponin throughout the procedure. Permeabilization was used in all cases, except when using anti-ErbB2 extracellular domain antibodies NeoMarkers 2, or anti-c-neu 5. Permeabilization was followed by quenching of the aldehyde groups in 50 mM NH_4Cl, and then the cells were incubated with primary antibody for 1 h at room temperature. The primary antibody was diluted in 1% BSA; in co-localization experiments, 0.1% immunoglobulin G of the same species as the secondary antibody was used instead of 1% BSA for rinsing steps and dilution of the primary antibody. After washes and incubation with secondary antibody, the cells were mounted in 10% polyvinyl alcohol, 30% glycerol, 1% *n*-propyl gallate, and Slow Fade™ (Molecular Probes, Invitrogen) at a dilution of 5:1. Laser confocal microscopy was performed with a Leica SP5 confocal microscope (Leica Microsystems GmbH, Germany). Stained cell monolayers were analyzed using a 63× oil immersion objective. The images were collected using the LAS AF software (Leica Microsystems GmbH).

POLARITY ASSAYS

The method for vectorial surface biotinylation followed was described before [Salas et al., 1997]. Briefly, cell monolayers grown to confluency on 24 mm Transwell-Clear™ filters (Corning Costar) were biotinylated on the apical or basolateral surfaces leaving tissue culture medium on the opposite side, 48 h after transient transfection with ErbB2 or empty vector tagged with GFP. After rinsing, the surface proteins of the cells were biotinylated at 4°C using a cell membrane-impermeable biotin derivative, sulfo-NHS-biotin (Invitrogen). Biotinylated proteins were affinity purified in batch with streptavidin-conjugated agarose beads (Pierce) at 4°C overnight. The biotinylated proteins were eluted from the beads in 2% SDS.

SDS–PAGE AND IMMUNOBLOTTING

For immunoblot analyses, proteins were enriched by three methods. Apical proteins by biotinylation and streptavidin pull-down. Alternatively, specific anti-ECD ErbB2 antibodies were added to the apical side, and the cells were then extracted and immunoprecipitated. For the rest of the assays, ErbB2-transfected cells were dissociated (according to kit manufacturer's instructions) and enriched by selection with the same antibodies using the CELLection kit (Invitrogen) and magnetic beads (Dynal) coated with anti-mouse IgG. The samples were run in SDS–PAGE, and blotted onto nitrocellulose or PVDF membranes. As additional controls for specific antibodies, lysates of EGF-treated ovary adenocarcinoma (SKOV-3), colorectal adenocarcinoma (SW620), and epidermoid carcinoma (A431) cells were purchased from Nanotools (Teningen, Germany). The signal of primary monoclonal or polyclonal antibodies was detected using secondary affinity-purified goat anti-mouse or anti-rabbit immunoglobulins coupled to peroxidase and a chemiluminescent system (Invitrogen) and exposed on X-ray film (Kodak). The intensity of the bands was estimated by digitizing the image (Scion Image) from X-ray film. After subtracting the background, all band intensities were compared against a control.

RESULTS

ErbB2 AND ErbB3 ARE SEPARATED BY RELOCALIZATION OF ErbB2 TO THE APICAL DOMAIN OF POLARIZED EPITHELIAL CELLS OVER-EXPRESSING ErbB2

MDCK and Caco-2 cells are two well-established systems to study cell polarity and trafficking [Le Bivic et al., 1990; Gilbert and Rodriguez-Boulan, 1991], including ErbB2 localization studies [Borg et al., 2000]. Despite the fact that Caco-2 is a cancer cell line, these cells undergo spontaneous enterocytic differentiation upon culture in vitro, and apicobasal polarity is similar to normal intestine cells [Gilbert and Rodriguez-Boulan, 1991]. In Caco-2 [Xu et al., 2005], and MDCK [Borg et al., 2000] cells, ErbB2 is not over-expressed and is detectable by immunofluorescence at the basolateral domain in polarized cells. We confirmed this in Caco-2 cell cultures (Fig. 1A), thus validating them as an appropriate platform to test the effects of ErbB2 over-expression. We first determined whether the up-regulation of ErbB2 expression causes relocalization of the molecule from the basolateral to the apical domain. We probed the apical surface of non-permeabilized confluent monolayers with two different antibodies against the extracellular domain of ErbB2. Caco-2 cells transfected with ErbB2 (+) showed relocalization of the receptor to the apical domain, where the exposed ECD was detected. This change of localization was better observed in the *x*–*z* plane (Fig. 1B). In addition, the lateral domains of the transfected cells were not stained although they are positive in permeabilized cells (Fig. 2B), suggesting that ErbB2 over-expression does not grossly affect tight junction competence in these cells. In order to exclude transfection as the cause of ErbB2 relocalization from the basolateral to the apical domain, non-permeabilized cells were transfected with empty vector expressing GFP. The antibodies against the ECD of ErbB2, applied on the apical surface of the unpermeabilized monolayer, showed no apical signal (Fig. 1C). The location of ErbB2 was further addressed by extracellular biotinylation experiments. ErbB2 (+) or mock-transfected (−) Caco-2 confluent cell monolayers grown on filters were treated with a non-permeable biotinylating reagent from either the apical or the basolateral side. The cells were then solubilized for affinity purification of the biotinylated cell surface proteins with streptavidin-conjugated agarose. Immunoblotting of the streptavidin precipitates with anti-ErbB2 antibodies demonstrated the surface to which the ErbB2 was exposed. As shown in Figure 1D, ErbB2 was present at the apical surface only in Caco-2 cells transfected with ErbB2 (+), but absent in mock-transfected cells (−). Conversely, the endogenous ErbB2 was found on the basolateral surface in mock-transfected Caco-2 cells (basolateral −),

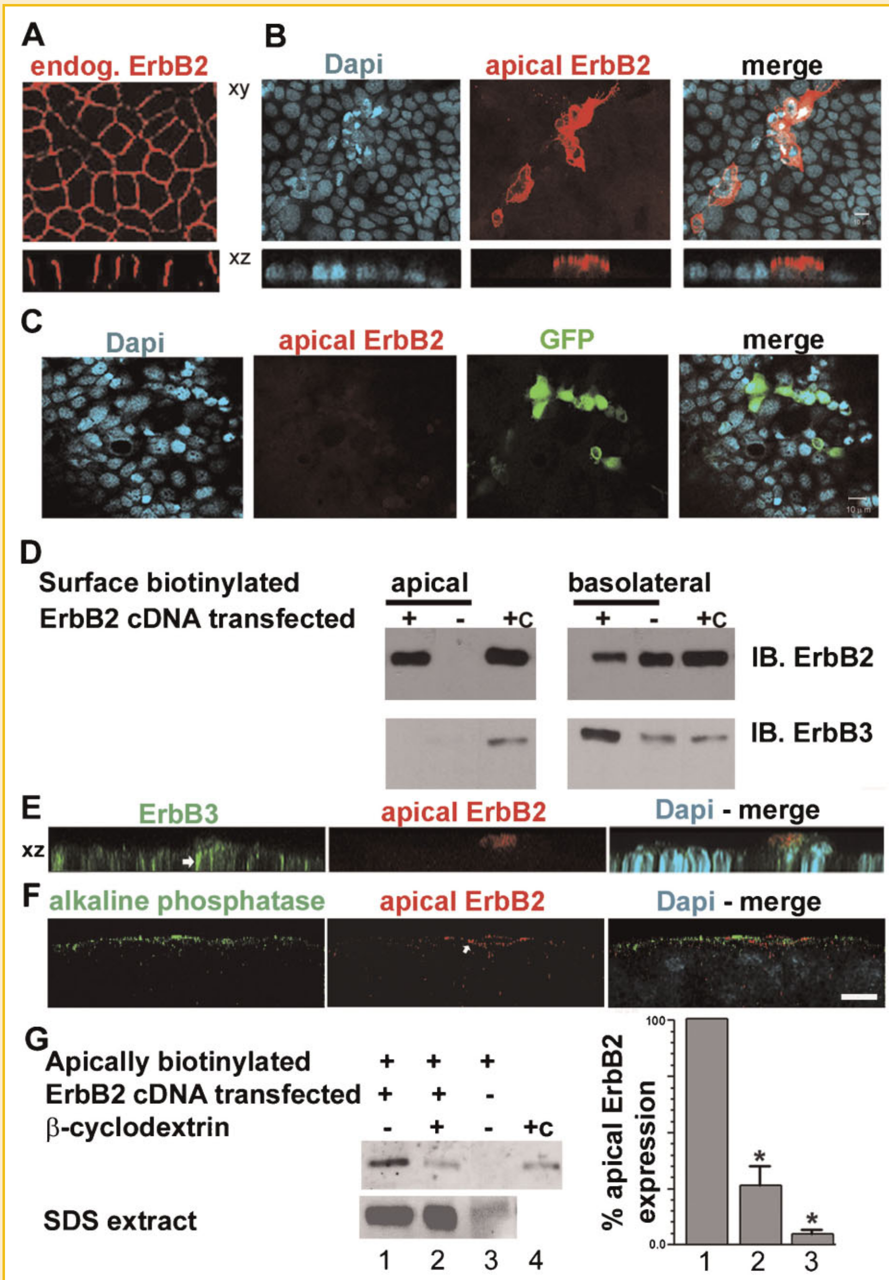

Fig. 1. Relocalization of ErbB2 to the apical surface of polarized Caco-2 cells by over-expression. A: Detection of endogenous ErbB2 on untransfected permeabilized Caco-2 cells. The cells were grown on filters for 10 days and then fixed and challenged with anti-ErbB2 ECD antibody (red). B: Apical localization of transfected (over-expressed) ErbB2. The cells were grown on filters and transfected with ErbB2 4 days after seeding the culture. These confluent non-permeabilized monolayers were fixed and processed with anti-ErbB2 ECD (red channel) from the apical side of the filter. A,B: Single confocal sections in the *x–y* plane (top panels), and 3D reconstructions of the confocal stack in the *x–z* plane (perpendicular to the monolayer, apical side up, bottom panels). C: Localization of ErbB2 in polarized mock-transfected Caco-2 cells (empty vector tagged with GFP). The confluent monolayers were transfected with vector expressing GFP as described in (A and B). The fixed, non-permeabilized monolayer was processed for immunofluorescence with anti-ErbB2 ECD antibody (red) only from the apical side of the filter. Scale bars, A–C, 10 μm. D: Detection of apical ErbB2 by extracellular biotinylation in ErbB2- (+) and mock-transfected cells (−). Caco-2 cells were grown on filters, transfected as described above and subjected to biotinylation from the apical or basolateral sides of the filter. Triton X-100 extracts were pulled-down with streptavidin–agarose and the SDS eluates separated by PAGE (left two lanes of each blot). A total SDS extract of a transfected monolayer was run in the right-hand side lane of each blot as a positive control for the antibody (+c). The immunoblot was performed with anti-ErbB2 antibody (top panels), or anti-ErbB3 antibody (bottom panels). The results shown are representative of three independent assays. E: Basolateral localization of ErbB3 in ErbB2-transfected cells by confocal immunofluorescence. Confluent Caco-2 monolayers were transfected as described in (B), and processed by sequential staining as follows: First, staining of non-permeabilized cells was performed with anti-ErbB2 ECD antibody (red). Then, the cells were permeabilized and processed with anti-ErbB3 antibody (green). The images are 3D reconstructions (*xz*) shown with the apical side up. F: Confocal immunofluorescence localization of intestinal alkaline phosphatase (iAP) in confluent Caco-2 monolayers cells by sequential staining as described in (E), except that anti-iAP antibody was used before permeabilization. The images 3D reconstructions (*xz*) shown with the apical side up. G: Cholesterol-sequestering methyl-β-cyclodextrin disrupts ErbB2 apical localization. ErbB2- or mock-transfected Caco-2 cells were exposed to 1 mM methyl-β-cyclodextrin for 24 h, biotinylated from the apical side, and lysed. Biotinylated proteins were pulled-down as described above, and ErbB2 was detected by immunoblot. The positive control for the ErbB2 band (+c) was performed as described above. Immunoblots from total SDS extracts of monolayers treated in parallel are shown in the bottom panel. For quantification (graph) ErbB2 band densities from the transfection/treatment conditions identified by the lane numbers, were calculated as percentages of the value for the transfected, untreated cells (100%), and shown averages ± standard deviations (for + cells only) from three independent experiments ($^{*}P < 0.05$).

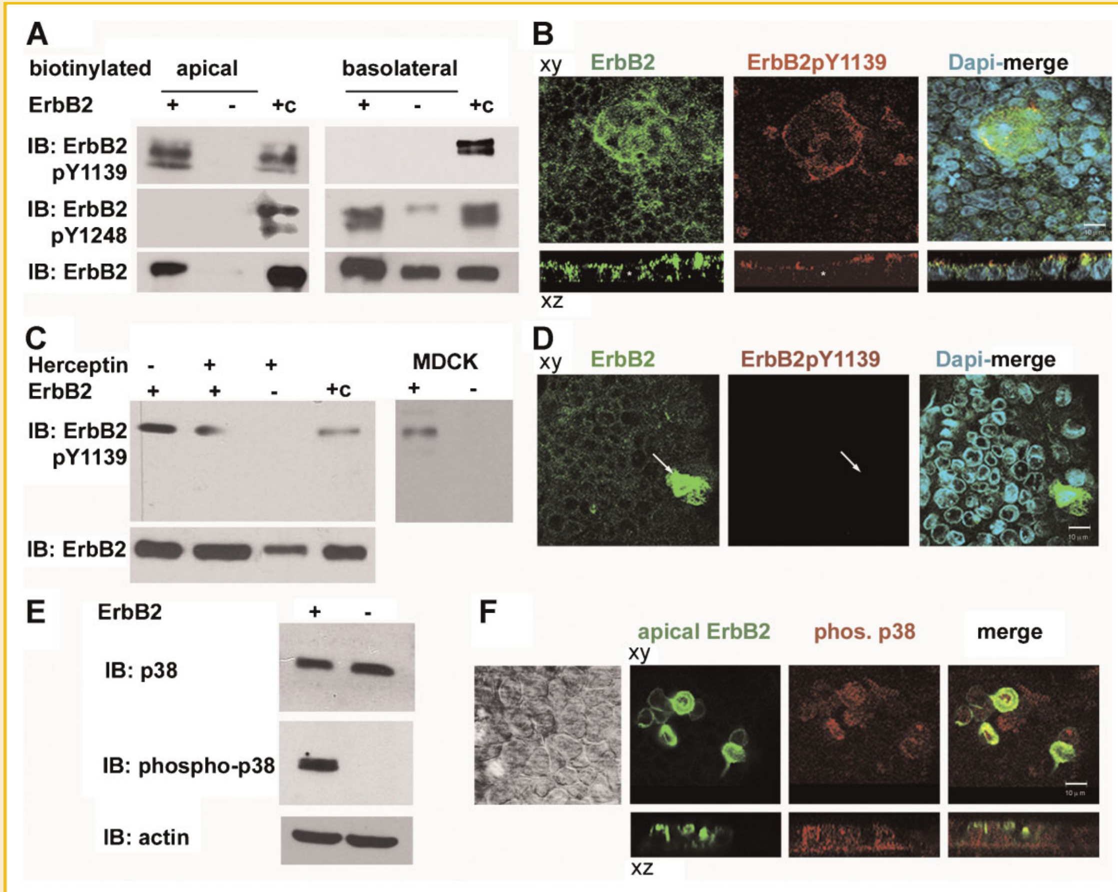

Fig. 2. Apical localization of ErbB2-activated tyrosine 1139 (pY1139) and phosphorylation of p38 in Caco-2 cells. A: Localization by vectorial biotinylation: Caco-2 cells were transfected with ErbB2 (+) or mock-transfected (−), biotinylated from the apical or basolateral side, and lysed. Biotinylated proteins were pulled-down with streptavidin-coupled beads. Total SDS extracts of SKOV3 cells were used as positive control for the antibody (+c). ErbB2 pY1139 and pY1248 were detected in the precipitates by SDS–PAGE and immunoblot with phospho site specific monoclonal antibodies. Total ErbB2 protein was localized in the same membranes by reprobing. B: Immunofluorescence localization of ErbB2 and its phosphorylated form, pY1139 ErbB2 by confocal microscopy in a confluent polarized Caco-2 cells. The cells were grown on filters, transfected with ErbB2 cDNA, permeabilized, and processed for immunofluorescence with anti-ErbB2 (green) and anti-ErbB2 pY1139 (red) antibodies. Confocal sections are shown in the *xy* plane and 3D reconstructions of the *xz* plane are shown apical side up (* non-transfected cell). C: Inhibition of apically localized pY1139 ErbB2 by apical Herceptin. Caco-2 cells were exposed to10 μg/ml Herceptin from the apical side for 24 h, biotinylated from the apical side, and lysed. Biotinylated proteins were pulled-down as described above, and ErbB2 pY1139 was detected by immunoblot. The control for locating the ErbB2 pY1139 band (+c) was performed as described above. Immunoblots from total SDS extracts of monolayers treated in parallel are shown in the bottom panel. In similar experiments, MDCK cells were transfected with ErbB2 (+) or mock vector (−), apically biotinylated and pulled-down with streptavidin. pY1139 was assayed for pY1139 ErbB2 by immunoblot. D: Apical Herceptin abrogates apical phosphorylation of Y1139 ErbB2. The experiments were performed as described in (B), but incubated with apical Herceptin as described in (C). The white arrow points at a group of ErbB2-transfected cells. E: p38 is activated in the ErbB2-transfected Caco-2 cells. ErbB2-transfected cells were labeled with anti-ErbB2ECD antibody from the apical side and enriched with magnetic beads. Equal amounts of protein in lysates from ErbB2- (+) and mock-transfected- (−) polarized Caco-2 cells were analyzed by immunoblot with anti-p38, anti-phospho-p38, and anti-actin antibodies (as a loading control). F: Confocal immunofluorescence localization of phospho-p38 in confluent Caco-2 monolayers cells by sequential staining. Staining of non-permeabilized cells was performed with anti-ErbB2 ECD antibody followed by FITC-conjugated secondary antibody (green). Then, the cells were permeabilized and processed polyclonal anti-phospho-p38 antibody (red); Note that only cells expressing ErbB2 at the apical surface of the cells have activated p38. The images are single confocal sections (*xy*) or 3D reconstructions (*xz*) shown with the apical side up. In blots, MW standards are shown in kDa. For immunofluorescence, scale bars represent 10 μm. The results shown are representative of at least three independent assays.

where it could not be discriminated from the over-expressed molecules in transfected cells because of the large excess of non-transfected cells.

THE SIGNALING INITIATED BY ErbB2 AT THE APICAL SURFACE OF POLARIZED Caco-2 CELLS DOES NOT INVOLVE ErbB3

To investigate whether over-expression and relocalization of ErbB2 causes the relocalization of ErbB3 to the apical domain as well, we assessed ErbB3 polarity in the same surface biotinylation experiments described above. The results were confirmed in independent experiments using antibodies against ErbB3. Figure 1D shows a representative immunoblot using an antibody against ErbB3 on the biotin pull-downs of apical or basolateral proteins from cells transfected with ErbB2 (+), or with an empty vector (−). Caco-2 cells showed no apical localization of ErbB3. These results were further confirmed by confocal microscopy. ErbB3 signal was absent from the apical domain of ErbB2 over-expressing cells (Fig. 1E). Thus, ErbB2 up-regulation does not relocalize ErbB3 to the apical surface

of polarized Caco-2 cells. Importantly, these experiments further show that ErbB2 over-expression does not increase tight junction permeability to the biotinylating agent, much smaller (MW <1,000) than IgG molecules. Clearly, the transfected cells maintain their polarity for ErbB3, and are not, therefore, generally depolarized. To further confirm this observation, we analyzed the status of an endogenous apical membrane protein in intestinal epithelia, alkaline phosphatase (Fig. 1F, green). It was found equally well polarized in non-transfected and transfected cells (Fig. 1F, arrow).

APICAL ErbB2 IN POLARIZED CELLS IS MAINLY LOCALIZED BY LIPID RAFTS

Previous studies in ErbB2 over-expressing cells indicate that ErbB2 protein clusters preferentially localize in lipid rafts, where the high concentration of ErbB2 molecules favors the formation of active homodimers [Nagy et al., 1999, 2002]. The possible association of apical ErbB2 with lipid rafts was tested by cholesterol sequestering using methyl-β-cyclodextrin followed by vectorial biotinylation of apical proteins as described above. Cholesterol sequestration has been extensively used to dissect the involvement of lipid rafts in apical membrane exocytosis [Keller and Simons, 1998; Hansen et al., 2000; Lipardi et al., 2000; Xiong et al., 2009]. Biotin pull-down of the apical proteins was followed by immunoblot with anti-ErbB2 antibody. As shown in Figure 1G, transfected Caco-2 cells treated with methyl-β-cyclodextrin, show a 71.5% decrease in the apical surface expression of ErbB2 as compared to untreated equally transfected cells. Because the total cellular expression of ErbB2 did not change under methyl-β-cyclodextrin treatment (Fig. 1G, SDS extract), we concluded that cholesterol chelation interfered with ErbB2 localization to the apical domain, which is consistent with lipid raft-mediated apical membrane traffic.

APICAL ErbB2 IS ACTIVATED AT Y1139 WHILE BASOLATERAL ErbB2 IS ACTIVATED AT Y1248

The phosphorylation state of the apical ErbB2 was analyzed by vectorial biotinylation of apical or basolateral proteins as described above, followed by streptavidin pull-down and immunoblot analysis. As shown in Figure 2A, ErbB2 was phosphorylated at phosphotyrosine 1139 only at the apical surface of the transfected cells (+), as compared with transfected (+) or mock-transfected cells (−) biotinylated from the basolateral side (Fig. 2A). Basolateral ErbB2 was successfully pulled-down but negative for pY1139 regardless of transfection. Conversely, phospho-Y1248 signal was found only in basolateral ErbB2 (Fig. 2A). Similar results were obtained in polarized, confluent MDCK cells (Fig. 2C, MDCK). The phosphorylation state of this tyrosine was further probed by confocal microscopy on transfected and permeabilized Caco-2 cell monolayers. Only ErbB2 over-expressing cells were found to display pY1139 signal (Fig. 2B, *xy*), and the signal was found only on the apical domain of transfected cells (Fig. 2B, *xz*).

HERCEPTIN PREVENTS PHOSPHORYLATION OF TYROSINE 1139 ON APICAL ErbB2

To further confirm the activation of ErbB2 at the apical domain we used the antibody Herceptin, which functionally abrogates ErbB2 phosphorylation [Sarup et al., 1991; Sliwkowski et al., 1999]. Because tight junctions are impermeable to antibodies, Herceptin was applied on the apical side only, to specifically modulate apical over-expressed ErbB2. The treatment was followed by vectorial biotinylation as described above. Apical Herceptin treatment resulted in the inhibition of phosphorylation of tyrosine 1139 on apical ErbB2 as shown by immunoblot (Fig. 2C, left hand side panels). Lysates of SKOV-3 cells known to express ErbB2 activated at tyrosine 1139 were used as positive controls (+c). This result was confirmed by confocal microscopy in confluent cell monolayers, permeabilized and processed for immunofluorecence after treatment with Herceptin on the apical side only. ErbB2 over-expressing cells (Fig. 2D, arrow, green channel) in a confluent monolayer, do not display a signal for ErbB2 activated at tyrosine 1139 (Fig. 2D, arrow, red channel).

MAPK p38 IS ACTIVATED IN POLARIZED EPITHELIAL CELLS TRANSFECTED WITH ErbB2

Previous studies have indicated that p38 is not activated in polarized Caco-2 cells [Ramsauer et al., 2006]. This was confirmed again by immunoblot from ErbB2-transfected and enriched Caco-2 cells (Fig. 2E +) or mock-transfected cells (Fig. 2E −). Only in polarized Caco-2 cells transfected with ErbB2 (+), p38 was activated at threonine 180/tyrosine 182. An immunoblot of total p38 confirmed the presence of the molecule in non-transfected cells. This observation was probed further by confocal microscopy on cells processed for immunofluorescence (Fig. 2F). Confluent, polarized Caco-2 monolayers, transfected with ErbB2, shown by phase contrast on the far left panel, were treated before permeabilization, with an ErbB2 antibody against the extracellular domain in order to detect the re localized apical ErbB2 (green channel). In all cases, cells expressing ErbB2 at the apical domain, also expressed activated p38 (red channel). However, ErbB2 and phospho-p38 did not co-localize, since over-expressed ErbB2 was cortical and phospho-p38 showed a cytoplasmic distribution (Fig. 2F, *xz* planes).

$p21^{RAS}$ IS NOT REQUIRED IN THE PATHWAY INITIATED BY THE ErbB2 ACTIVATION OF TYROSINE 1139 AND SUBSEQUENT BINDING OF Grb2

Previous studies have indicated that phosphotyrosine 1139 binds Grb2, an adaptor protein linked to downstream signaling from receptor tyrosine kinases. To test this possibility in our system, Caco-2 cells transfected with ErbB2 were incubated with anti-ErbB2 ECD antibody from the apical side, and then extracted and processed for immunoprecipitation (Fig. 3A, apical ErbB2). Immunoblots of anti-apical ErbB2 immunoprecipitates from ErbB2-transfected Caco-2 cells (+), were positive for Grb2, supporting the notion that this adaptor protein is utilized by apical ErbB2 molecules. This is important because it represents a difference in signaling due to ErbB2 over-expression in polarized cells as compared to non-polarized cells where the downstream signaling is mostly mediated by Erk [Yarden and Sliwkowski, 2001; Ramsauer et al., 2006]. Recent studies have shown that Grb2 mediates not only the canonical Sos/Ras pathway, associated with cell proliferation [Downward, 1994], but also Ras-independent pathways associated with morphological changes [Puto et al., 2003]. To determine whether Grb2 bound to apical ErbB2 is activating Ras, we used a pull-down assay for active

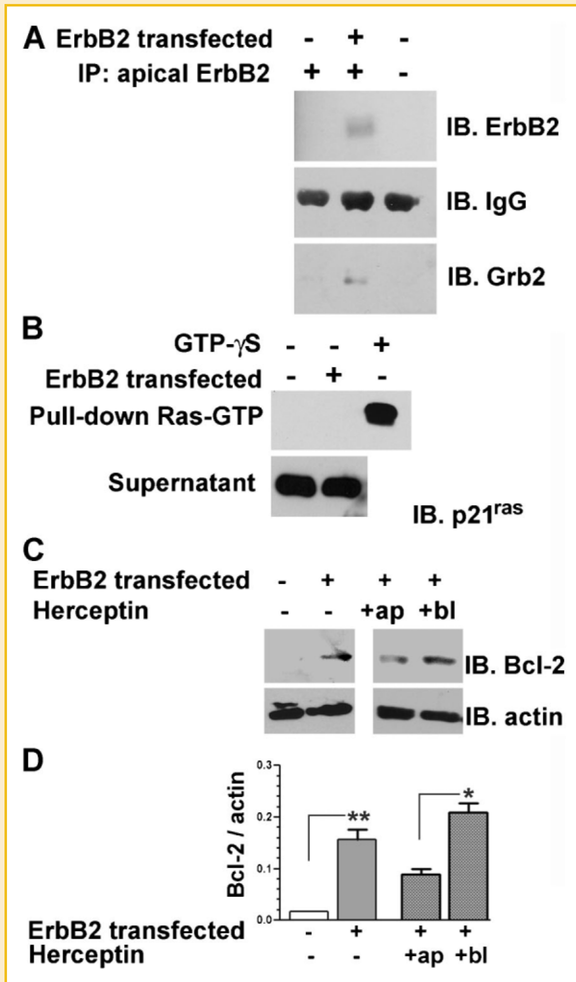

Fig. 3. Analysis of downstream effectors induced by the over-expression of ErbB2 in polarized Caco-2 cells. A: Co-immunoprecipitation of Grb2 with anti-ErbB2 in the apical side. The precipitates were obtained by antibody labeling and separation using magnetic beads. CACO-2 cells transfected (+) or not (mock transfected, empty vector, −) were incubated from the apical side with anti-ErbB2 ECD antibody, extracted in Triton X-100 and immunoprecipitated (left two lanes). An IP control (−) was processed similarly but with a non-immune IgG (right hand side lane). The eluates were immunoblotted with anti-ErbB2 Ab, secondary antibody anti-immunoprecipitating IgG, or anti-Grb2 Ab. This figure is representative of at least three separate experiments. B: ErbB2 activation does not activate p21*ras*. Equal amounts of total protein in extracts from ErbB2-transfected and enriched for ErbB2 ECD in the apical side as described above (+), mock-transfected polarized Caco-2 cells (−) (left two lanes), and non-transfected controls activated by treatment with GTPγS (control) were used pulled-down with GTP-p21*ras* affinity beads (glutathione *S*-transferase fusion protein corresponding to the human Ras-binding domain of Raf-1 bound to glutathione–agarose beads). After SDS–PAGE, membranes were probed with an anti-p21*ras* antibody. Ras-GDP in the supernatants was used as a control. The results shown are representative of three independent experiments. C: ErbB2 activation up-regulates Bcl-2 but is abrogated by apical Herceptin. Caco-2 cells were transfected or not with ErbB2, and some monolayers treated with Herceptin either from the apical side (ap+) or the basolateral side (bl+). Transfected cells were enriched for apical ErbB2 ECD on magnetic beads. Equal amounts of protein in lysates from those cells (ErbB2 +) or from non-enriched non-transfected cells (ErbB2 −) were analyzed by immunoblot with anti-Bcl2 antibody. The membranes were reprobed for actin as a loading control. The results presented are representative of three separate experiments. D: The ratios of Bcl-2/actin bands were normalized to the value for the non-transfected (−) cells. The values are averages ± standard deviations (for + cells only) from three independent experiments ($^{**}P < 0.005$; $^{*}P < 0.05$).

Ras (Ras-GTP) followed by immunoblot against p21^{ras}. As shown in Figure 3B, active Ras was absent in the extracts of the ErbB2-transfected cells (+), supporting the previous data on phosphorylation of tyrosine 1139 (treatment with GTP-γS was used as a positive control for the assay). To demonstrate the presence of the inactive protein in the samples assayed, the supernatants were also probed with the antibody against p21^{ras} (Fig. 3B, bottom panels). These results, therefore, support the activation of a Ras-independent pathway by Grb2 in Caco-2 cells over-expressing ErbB2.

Bcl-2 IS UP-REGULATED IN ErbB2 OVER-EXPRESSING CELLS

To determine whether Bcl2 is up-regulated in cells over-expressing ErbB2, confluent Caco-2 monolayers were probed with mouse monoclonal antibodies against the ErbB2 ECD on the apical side only, as indicated above, and the cells were enriched on anti-mouse antibody-coated magnetic beads. The ErbB2-transfected (+) cells or identical amounts of non-transfected cells (−), were solubilized, immunoprecipitated, and challenged in Western blot with anti-Bcl2 antibody. As shown in Figure 3C,D (left panels and bars), ErbB2-transfected/enriched Caco-2 cells (+) showed an approximate sevenfold increase in the level of expression of Bcl-2 as compared to untransfected cells (−). The membranes were stripped and reprobed with anti-actin antibody, and were used as a control to normalize the band intensity readings.

APICAL HERCEPTIN TREATMENT DOWN-REGULATES Bcl-2 IN ErbB2-EXPRESSING CELLS

In parallel experiments, performed along with those described in the previous section, Herceptin was applied on the apical surface of confluent polarized ErbB2-transfected Caco-2 cells, followed by immunoprecipitation and Western blot as described above. As shown in Figure 3C,D (right hand side panel and bars), transfected Caco-2 cells treated with Herceptin on the apical side (+ap), showed a 57% decrease in the level of expression of Bcl-2 as compared to equally transfected cells treated with Herceptin from the basolateral side (+bl). The latter showed Bcl-2 expression levels similar to transfected cells not treated with Herceptin. These results suggest that Bcl-2 up-regulation is dependent on apically localized ErbB2.

DISCUSSION

The majority of carcinomas arise from polarized epithelial cell lineages, with functionally and biochemically distinct apical and basolateral domains. Loss of cell polarity is a hallmark in the oncogenic process. However, many cancers cells retain various degrees of apico-basal polarity in vivo and in culture, and pre-neoplastic cells are often polarized. The main conclusion from this work is that in its early stages in still-polarized epithelial cells, over-expression of ErbB2 causes the partial relocalization of ErbB2 molecules to the apical domain, possibly as homodimers, away from ErbB3 which remains basolateral. This change of localization results in specifically apical phosphorylation of Y1139, Grb2 binding, and signaling mediated by p38 phosphorylation and Bcl-2 up-regulation, possibly conducive to cell survival.

Essential to this model, is the fact that transient ErbB2 over-expression did not cause loss of epithelial cell polarity: ErbB3 and alkaline phosphatase remained in their respective basolateral and apical localizations in transfected cells and the tight junctions remained impermeable to the small extracellular biotin reagent and to antibodies. This result challenges findings from other laboratories in MCF-10A, which were found to lose polarity in stable ErbB2 transfections [Debnath et al., 2002; Aranda et al., 2006]. Two non-mutually excluding explanations may account for the different results. First MDCK and Caco-2 cells are robustly polarized epithelial cells in monolayers. MCF-10A cells, on the other hand, require 3D cultures to polarize. Second, the results reported here correspond to short-term over-expression of ErbB2. This is a model more likely to represent initial stages of transformation, with still lower rates of proliferation, such as pre-neoplastic lesions. Stable ErbB2 transfection in rapidly dividing cells, as used in the previous studies, may mimic more advanced stages of neoplasia when epithelial polarity is usually lost. The apical relocalization of ErbB2 and activation, has been observed in pre-neoplasia of the pancreas [Chaturvedi et al., 2008] and gall bladder [Miyahara et al., 2008] as a result of the gradual increase of the mucin4 levels which favors their interaction and correlates with disease progression. The results in this article open the possibility that in pre-neoplastic conditions ErbB2 over-expression alone may shift the nature of the downstream signaling by relocalization to the apical domain of still polarized epithelial cells. Future studies on human biopsies will be required to test this working hypothesis.

The relocalization of ErbB2, combined with the increased number of available molecules for binding, may be a factor in overcoming the steric and ionic constraints associated with homodimer formation [Garrett et al., 2003; Franklin et al., 2004]. Thus, the relocalization of ErbB2 may effectively cause a shift from mainly heterodimer formation, such as ErbB2–ErbB3, a very effective proliferative unit [Holbro et al., 2003] to homodimer formation, at the apical surface of the polarized cell. While our data cannot dissect the dimerization state of ErbB2 at the apical domain, the methyl-β-cyclodextrin experiments strongly suggest that ErbB2 utilizes lipid rafts as a mechanism for apical sorting and intracellular transport. Because it is known that ErbB2 partition into lipid rafts is primarily in the form of monomers, and because the lack of apical ErbB3, this also suggests the possibility that apical ErbB2 may be either in the form of monomers or homodimers.

Two tyrosines in the cytoplasmic domain or ErbB2 are known to be pro-tumorigenic: Y1139 and Y1248. Work from other laboratories using phospho-mimetic mutants has clearly shown different docking proteins for these two phospho-sites (Grb2 and Shc, respectively), and different downstream targets and phenotypes [Dankort and Muller, 2000]. Our results indicate that apical (over-expressed) ErbB2 is phosphorylated on tyrosine 1139, which is not phosphorylated on basolateral ErbB2, but not in Y1248. Conversely, basolateral ErbB2 is phosphorylated in Y1248 but not in Y1139. Apical ErbB2, relocalized by mucin4 over-expression, on the other hand, is phosphorylated in both Y1248 and Y1139. Phospho-Y1139 docking of Grb2 is absent in polarized non-transfected Caco-2 cells, and only observed either under mucin4 over-expression [Ramsauer et al., 2006], or by ErbB2 over-expression (this work). GrbB2 is an adaptor protein commonly associated with cell proliferation pathways via Ras. However, recent reports have demonstrated that Grb2 can also mediate Ras-independent pathways related to survival [Puto et al., 2003], and morphological changes consistent with metaplasia [Khoury et al., 2001], in agreement with the results presented in this study, where the tyrosine 1139 phosphorylation is associated with the activation of p38 and up-regulation of Bcl-2. In summary, we conclude that ErbB2 over-expression in polarized cells not only has a quantitative effect, by increasing the signaling downstream of the pY1248-Shc adaptor for basolateral ErbB2, but also creates a second, previously non-existent signaling pathway, originating at the apical pY1139–ErbB2–Grb2 interaction.

The p38 pathway, originally associated with stress response and apoptosis, has recently been implicated in the regulation of differentiation mechanisms and cell-cycle control [Cuenda and Cohen, 1999; Maher, 1999; Zetser et al., 1999; Houde et al., 2001]. Our results indicate that p38 as well as Bcl-2 are activated in cells where ErbB2 has been relocalized to the apical. Importantly, over-expression of ErbB2 in non-polarized cells, where it cannot be segregated from ErbB3, does not result in Y1139 phosphorylation or p38 activation, but rather in alternative Erk activation [Carraway and Cantley, 1994]. These results further highlight that the atypical localization of ErbB2 in polarized cells is one of the initial response to the up-regulation of ErbB2, and initiates a new survival signaling pathway via the activation of tyrosine 1139, Grb2 adaptor, p38, and Bcl-2. This mechanism may be conducive to a heightened transformation potential, consistent with initial pre-neoplastic stages.

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