J. Biochem. **143,** 793–801 (2008)
doi:10.1093/jb/mvn032

Erbin-regulated Sensitivity of MCF-7 Breast Cancer Cells to TRAIL via ErbB2/AKT/NF-κB Pathway

Ning Liu, Jindan Zhang, Jinchun Zhang, Shilian Liu, Yanxin Liu and Dexian Zheng*

National Laboratory of Medical Molecular Biology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences & Peking Union Medical College, 5 Dong Dan San Tiao, Beijing 100005, China

Received December 28, 2007; accepted February 19, 2008; published online March 3, 2008

We have reported that Erbin expression was down-regulated in the Jurkat leukaemia T lymphocytes treated with the recombinant soluble tumour necrosis factor-related apoptosis-inducing ligand (rsTRAIL). Herein, we studied the expression and the regulation of Erbin and its binding partner, ErbB2, in the MCF-7 breast cancer cell line. We showed that the expressions of Erbin and ErbB2 were modulated by PKCδ inhibitor, rottlerin, in the TRAIL-resistant MCF-7 cell line. The affinity of Erbin–ErbB2 interaction was reduced by ErbB2 phosphorylation. Inhibiting the expression of Erbin facilitated the sensitivity of the MCF-7 cells to TRAIL via suppressing the ErbB2/AKT/NF-κB signalling pathway.

Key words: apoptosis, breast cancer, erbin, ErbB2, rsTRAIL.

Abbreviations: rsTRAIL, recombinant soluble tumor necrosis factor-related apoptosis-inducing ligand; LAP, LRR (leucine-rich repeat) and PDZ; TNF, Tumor necrosis factor; NF-κB, nuclear factor-kappa B; c-FLIP, FLIC (Fas associated death domain-like IL-1β-converting enzyme)-like inhibitory protein; IAP, inhibitor of apoptosis; EGF, epidermal growth factor.

Erbin was originally identified as a binding protein to ErbB2 (also known as HER-2 or Neu) (*1*). This protein belongs to the LAP [LRR (leucine-rich repeat) and PDZ (known as motifs of 80–90 amino acids that often bind to specific sequences at the extreme C termini of target proteins)] protein superfamily, which consists of 16 LRR and a single PDZ domain in its C terminus (*1*). It has been reported that the PDZ domain of Erbin binds with various proteins including ErbB2, Nod2 (*2*), p120 catenin (p0071, δ-catenin and ARVCF peptide) (*3–6*), PSD-95 (*7*), β_4-integrin and bullous pemphigoid antigen-1 (*8*). Erbin inhibits Ras-mediated activation of ERK pathway by disrupting the Sur-8-Ras-Raf complex (*9*, *10*). It is reported that Erbin participates in inflammatory responses by interacting with Nod2 and inhibiting Nod2-dependent activation of NF-κB and cytokine secretion (*2*). More and more studies indicated that Erbin plays important roles in cell polarization, receptor localization and signal transduction (*11*).

ErbB2 is a transmembrane receptor tyrosine kinase, which is a member of the EGFR family (*12*). ErbB2 heterodimerizes with other members of the EGFR family and promotes the transduction of proliferative and survival signals. Overexpression of ErbB2 correlates with poor prognosis and resistance to chemotherapy in multiple malignancies, including breast and ovarian cancers (*13*, *14*). Therefore, ErbB2 has been identified as a valuable molecular target for the treatment and diagnosis of these cancers. Because the constitutive activity of ErbB2 kinase is rarely found in ErbB2-overexpressing tumours (*15*, *16*), there are increasing studies focusing on identification of the novel models to regulate ErbB2 stability. Borg *et al*. (*1*) reported that the Erbin PDZ domain binds preferentially to the C terminus of ErbB2, which is non-Tyr1248-phosphorylated. Importantly, phosphorylation of Tyr1248 following ErbB2 activation is a critical event for the mitogenic signalling and oncogenicity of this receptor (*17*). Moreover, Tyr1248 plays an important role in the basolateral localization of ErbB2.

Tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a member of the TNF superfamily with the ability to induce apoptosis in a wide variety of transformed cell lines of diverse organs, but almost no toxic to most normal cells. TRAIL binding triggers receptor (DR4/DR5) trimerization and subsequent recruitment of various signalling proteins to the receptors. TRAIL not only transduced apoptotic signals through the activation of caspase cascade, but also induced nuclear factor-kappa B (NF-κB) activation, which could up-regulate expression of the genes of apoptosis inhibitors, such as c-FLIP [FLIC (Fas associated death domain-like IL-1β-converting enzyme)-like inhibitory protein] and IAP (inhibitor of apoptosis) protein family. Up to date, the exact causation, why some cancers are resistant to TRAIL cytotoxicity, is not well understood. The reports in the literatures (*18*) suggest that dysregulation of proliferation and apoptosis signal pathway may play major roles in the TRAIL killing-resistant tumour cells.

Our previous experiments demonstrated that Erbin mRNA expression was down-regulated in the Jurkat leukaemia T lymphocytes treated with the recombinant soluble TRAIL (rsTRAIL). In the present study, we further investigated the expression and the interaction of Erbin and ErbB2 proteins in TRAIL-resistant MCF-7

*To whom correspondence should be addressed. Tel: 8610 6529 6409, Fax: 8610 6510 5102, E-mail: zhengdx@pumc.edu.cn or zhengdx@tom.com

breast cancer cell line. Our data demonstrated that inhibiting the expression of Erbin facilitated TRAIL-induced MCF-7 cell death via suppressing ErbB2/AKT/NF-κB pathway.

MATERIALS AND METHODS

Materials—The rsTRAIL (a.a. 95–281, non-tagged) was prepared as previously described by Liu *et al*. (*19*). Protease inhibitors were purchased from Roche Molecular Biochemical (Basel, Switzerland). Polyclonal antibodies against Erbin, ErbB2, p-ErbB2, p-IκBα and IκBα were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Monoclonal antibody against β-actin was from Sigma (St Louis, MO, USA). Horseradish peroxidase (HRP)-conjugated anti-rabbit, anti-goat or anti-mouse IgG complex were provided by Zhongshan Co. (Beijing, China).

Cell Culture—Human breast cancer MCF-7 cells from American Type Culture Collection (Manassas, VA, USA) were maintained and cultured in RPMI 1640 medium containing 10% fetal calf serum (FCS, GIBCO-BRL) and 100 U/ml penicillin G, 100 μg/ml streptomycin and 1 mM L-glutamine at 37°C in a humidified 5% CO_2.

Western blot assay—The 2×10^5 cells were homogenized in lysate buffer (1× PBS, 1% NP40, 14 μg/ml pepstatin A and protease inhibitor cocktail tablets) (Roche, Mannheim, Germany). The protein concentration was determined by Bradford method. The lysates were subjected to SDS–PAGE then were transferred onto PVDF (polyvinylidene difluoride) membranes (Amersham Biosciences, Sweden). The membrane was blocked with 5% non-fat dry milk in TBS-T (20 mM Tris–HCl, pH 8.0, 100 mM NaCl and 0.1% Tween-20) for 1 h at room temperature and then incubated with the indicated primary antibodies in TBS-T containing 5% non-fat dry milk at 4°C overnight and followed with HRP-conjugated secondary antibodies. The specific proteins were visualized by using the ECL plus Western Blotting Detection System according to the manufacturer's instructions (Amersham Biosciences). All specific proteins bands were digitally scanned using an AlphaImager 2200 system (Alpha Innotech Corp., CA, USA). The ratios of each protein over the internal control, β-actin, were obtained for semi-quantitative analysis.

Down-regulation of Erbin and ErbB2 by siRNA—The nucleotide sequence of erbin siRNA and erbB2 siRNA was 5′-UAG ACU GAC CCA GCU GGA AdTdT-3′ as previously described (*20*) and 5′-CUC UCA CAC UGA UAG ACA C-3′, which are consistent of ~50% G/C. The NCBI sequence bank against these segments of DNA were searched using the BLAST program, which confirmed no match to any genes other than the target genes, verifying the specificity of the target region by the siRNA. The siRNAs were chemically synthesized by Genechem Co. Ltd. (Shanghai, China). A scrambled siRNA was also synthesized and used as a negative control. The cells in 35 mm dishes were transfected with the siRNA duplex using Lipofectamine™ 2000 (Invitrogen, CA, USA) and incubated for 48 h followed by lysing the cells with lysis buffer. Down-regulation of Erbin and ErbB2 by siRNA in the cell lysate was confirmed by western blot assay. Apoptosis of the cells was detected by Annexin V-FITC Apoptosis Detection Kit according to the manufacturer's instructions and followed by flow cytometry.

Immunoprecipitation and Immunoblotting—The cell lysates (~1 mg proteins) were incubated with or without the antibody against ErbB2 overnight at 4°C and subsequently with protein A-agarose beads at 4°C for 4 h on a rotating platform. After centrifugation, beads were collected and washed five times with NETN buffer (20 mM Tris–Cl, pH 8.0, 1 mM EDTA, 100 mM NaCl, 0.5% NP-40). The beads were heated in loading buffer at 90°C for 10 min and then subjected to SDS–PAGE. The proteins in the gel were transferred onto PVDF membranes. The specific proteins were visualized by using the ECL plus western blotting detection system.

Apoptosis Detection—The cells after treatment were stained using Annexin V -FITC apoptosis detection kit according to the manufacturer's instructions (BD Biosciences Pharmingen, CA, USA). Briefly, 2×10^5 cells were washed once with ice cold phosphate-buffered saline (PBS, pH 7.4) and re-suspended in 200 μl binding buffer. The cells were incubated with Annexin V- FITC for 30 min and then propidium iodidement (PI) for 5 min at room temperature. The apoptotic cells were analysed with flow cytometry (FACS Calibur, Becton Dickinson).

Dual Firefly and Renilla Luciferase Reporter Gene Assay—The pGL_2 NF-κB-Luc reporter plasmid consisting three copies of NF-κB binding sequence and one copy of firefly luciferase gene was constructed as described by Shigeno *et al*. (*21*). The MCF-7 cells were grown in 96-well plates in triplicate and co-transfected with or without Erbin-siRNA plus the pGL_2 NF-κB-Luc reporter plasmid. The phRL-TK (Promega), which expresses *Renilla* luciferase under the control of the TK promoter, was co-transfected as a control to monitor the transfection efficiency. Twenty-four hours after transfection, the cells were incubated in fresh media with or without rsTRAIL (1 μg/ml) for 4 h. The cells were lysed, and the activities of the two different luciferases were detected with their respective substrates with a Dual-Glo luciferase assay kit according manufacturer's instruction (Promega).

Real-time PCR—Total RNA was extracted from the cells with TRIzol reagent (Invitrogen). The cDNA was synthesized from 1 μg total RNA with M-MLV reverse transcriptase (Promega). The real-time PCR primers used to detect ErbB-2 were: 5′-CAT TTC TGC CGG AGA GCT TTG-3′ (sense) and 5′-ATT CGT CCC CGG ATT ACT TGC-3′ (anti-sense). The primers for β-actin were: 5′-ATG GTG GGA ATG GGT CAG AAG-3′ (sense) and 5′-CAC GCA GCT CAT TGT AGA AGG-3′ (anti-sense). The relatively real time quantitative PCR was carried out in a total volume of 20 μl containing 2 μl of diluted cDNA, 10 μl of 2× SYBR Green I mix buffer (ABI, Foster City, CA, USA), and 1 μM of each primer at 50°C for 2 min, 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 1 min by using Applied Biosystems 7500 (ABI). The data were quantified using Sequence Detection Software version 1.2 (ABI).

Statistical Analysis—All data presented in this article were expressed as mean values ± SD, and the Student's *t*-test was used for evaluating statistical significance. $P < 0.05$ were considered to be statistically significant.

All data were obtained from three independent experiments at least, $^{*}P < 0.05$, $^{**}P < 0.01$.

RESULTS

The Expressions of Erbin and ErbB2 were Regulated by Rottlerin in MCF-7 Breast Cancer Cells—We previously reported that the MCF-7 breast cancer cell line is resistant to TRAIL cytotoxicity, and the PKCδ inhibitor, rottlerin, sensitized the MCF-7 breast cancer cells to TRAIL cytotoxicity (*22*). To investigate the interaction of Erbin and ErbB2 in breast cancer, the MCF-7 cells were treated with PKCδ inhibitor rottlerin (15 μM), rsTRAIL (1 μg/ml) and rottlerin plus rsTRAIL, respectively, then ErbB2 and Erbin expression were analysed by western blot assay. As shown in Fig. 1D and E, rsTRAIL treatment did not affect the expression of ErbB2 and Erbin (Fig. 1A), whereas rottlerin suppressed the two protein expression significantly (Fig. 1B), and the combination of rottlerin and rsTRAIL further inhibited both ErbB2 and Erbin expression in a time-dependent manner (Fig. 1C), suggesting that PKCδ could regulate Erbin and ErbB2 expression and the sensitivity of MCF-7 cells to TRAIL-induced cell death.

ErbB2 Expression, but not Erbin, was Regulated by Active Caspase-8—Benoit *et al*. (*23*) reported that ErbB2 in NF-κB-defective cells could be cleavaged by TNFα-activated caspase-8. We further investigated whether caspase-8 was activated in the MCF-7 cells in the presence of rottlerin and/or rsTRAIL. Western blot analysis (Fig. 2) showed that rottlerin did not activate caspase-8, but rsTRAIL did and therefore cleaved caspase-8 substrate PARP, and the combination of rsTRAIL and rottlerin further activated caspase-8 and resulted more PARP degradation. However, in the presence of specific caspase-8 inhibitor, Z-IETD-fmk, rsTRAIL plus rottlerin-induced caspase-8 activation was blocked markedly. Interestingly, the combination of rsTRAIL and rottlerin inhibited both ErbB2 and Erbin expression, and Z-IETD-fmk could reconstitute the expression of ErbB2, but not Erbin, in the MCF-7 cells. These results indicated that ErbB2 expression, but not

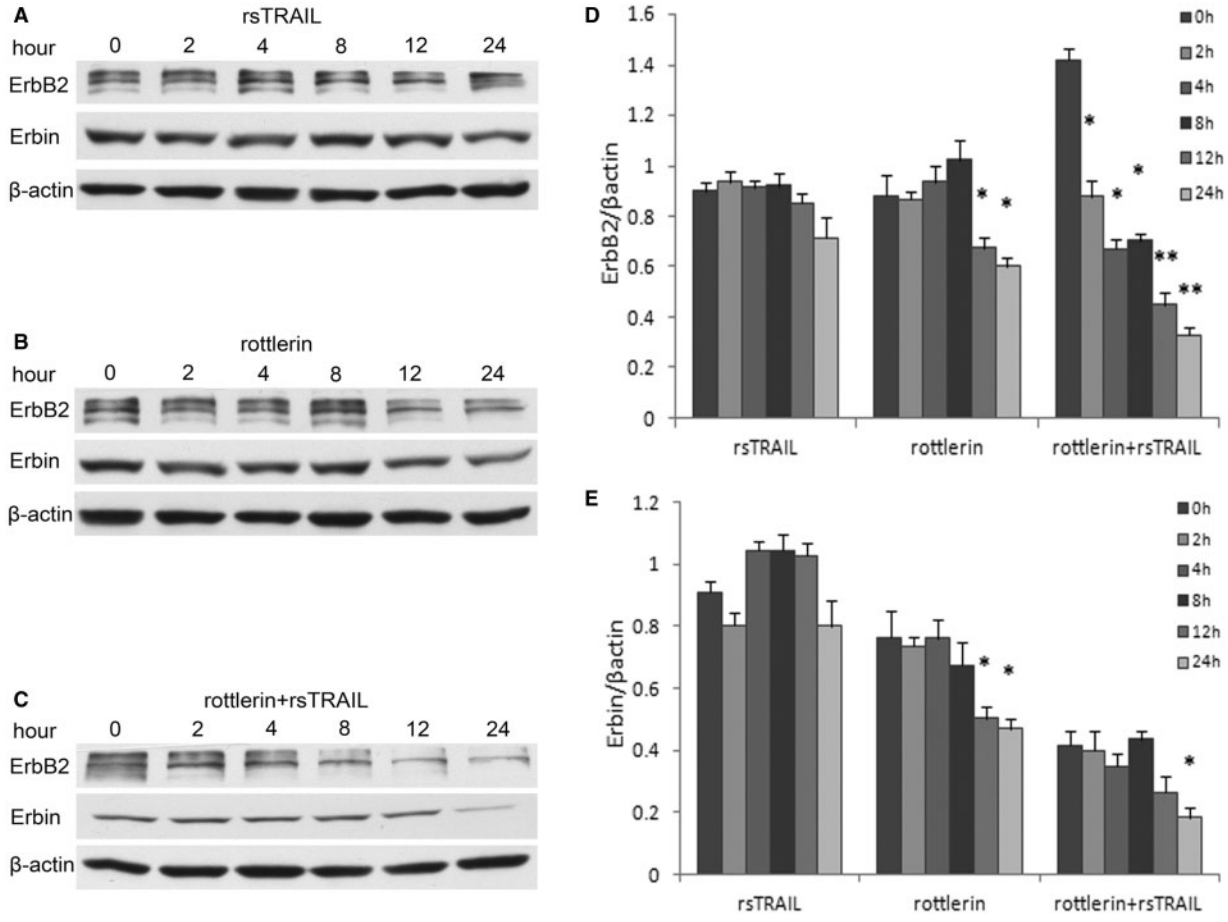

Fig. 1. **Rottlerin inhibited both Erbin and ErbB2 expression in the MCF-7 cells in a time-dependent manner.** The MCF-7 cells (2×10^5) were treated with rsTRAIL (A), rottlerin (B) and rottlerin plus rsTRAIL (C) for the indicated time course. The cells were lysed with lysis buffer and subjected to SDS-PAGE. The proteins in the gel were transferred onto PVDF membranes. The specific proteins were probed with individual antibody against Erbin or ErbB2 and visualized by using the ECL plus Western Blotting Detection System according to the manufacturer's instructions. The β-actin was used as control. (D and E) ErbB2 and Erbin expression were quantitative analysis compared with the internal control, β-actin. The ratios were statistically analysed with the Student's *t*-test.

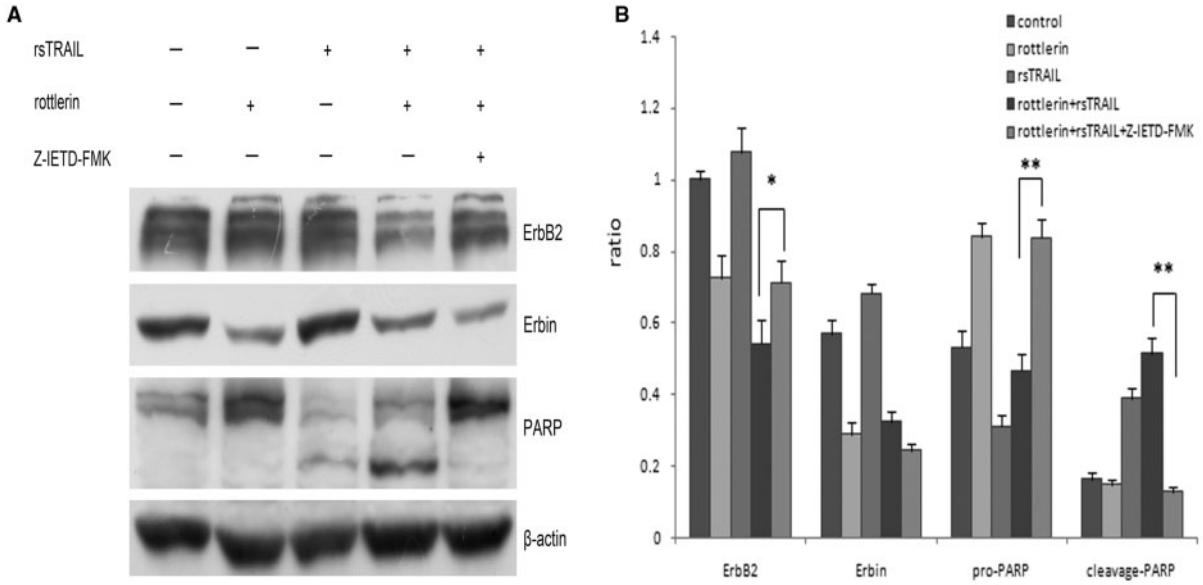

Fig. 2. **Rottlerin and rsTRAIL inhibited ErbB$_2$ expression in MCF-7 cells in caspase-dependent manner.** The MCF-7 cells were pretreated with 15 μM rottlerin and/or 10 μM Z-IETD-FMK for 1 h, then treated with 1 μg/ml rsTRAIL for 16 h. The cells were lysed with lysis buffer and the lysates were subjected to SDS-PAGE. Western blot analysis (A) and statistical analysis (B) were carried out as mentioned earlier.

Erbin, was regulated by active caspase-8 in the breast cancer cells.

ErbB2 Phosphorylation was Suppressed and Interaction of Erbin/ErbB2 was Enhanced in the Cells Treated with Rottlerin Plus rsTRAIL—Next, we investigated the interaction between ErbB2 and Erbin in the MCF-7 cells. The cells were treated by the combination of rsTRAIL (1 μg/ml) and rottlerin (15 μM) for a time course followed by co-immunoprecipitation with anti-ErbB2 antibody and western blot assay by using the antibodies against Erbin, ErbB2 and phosphorylated ErbB2, respectively. As shown in Fig. 3A, co-immunoprecipitated Erbin by anti-ErbB2 antibody was increased with the reduction of phosphorylated ErbB2 in the MCF-7 cells treated with rottlerin plus rsTRAIL in a time-dependent manner, suggesting that the combination of rottlerin and rsTRAIL suppressed ErbB2 phosphorylation but enhanced the affinity of Erbin-ErbB2 interaction.

To validate this result, the MCF-7 cells were treated with epidermal growth factor (EGF, 100 ng/ml), which is known to stimulate the tyrosine phosphorylation of ErbB2 (*24*). Then co-immunoprecipitation and western blot analyses were carried out to detect the expressions of Erbin and phosphorylated ErbB2 dynamically. As shown in Fig. 3B, ErbB2 was indeed quickly phosphorylated and reached a peak at 15 min then declined gradually, while Erbin was at lowest amount and then increased afterwards in the 60 min time course, confirming that phosphorylated ErbB2 declined the affinity of the Erbin–ErbB2 interaction significantly.

Down-regulation of Erbin by siRNA Facilitated Apoptosis of MCF-7 Cells—To further investigate the function of Erbin and ErbB2, the MCF-7 cells were transfected with Erbin-siRNA or ErbB2-siRNA and treated with rottlerin, rsTRAIL and rottlerin plus rsTRAIL, respectively. Down-regulation of Erbin and ErbB2 (data not shown) by siRNA in MCF-7 cells was confirmed by western blot assay. As shown in Fig. 4A, Erbin protein expression was significantly suppressed by Erbin-siRNA transfection in the cells compared with the scrambled-siRNA transfected cells. Apoptosis of the cells was detected by Annexin V-FITC Apoptosis Detection Kit followed by flow cytometry. As shown in Fig. 4C, the apoptosis ratios of the cells transfected with Erbin-siRNA or ErbB2-siRNA and then treated with rsTRAIL, rsTRAIL plus rottlerin were significantly more than cells which were transfected with the scrambled-siRNA ($P < 0.01$). Cell viability was also confirmed that knockdown of Erbin by RNAi decreased the cell viability (Fig. 4E). Taken together, these data demonstrated that down-regulation of Erbin by siRNA facilitated apoptosis of MCF-7 cells triggered by rsTRAIL, rsTRAIL plus rottlerin, significantly.

Erbin-regulated Sensitivity of MCF-7 Cells to TRAIL via ErbB2/AKT/NF-κB Signalling Pathway—To investigate the mechanism by which Erbin regulates the sensitivity of the MCF-7 cells to TRAIL cyotoxicity, the cells were transfected with or without Erbin-siRNA and then treated with rsTRAIL, the expressions of Erbin, ErbB2, AKT and phosphorylated AKT (p-AKT) were analysed by western blot using the specific antibodies. As shown in Fig. 5A, reduced ErbB2 expression and suppressed p-AKT were observed while Erbin was down-regulated by Erbin-siRNA. However, ErbB2 mRNA expression evaluated by real-time PCR did not change in the cells transfected with Erbin-siRNA compared to the control (Fig. 5C), suggesting that Erbin regulated the expression of ErbB2 at protein level. This result suggested that Erbin modulated the expression of ErbB2 and phosphorylated AKT.

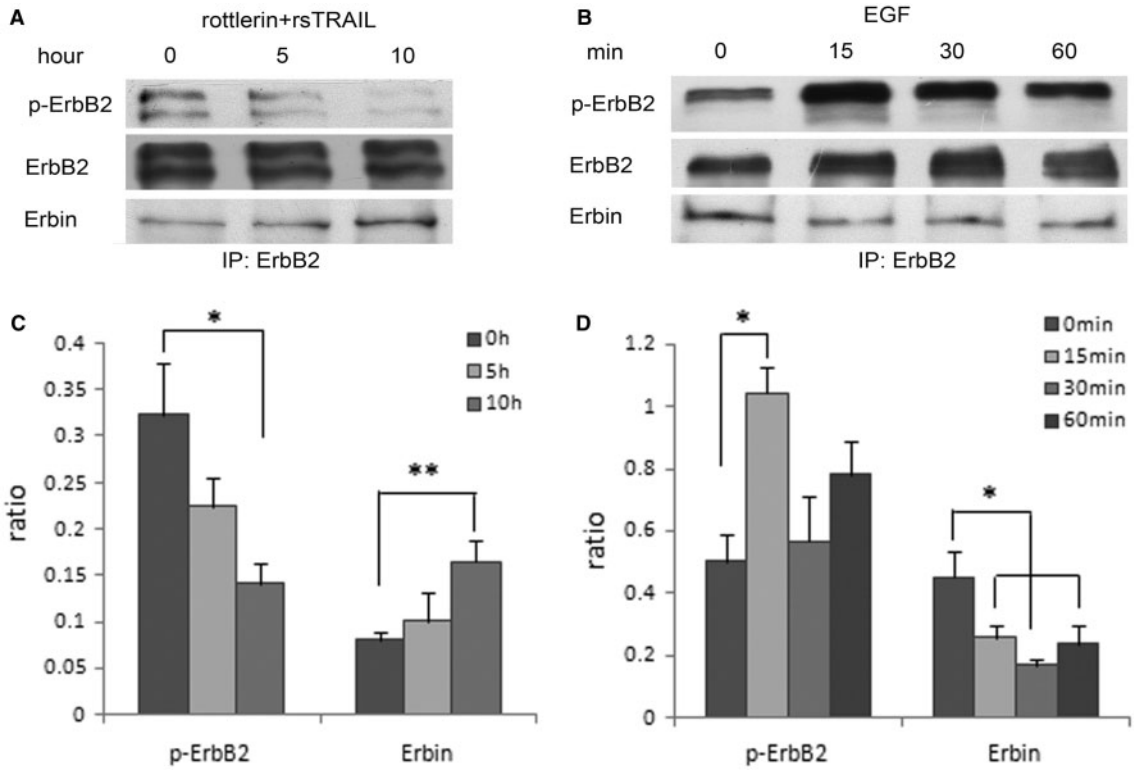

Fig. 3. **Rottlerin and rsTRAIL suppressed ErbB$_2$ phosphorylation and increased its interaction with Erbin.** (A) The cells were treated with rottlerin plus rsTRAIL for indicated time and lysed with lysis buffer. The lysates containing 1.0 mg proteins were incubated with ErbB2 antibody and subsequently with protein A-agarose beads. The beads were washed five times with NETN buffer and heated in loading buffer at 90°C for 10 min, then subjected to SDS–PAGE. The ErbB2, phosphorylated ErbB2 and Erbin were probed with individual antibody, respectively, and visualized in ECL plus Western blotting detection system according to the manufacturer's instructions. (B) The cells were treated with epidermal growth factor (EGF, 100 ng/ml) as an internal control. Then co-immunoprecipitation and western blot analyses were carried out as earlier. (C and D) phosphorylated ErbB2 and Erbin expression were quantitative analysis compared with ErbB2. The ratios were statistically analysed with the Student's *t*-test.

It is well known that NF-κB plays a key role in dynamic equilibrium between apoptosis and proliferation in various cells. We further checked the influence of Erbin on the activity of NF-κB. Western blot analysis showed that phosphorylated IκB (p-IκB, the inhibitor of NF-κB) was lowered in the cells transfected with Erbin-siRNA no matter treated with or without rsTRAIL (Fig. 5D). To investigate the regulation of NF-κB activity by Erbin, we co-transfected MCF-7 cells with or without Erbin-siRNA and pGL$_2$-NF-κB-Luc reporter plasmid, and treated with rsTRAIL. The activity of NF-κB was evaluated by the luciferase activity in the cell lysate. As shown in Fig. 5F, NF-κB activity was declined in the MCF-7 cells transfected with Erbin-siRNA and further down-regulated by rsTRAIL treatment. Taken together, these data suggested that Erbin regulated the sensitivity of the breast cancer cells to rsTRAIL cytotoxicity via ErbB2/AKT/NF-κB pathway.

DISCUSSION

TRAIL could specially induce apoptosis of cancer cells without toxicity to the most normal cells. However, there are about 50% of cancer cell lines which were insensitive to TRAIL. The exact molecular mechanism of TRAIL-resistant was not clear, more and more evidences demonstrated that TRAIL-mediated signal transduction pathways were the pivotal events, which are cell-specific in the various cells. Apoptotic or survival signals determine the fate of cells. We and others have reported that MCF-7 breast cancer cells are resistant to TRAIL cytotoxicity (*22*). Rottlerin, a PKCδ inhibitor, sensitizes the cells to TRAIL-induced apoptosis, suggesting that PKCδ might be one of the factors protecting the cells from TRAIL killing. And we have found that both Erbin and ErbB2 expressed at a higher level in the breast cancer than the matched normal tissue (data not shown). In the present study, we explored the function of Erbin and its interaction with ErbB2 in TRAIL-triggered signalling pathway in the MCF-7 cells to understand the regulation of the cell death and the significance for the treatment of TRAIL-resistant breast cancers.

We further investigate the interaction of Erbin and ErbB2 and observed that treatment of MCF-7 cells with PKCδ inhibitor, rottlerin, at the presence or absence of rsTRAIL down-regulated the expressions of both Erbin and ErbB2 proteins, and Z-IETD-fmk, a specific caspase-8 inhibitor, reconstituted the expression of ErbB2, but not Erbin, indicated that rottlerin and rsTRAIL activated caspase-8 and induced caspase-8-dependent ErbB2

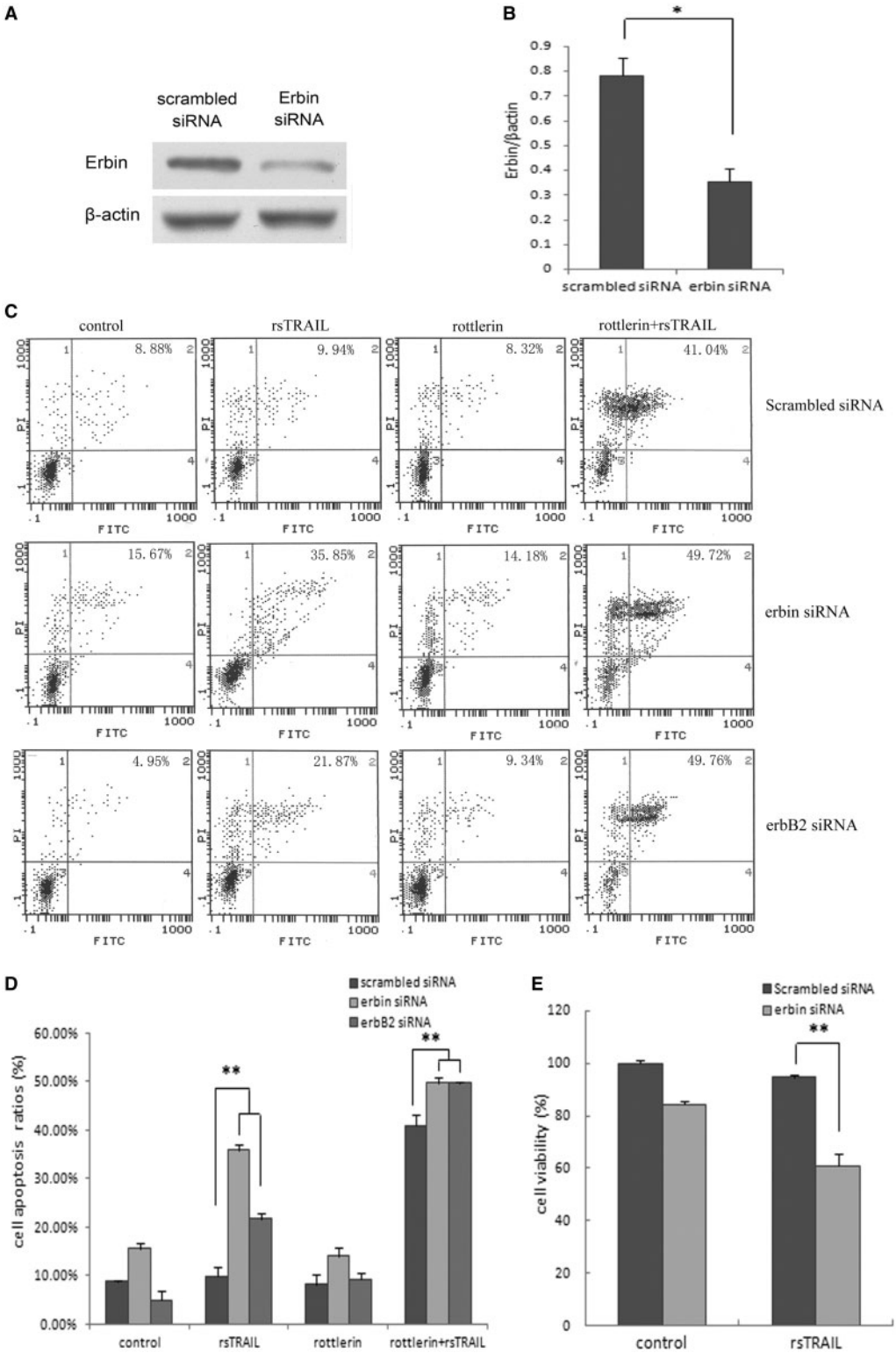

Fig. 4. **Erbin-siRNA facilitated apoptosis of MCF-7 in presence of rsTRAIL.** The 21-nucleotide *erbin* siRNAs were chemically synthesized and a scrambled siRNA was used as a negative control. (A) The cells in 35 mm dishes were transfected with the Erbin-siRNA duplex and incubated for 48 h. The cells were lysed with lysis buffer. The lysates were subjected to SDS–PAGE and western blot assay to confirm the down-regulation of Erbin. (B) Erbin expression were quantitative analysis compared with the internal control, β-actin. (C) The cells transfected with the Erbin-siRNA or ErbB2-siRNA were treated with rottlerin, rsTRAIL and rottlerin plus rsTRAIL, respectively. Apoptosis of the cells was detected by Annexin V-FITC Apoptosis Detection Kit according to the manufacturer's instructions and followed by flow cytometry. (D) Statistical analysis of apoptosis ratio. (E) The cells transfected with the Erbin-siRNA were treated with or without rsTRAIL. Cell viability was determined by the MTT assay.

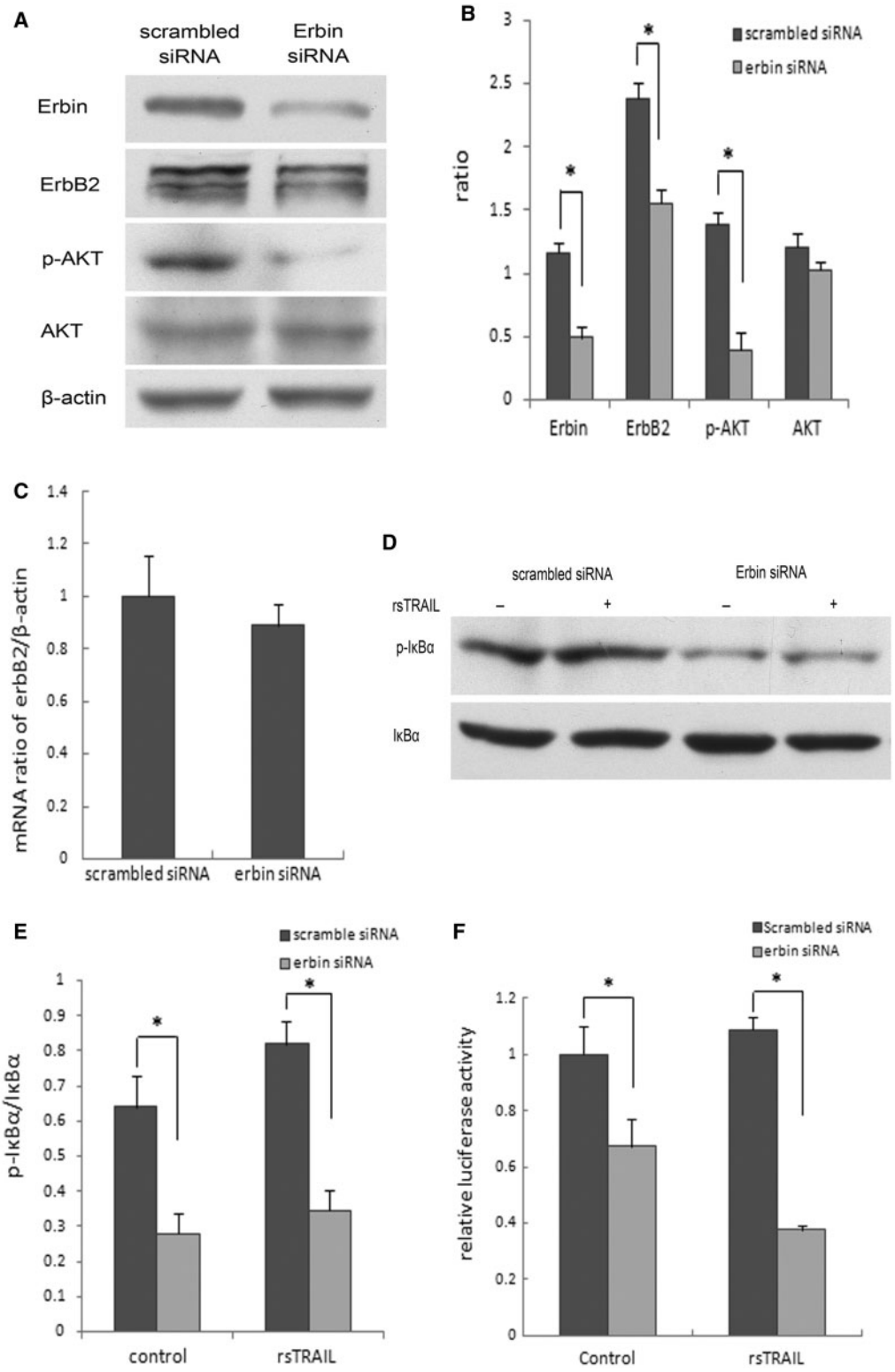

Fig. 5. **Erbin regulated ErbB$_2$ expression via ErbB$_2$/AKT/NF-κB pathway.** (A) The cells were transfected with Erbin-siRNA or scrambled siRNA and treated with rsTRAIL, and the expressions of Erbin, ErbB2, AKT and phosphorylated AKT (p-AKT) were analysed by western blotting using the specific antibodies. (B) The proteins expression were quantitative analysis compared with the internal control, β-actin. (C) ErbB2 mRNA expression was analyed by real-time PCR. (D) IκB phosphorylation (p-IκB) in the cells was analysed by western blot. (E) The expression of phosphorylated IκB was quantitative analysis compared with the internal control, IκB. (F) The activity of NF-κB in the cell lysate was evaluated by the luciferase reportor gene assay.

degradation, but not Erbin. This result is consistent with the report by Benoit *et al*. (*23*) that ErbB2 (HER2) is a substrate for caspase-8 and that TNF-α stimulation leads to an early caspase-8-dependent ErbB2 cleavage in MCF7 A/Z breast adenocarcinoma cells. Moreover, we observed that not only total amount of ErbB2 but also phosphorylated of ErbB2 were decreased in MCF-7 cells treated with rottlerin and rsTRAIL. Down-regulation of ErbB2 expression by siRNA enhanced rsTRAIL-induced apoptosis in the MCF-7 cells, indicated that ErbB2 phosphorylation plays an important role in apoptosis induction in MCF-7 cells, suggesting that ErbB2 cleavage contributes to the apoptosis pathway induced by the combination of rottlerin and TRAIL. In addition, we found that Erbin was increased in the Erbin/ErbB2 complex in the co-immunoprecipitation assay while the phosphorylated ErbB2 was decreased in the MCF-7 cells treated by rottlerin plus rsTRAIL, suggesting that Erbin might bind with nonphosphorylated ErbB2 preferentially. It is well known that protein phosphorylation could facilitate protein degradation via ubiquitination (*25*, *26*). Whether the interaction of Erbin and ErbB2 involves in ubiquitination or not is remained to be further investigated.

Erbin could interact with various proteins and functions in cell polarization, receptor location and signal transduction (*11*). The subcellular localization of Erbin in normal human skin is similar to that of ErbB2 and varies with cell differentiation. Disturbed expression or function of Erbin is implicated in developing the malignant phenotype of the basal cell carcinoma (*27*). To further evaluate the function of Erbin in MCF-7 breast cancer cells, the Erbin expression was down-regulated by RNAi technology and the consequent data demonstrated that the sensitivity of MCF-7 cells to TRAIL was markedly enlarged. Meanwhile, the expression of ErbB2, phosphorylated AKT and NF-κB activity were suppressed in the cells. These results suggested that Erbin could be a positive regulator for ErbB2 expression and inhibited apoptosis of MCF-7 cells induced by rsTRAIL via ErbB2/AKT/NF-κB signal pathway. As we have found that phosphorylated AKT and phosphorylated IκB were markedly reduced in Erbin-RNAi-treated cells, so we conjectured maybe there are two mechanisms of Erbin regulation to ErbB2/AKT/NF-κB signal pathway. Firstly, Erbin regulated the amount of ErbB2 protein, then influenced the activity of AKT and NF-κB. Second, Erbin maybe involved in the regulation of ubiquitination/proteasome pathway to participate in the degradation of phosphorylated protein. The exact mechanism needs to be further investigated. However, Huang *et al*. (*20*) demonstrated that overexpression of Erbin leads to inhibition of NGF-induced neuronal differentiation of PC12 cells, whereas down-regulation of endogenous Erbin by specific siRNA exhibits an opposite effect, indicating that Erbin is a negatively regulator for the MAP kinase pathway. McDonald *et al*. (*2*) provided evidences that Erbin overexpression inhibited Nod2-dependent NF-κB activation and cytokine secretion, whereas Erbin$^{-/-}$ mouse embryo fibroblasts showed an increased sensitivity to muramyl dipeptide. These contradictive results may illuminate that Erbin's function is cell-specific.

In summary, this study demonstrated that the expressions of Erbin and ErbB2 were modulated by PKCδ in MCF-7 breast cancer cells, which are resistant to TRAIL cytotoxicity. The affinity of Erbin–ErbB2 interaction was reduced by ErbB2 phosphorylation and Erbin could regulate the sensitivity of the MCF-7 cells to TRAIL through ErbB2/AKT/NF-κB pathway. These data might throw lights on the strategy for personalized therapy of breast cancers.

This work was funded by Nature Science Foundation of China (No. 30571687 to D.Z.).

REFERENCES

1. Borg, J.P., Marchetto, S., Le Bivic, A., Ollendorff, V., Jaulin-Bastard, F., Saito, H., Fournier, E., Adelaide, J., Margolis, B., and Birnbaum, D. (2000) ERBIN: a basolateral PDZ protein that interacts with the mammalian ERBB2/HER2 receptor. *Nat. Cell Biol.* **2**, 407–414
2. McDonald, C., Chen, F.F., Ollendorff, V., Ogura, Y., Marchetto, S., Lecine, P., Borg, J.P., and Nunez, G. (2005) A role for Erbin in the regulation of Nod2-dependent NF-κB signaling. *J. Biol. Chem.* **280**, 40301–40309
3. Ohno, H., Hirabayashi, S., Iizuka, T., Ohnishi, H., Fujita, T., and Hata, Y. (2002) Localization of p0071-interacting proteins, plakophilin-related armadillo-repeat protein- interacting protein (PAPIN) and ERBIN, in epithelial cells. *Oncogene* **21**, 7042–7049
4. Izawa, I., Nishizawa, M., Tomono, Y., Ohtakara, K., Takahashi, T., and Inagaki, M. (2002) ERBIN associates with p0071, an armadillo protein, at cell-cell junctions of epithelial cells. *Genes Cells* **7**, 475–485
5. Jaulin-Bastard, F., Arsanto, J.-P., Le Bivic, A., Navarro, C., Vely, F., Saito, H., Marchetto, S., Hatzfeld, M., Santoni, M.-J., Birnbaum, D., and Borg, J.-P. (2002) Interaction between Erbin and a Catenin-related protein in epithelial cells. *J. Biol. Chem.* **277**, 2869–2875
6. Laura, R.P., Witt, A.S., Held, H.A., Gerstner, R., Deshayes, K., Koehler, M.F., Kosik, K.S., Sidhu, S.S., and Lasky, L.A. (2002) The Erbin PDZ domain binds with high affinity and specificity to the carboxyl termini of delta-catenin and ARVCF. *J. Biol. Chem.* **277**, 12906–12914
7. Huang, Y.Z., Wang, Q., Xiong, W.C., and Mei, L. (2001) Erbin is a protein concentrated at postsynaptic membranes that interacts with PSD-95. *J. Biol. Chem.* **276**, 19318–19326
8. Favre, B., Fontao, L., Koster, J., Shafaatian, R., Jaunin, F., Saurat, J.H., Sonnenberg, A., and Borradori, L. (2001) The hemidesmosomal protein bullous pemphigoid antigen 1 and the integrin beta 4 subunit bind to ERBIN. Molecular cloning of multiple alternative splice variants of ERBIN and analysis of their tissue expression. *J. Biol. Chem.* **276**, 32427–32436
9. Huang, Y.Z., Zang, M., Xiong, W.C., Luo, Z., and Mei, L. (2003) Erbin suppresses the MAP kinase pathway. *J. Biol. Chem.* **278**, 1108–1114
10. Dai, P., Xiong, W.C., and Mei, L. (2006) Erbin inhibits RAF activation by disrupting the Sur-8-Ras-Raf complex. *J. Biol. Chem.* **281**, 927–933
11. Bryant, P.J. and Huwe, A. (2000) LAP proteins: what's up with epithelia? *Nat. Cell Biol.* **2**, E141–E143
12. Yarden, Y. (2001) Biology of HER2 and its importance in breast cancer. *Oncology* **61** (Suppl 2), 1–13
13. Klapper, L.N., Kirschbaum, M.H., Sela, M., and Yarden, Y. (2000) Biochmical and clinical implications of the ErbB/HER signaling network of growth factor receptors. *Adv. Cancer Res.* **77**, 25–79

14. Ouyang, X., Gulliford, T., Zhang, H., Smith, G., Huang, G., and Epstein, R.J. (2001) Association of ErbB2 Ser1113 phosphorylation with epidermal growth factor receptor co-expression and poor prognosis in human breast cancer. *Mol. Cell Biochem.* **218**, 47–54
15. Lemoine, N.R., Staddon, S., Dickson, C., Barnes, D.M., and Gullick, W.J. (1990) Absence of activating transmembrane mutations in the c-erbB-2 proto-oncogene in human breast cancer. *Oncogene* **5**, 237–239
16. Lenferink, A.E., Pinkas-Kramarski, R., van de Poll, M.L., van Vugt, M.J., Klapper, L.N., Tzahar, E., Waterman, H., Sela, M., van Zoelen, E.J., and Yarden, Y. (1998) Differential endocytic routing of homo- and hetero-dimeric ErbB tyrosine kinases confers signaling superiority to receptor heterodimers. *EMBO J.* **17**, 3385–3397
17. Dittmar, T., Husemann, A., Schewe, Y., Nofer, J., Niggemann, B., Zanker, K.S., and Brandt, B.H. (2002) Induction of cancer cell migration by epidermal growth factor is initiated by specific phosphorylation of tyrosine 1248 of c-erbB-2 receptor via EGFR. *FASEB J.* **16**, 1823–1825
18. Macfarlane, M. (2003) TRAIL-induced signaling and apoptosis. *Toxicol. Lett.* **139**, 89–97
19. Liu, Y.X., Zhu, X., and Ma, Z.Y. (1999) Expression, purification of and biological activity of rsTRAIL in E. coli. *Chinese Sci. Bull.* **44**, 1306–1309
20. Huang, Y.Z., Zang, M., Xiong, W.C., Luo, Z., and Mei, L. (2003) Erbin suppresses the MAP kinase pathway. *J. Biol. Chem.* **278**, 1108–1114
21. Shigeno, M., Nakao, K., Ichikawa, T., Suzuki, K., Kawakami, A., Abiru, S., Miyazoe, S., Nakagawa, Y., Ishikawa, H., Hamasaki, K., Nakata, K., Ishii, N., and Eguchi, K. (2003) Interferon-alpha sensitizes human hepatoma cells to TRAIL-induced apoptosis through DR5 upregulation and NF-kappa B inactivation. *Oncogene* **22**, 1653–1662
22. Zhang, J.D., Liu, N., Zhang, J.C., Liu, S.L., Liu, Y.X., and Zheng, D.X. (2005) PKCδ protects human breast tumor MCF7 cells against tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis. *J. Cell Biochem.* **96**, 522–532
23. Benoit, V., Chariot, A., Delacroix, L., Deregowski, V., Jacobs, N., Merville, M.P., and Bours, V. (2004) Caspase-8-dependent HER-2 cleavage in response to tumor necrosis factor α stimulation is counteracted by nuclear factor κB through c-FLIP-L expression. *Cancer Res.* **64**, 2684–2691
24. Huang, Y., Kim, S.-O., Jiang, J., and Frank, S.J. (2003) Growth hormone-induced phosphorylation of epidermal growth factor (EGF) receptor in 3T3-F442A cells. *J. Biol. Chem.* **278**, 18902–18913
25. Waterman, H., Levkowitz, G., Alroy, I., and Yarden, Y. (1999) The RING finger of c-Cbl mediates desensitization of the epidermal growth factor receptor. *J. Biol. Chem.* **274**, 22151–22154
26. Lamaze, C. and Schmid, S.L. (1995) Recruitment of epidermal growth factor receptors into coated pits requires their activated tyrosine kinase. *J. Cell Biol.* **129**, 47–54
27. Lebeau, S., Masouye, I., Berti, M., Augsburger, E., Saurat, J.H., Borradori, L., and Fontao, L. (2005) Comparative analysis of the expression of ERBIN and ErbB2 in normal human skin and cutaneous carcinomas. *Br. J. Dermatol.* **152**, 1248–1255