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# The Protein ERp57 Contributes to EGF Receptor Signaling and Internalization in MDA-MB-468 Breast Cancer Cells

Elisa Gaucci,[1,2] Fabio Altieri,[1,2] Carlo Turano,[2] and Silvia Chichiarelli[1]*

[1]*Department of Biochemical Sciences "A. Rossi Fanelli", Sapienza University of Rome, Piazzale Aldo Moro 5, Rome, 00185, Italy*

[2]*Istituto Pasteur-Fondazione Cenci Bolognetti, Sapienza University of Rome, Piazzale Aldo Moro 5, Rome, 00185, Italy*

## ABSTRACT

The disulfide isomerase ERp57 is a soluble protein mainly located in the endoplasmic reticulum, where it acts in the quality control of newly synthesized glycoproteins, in association with calreticulin and calnexin. It has been also detected in other cell compartments, such as the cytosol, the plasma membrane and the nucleus. In these locations it is implicated in various processes, participating in the rapid response to calcitriol, modulating the activity of STAT3 and being requested for the pre-apoptotic exposure of calreticulin on the plasma membrane. In the present work, the involvement of ERp57 in the activity of the EGF receptor was evaluated for the first time. EGFR is a tyrosine kinase receptor, which is able to activate numerous signaling cascades, leading to cell proliferation and inhibition of apoptosis. In the MDA-MB-468 breast adenocarcinoma cells, which overexpress EGFR, ERp57 expression has been knocked down by siRNA and the effects on EGFR have been studied. ERp57 silencing did not affect EGFR protein expression, cell membrane exposure or EGF binding, whereas the internalization and the phosphorylation of the receptor were impaired. The implication of ERp57 in the activity of EGFR, whose upregulation is known to be associated with tumors, could be relevant for cancer therapy. J. Cell. Biochem. 114: 2461–2470, 2013. 

**KEY WORDS:** SIGNAL TRANSDUCTION; RECEPTOR ENDOCYTOSIS; EGFR ACTIVATION; RNA INTERFERENCE; IMMUNOFLUORESCENCE

The epidermal growth factor receptor (EGFR or ErbB1) belongs to the superfamily of receptor tyrosine kinases (RTK), which are characterized by an extracellular ligand binding domain, a single chain transmembrane domain, an intracellular tyrosine kinase domain and a carboxyl tail devoid of kinase activity. The other members of the EGFR group of RTK are ErbB2 (which lacks the ligand binding domain), ErbB3 (which lacks the kinase domain) and ErbB4.

The EGF receptor can bind numerous molecules: EGF, amphiregulin, betacellulin, transforming growth factor α (TGFα), heparin-binding EGF-like growth factor (HB-EGF), epigen and epiregulin. The ligands are often present as transmembrane inactive precursors, which are then cleaved by a metallopeptidase, such as those belonging to the ADAM (a disintegrin and metalloproteinase) family, releasing the extracellular moiety which acts as a soluble ligand [reviewed in Sanderson et al., 2006]. The interaction with the ligand induces a conformational change in the receptor molecule, which discloses an otherwise hidden dimerization domain [Ferguson et al., 2003]. The activated kinase domain of one monomer phosphorylates several tyrosine, serine or threonine residues of the carboxyl tail of the other monomer, leading to the activation of downstream signaling molecules, typically containing Src homology 2 (SH2) or phosphotyrosine binding (PTB) domains, which mediate mainly pro-survival and anti-apoptotic cellular responses. The major signaling pathways include Ras-MEK-ERK, PI3K-Akt, as well as PLCγ-PKC [Jorissen et al., 2003]. Also STAT family proteins can be activated by EGFR [Park et al., 1996]. Negative regulation is also possible, by binding to molecules which trigger EGFR dephosphorylation or degradation [Wilson et al., 2009].

In addition to receptor clustering, EGFR may undergo internalization, in clathrin-coated pits or in caveolae. A hypothesis has been made about the mechanism of endocytosis according to the abundance of ligand [Sigismund et al., 2008]. Once internalized, it can be recycled to the cell surface, through slow or fast routes [Gáborik and Hunyady, 2004], degraded in lysosomes or even translocated to the nucleus [Lo et al., 2005]. Signaling can proceed also from the endosomal compartment. Adaptor proteins, like Shc

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*Correspondence to: Silvia Chichiarelli, Department of Biochemical Sciences "A. Rossi Fanelli", Sapienza University of Rome, Piazzale Aldo Moro 5, Rome 00185, Italy. E-mail: silvia.chichiarelli@uniroma1.it

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and Grb2, and all of the Ras-MAPK pathway components have been found to reside also on endosomes, interacting with the activated EGFR [Pol et al., 1998; Jiang and Sorkin, 2002]. It has been hypothesized that the internalization pathway of receptors and their intracellular trafficking via distinct endocytic structures could control the duration and strength of signals and enable the interaction of the receptors with different molecules [Polo and Di Fiore, 2006].

The de-regulation of EGFR signaling is often correlated to cancer [reviewed in Zandi et al., 2007]. In the past, EGFR was believed to play a minor role in breast malignancies. Emerging evidences highlight, on the contrary, a possible important role.

In the present work, evidences have been provided about the implication of the protein ERp57 in the signaling and internalization of EGFR. ERp57 is a member of the disulfide isomerase family and resides mainly in the endoplasmic reticulum, as a soluble protein. In this location it interacts with the lectins calreticulin and calnexin, taking part to the quality control of newly synthesized glycoproteins, and in the assembly of the major histocompatibility complex class I (MHC I) [reviewed in Turano et al., 2011]. A role has been also recently elucidated in the entry of viruses in the cell [Schelhaas et al., 2007; Walczak and Tsai, 2011].

ERp57 has been nonetheless detected in other cell compartments. Recently, cytoplasmic ERp57 has been demonstrated to translocate to the nucleus after tumor necrosis factor-α treatment [Grindel et al., 2011]. Concerning the membrane, ERp57 has been found on the acrosome-reacted sperm, consistently with a role in the egg-sperm fusion [Ellerman et al., 2006]. Together with calreticulin, ERp57 has been detected on the surface of cells that are treated with immunogenic cell death inducers [Obeid et al., 2007]. Furthermore, ERp57 (also called MARRS, which stands for membrane-associated rapid response steroid binding protein) has been proposed as the membrane receptor of 1,25-dihidroxyvitamin $D_3$, responsible for the rapid, transcription-independent responses to the hormone [Nemere et al., 2004]. Particularly important is the interaction with the signal transducer and transcription factor STAT3, which has been detected in the cytosol [Ndubuisi et al., 1999], in the lipid raft fraction of the cell membrane [Guo et al., 2002], as well as in the nucleus [Eufemi et al., 2004].

The study here presented has been conducted in MDA-MB-468 breast cancer cells. The expression of the protein ERp57 has been knocked down by means of RNA interference and the influence on the activity of EGFR has been investigated. In particular, the phosphorylation of the receptor and its internalization, as well as the effect on the downstream pathways have been explored.

## MATERIALS AND METHODS

### MATERIALS

Reagents for cell culture were from PAA. EGF (epidermal growth factor) and biotin were from Sigma. The anti-ERp57 antibody was prepared as a rabbit antiserum by Eurogentec (Seraing, Belgium), using as antigen the human recombinant protein prepared in our laboratory [Altieri et al., 1993], and partially purified on immobilized protein G (Sigma). Its specificity has been verified, as previously reported [Coppari et al., 2002]. For some experiments a commercial mouse monoclonal antibody was employed (Santa Cruz, sc 23886). The rabbit polyclonal anti-EGFR antibody was obtained from Santa Cruz, while the rabbit anti-pY1173 and anti-pY1068 were from Abcam. Mouse monoclonal anti-intracellular (activated) EGFR was from Millipore (clone 74). The antibody for STAT3 phosphorylated on tyrosine 705 was from Cell Signaling and anti-Actin from Sigma. For the downstream signaling pathway, antibodies from a Cell Signaling kit (Phospho-EGF Receptor Pathway Antibody Sampler Kit, #9789) were used. Secondary antibodies for Western blotting and immunofluorescence were from Jackson Immunoresearch.

The immunofluorescence images have been processed similarly with the image processing program ImageJ 1.37v (LSC_lite Software). In synthesis, the merged RGB images were split into three grayscale images containing the red, blue and green components. The red and green components are shown in the figures.

### CELL CULTURE

Human breast adenocarcinoma cells (MDA-MB-468) and human cervical adenocarcinoma (HeLa) were obtained from ATCC and grown to 60–70% confluence in Dulbecco's minimal essential medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 1% w/v sodium pyruvate, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in 5% $CO_2$. For EGF stimulation, the cells were serum-starved o.n., and then treated with EGF at a final concentration of 100 ng/ml.

### SIRNA KNOCKDOWN OF ERp57

The silencing of the protein ERp57 was obtained by administration of specific small interfering RNA (Hs_GRP58_6_HP validated siRNA, QIAGEN). The day before transfection, cells were seeded in complete culture medium. The siRNA was used at 10 nM final concentration with INTERFERin (Polyplus transfection) transfection reagent. The transfection was carried out according to the manufacturer's instructions. The cells were incubated with the transfection complexes for 96 h (MDA-MB-468) or 48 h (HeLa) under their normal growth conditions. Cells treated with scrambled, non-specific siRNA duplex (Negative control siRNA, QIAGEN) were used as control reference.

### REAL-TIME PCR

Total RNA was isolated with TRIzol (Invitrogen) following the manufacturer's instructions. The reverse transcription was carried out with SideStep™ II QPCR cDNA Synthesis Kit (Stratagene) and ERp57 expression was evaluated by Real Time PCR, using a MJ MiniOpticon Detection System (BioRad) with SensiMix Plus SYBR (Quantace). The protocol used was: denaturation (95°C for 5 min), amplification repeated 40× (95°C for 30 s, 55°C for 30 s, 72°C for 30 s). RPS27A and GAPDH were used as reference genes for normalization and the relative quantification was analyzed using the Gene Expression Analysis for iCycler iQ Real Time PCR Detection System Software, Version 1.10 (BioRad Laboratories, Ltd). All the primers were from QIAGEN.

### BIOTINYLATION OF PLASMA MEMBRANE PROTEINS

The biotinylation of plasma membrane proteins of MDA-MB-468 cells was performed according to the procedure described in Xiao et al. [1999], modified where necessary. siRNA- (Sil) or scrambled

RNA-treated (Scr) cells were washed three times with cold PBS, pH 8, and resuspended at a concentration of $10^6$/ml in 1 mg/ml Sulfo-NHS-SS-biotin (Pierce) in PBS, pH 8. The reaction was carried out at r.t. for 15 min in gentle rotation. Cells were then washed with 50 mM glycine in PBS, pH 8, to inactivate the unreacted biotinylating reagent, then three times with cold PBS, pH 8. Sil and Scr cell pellets were resuspended in 50 μl resuspension buffer (10 mM Tris, pH 8, 150 mM NaCl, 1% SDS) and diluted to 500 μl with solubilization buffer (SB: 10 mM Tris, pH 8, 150 mM NaCl, 0.5% Triton-X100 and proteases inhibitors). The samples were sonicated (3 × 30 s strokes, Labsonic M, Sartorius) and centrifuged at 5,000*g* for 10 min at 4°C; 1/10 of the recovered supernatant was retained as Input. A 50% slurry of streptavidin (Ultralink Streptavidin Plus, Pierce) was washed twice with SB containing 0.1% SDS and then added to the Sil or Scr lysate, after protein quantification with the BioRad protein assay. The mixture was incubated o.n. at 4°C with rotation. The supernatant of the resin was collected and the resin washed three times with SB containing 0.1% SDS, three times with 0.5 M NaCl containing 0.5% v/v Triton X-100, and twice with 10 mM Tris, pH 8. The bound biotinylated proteins were eluted with 0.5 ml of 50 mM dithiothreitol (DTT) in 10 mM Tris, pH 8, for at least 4 h at r.t. Sil and Scr Inputs and eluates were analyzed by SDS–PAGE followed by Western blotting for the analysis of the EGFR distribution.

### EGF-ALEXA555 BINDING EXPERIMENTS

Experiments of EGF binding on the cell surface receptor were performed according to Kharchenko et al. [2007] and Bild et al. [2002]. MDA-MB-468 cells on glass coverslips were treated with siRNA (Sil) or scrambled RNA (Scr), as previously described, or left untreated (C). After an o.n. serum starvation, cells were washed twice with cold working medium (WM: DMEM supplemented with 20 mM Hepes, pH 8, 1% w/v bovine serum albumin (BSA)), then placed on ice and added with 0.5 μg/ml EGF conjugated with the fluorophor AlexaFluor® 555 (EGF-Alexa555, Molecular Probes, Invitrogen) for 50 min. Unbound EGF-Alexa555 was then washed out by three rinses with cold WM. The endocytosis of pre-bound EGF was stimulated by shifting the temperature to 37°C for 20 min. After washing with PBS, cells were fixed with freshly prepared 4% formaldehyde for 20 min and washed again with PBS. Glass coverslips were mounted with Vectashield Mounting Medium (Vector Laboratories) and analyzed by a Leica DM-IRE2 confocal microscope.

### IMMUNOFLUORESCENCE

Sil or Scr MDA-MB-468 cells on glass coverslips were treated with 100 ng/ml EGF for 50 min at 4°C. The unbound EGF was then washed out by three rinses with cold DMEM. Endocytosis of pre-bound EGF was stimulated by shifting the temperature to 37°C for 40 min. For double immunostaining, the cells were fixed with 4% formaldehyde for 20 min, washed twice with PBS, and permeabilized with 0.1% Triton X-100 for 15 min. After 1 h blocking with 1% w/v BSA, cells were immunostained with anti-EGFR or anti-intracellular (activated) EGFR and anti-ERp57 for 1 h. After washing three times with PBS, cells were incubated with TRITC- and FITC-conjugated secondary antibodies for 1 h, then washed again three times with PBS. The glass microscope slides, mounted with Vectashield (Vector Laboratories), were examined by a Leica DM5000B fluorescence microscope.

### BIOTINYLATION-BASED INTERNALIZATION ASSAY

The internalization assays were performed according to Grampp et al. [2007], modifying the procedure where necessary. The biotinylation step was performed as described in the *Biotinylation of plasma membrane proteins* paragraph. After the labeling step, the cells were washed once with 50 mM glycine to quench the reaction, then twice with serum-free DMEM to eliminate the unreacted biotin. Half of the biotin-labeled cells were stimulated with 100 ng/ml EGF for 30 min at r.t., and half were left untreated as a control. The cell surface biotin was cleaved off with a glutathione solution (75 mM reduced glutathione, 75 mM NaCl, 10 mM EDTA, 1% BSA), twice for 15 min on ice. The cells were then resuspended in 50 μl of 10 mM Tris, pH 8, 150 mM NaCl, supplemented with 1% SDS, and the homogenate was diluted to 500 μl with solubilization buffer (10 mM Tris, pH 8, 150 mM NaCl, 0,5% Triton X-100, proteases inhibitors). Samples were sonicated (3 × 30 s strokes) and centrifuged at 5,000*g* for 10 min at 4°C. In order to recover the biotinylated proteins, the samples were incubated with a monomeric avidin resin (Pierce), prepared according to the manufacturer's instructions. The eluted proteins were subjected to SDS–PAGE and Western blotting.

## RESULTS

### ERp57 SIRNA SILENCING

To study the involvement of the protein ERp57 in the endocytosis of EGFR, experiments were performed on MDA-MB-468 breast cancer cells, in which the ERp57 protein content was decreased by means of RNA interference. A whole cell lysate from silenced cells was prepared after 24, 48, and 72 h of incubation with the specific siRNA and analyzed by Western blotting with anti-ERp57. The amount of ERp57 in silenced cells was compared with that of untreated cells. As shown in Figure 1A, ERp57 was already diminished after 48 h of treatment with siRNA and remained at a low level after 72 h. We have checked the knockdown also at a longer incubation time. The decrease of ERp57 was even more marked after 96 h of treatment (Fig. 1B). Thus, all subsequent experiments which needed ERp57 silencing were performed after at least 90 h of treatment with the specific siRNA, to ensure the highest depletion.

The ERp57 expression was also checked by Real Time PCR. After 96 h of silencing the ERp57 mRNA was demonstrated to be very low (Fig. 1C).

### EGFR EXPRESSION AND EXPOSURE ON THE CELL MEMBRANE

To assess the expression of EGFR in silenced cells, as well its exposure on the cell membrane, the biotinylation of plasma membrane proteins of silenced (Sil) and scrambled (Scr) MDA-MB-468 cells was performed. After treatment with the specific siRNA or with scrambled RNA, cells were recovered and incubated with the biotinylating agent Sulfo-NHS-SS-biotin, which labels only the proteins exposed on the plasma membrane. After washing to remove the unspecific labeling, cells were lysed and total proteins in Sil and Scr samples were quantified with the BioRad protein assay. An equal amount of proteins from the two samples was then incubated with a streptavidin resin, to accomplish the binding of biotinylated membrane proteins. A 1/10 aliquot (Input) was instead retained for subsequent analyses. After incubating o.n. at 4°C, the unbound fractions, corresponding to

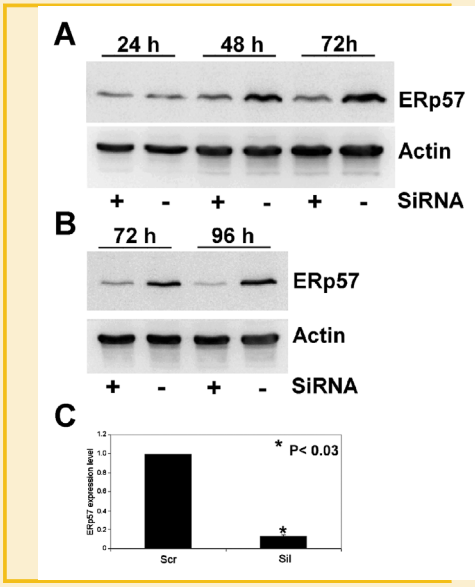


**Fig. 1.** MDA-MB-468 cells were incubated with specific ERp57 siRNA or left untreated. SDS–PAGE and Western blotting with anti-ERp57 were performed on total cell lysate recovered after 24, 48 and 72 h (A), or 72 and 96 h (B) of treatment. Actin was detected as a loading control. (C) Graphical display of the Real time PCR expression profile of the ERp57 gene in scrambled (Scr) or silenced (Sil) cells after 96 h incubation. For normalization, the expression of the reference genes GADPH and RSP27A in the same sample was used. The relative expression in the Scr sample is given as 1.

internal, unlabeled proteins, were recovered and, after washing, the biotinylated streptavidin-bound proteins were eluted with the reducing agent DTT. Sil and Scr inputs and eluates were analyzed by SDS–PAGE and Western blotting for the EGFR distribution. As shown in Figure 2, no difference in EGFR total content and only a slight increase in its membrane exposure, but not statistically significant, could be detected in Sil compared to Scr. The difference between input and eluate band intensities can be explained considering that the input volume loaded for electrophoresis was about 1/18 of the total volume incubated with the streptavidin resin.

## EGF BINDING

The possible ligand binding impairment of EGFR in silenced cells was explored by means of EGF conjugated with the fluorophor AlexaFluor555. In these experiments, Sil, Scr or control (C) MDA-MB-468 cells, grown on glass microscope coverslips, were treated with EGF-Alexa555 at 0°C, a condition which allows the binding of the growth factor to the cell surface receptor, but which is non permissive for internalization. Then, the temperature was switched to 37°C to induce the ligand-receptor complex internalization. In Figure 3A, the fluorescence of EGF-Alexa555 is shown. To visualize the fluorescence on the cell surface before endocytosis, cells were fixed immediately after the pre-binding on ice, without stimulating the internalization. At 0°C, EGF-Alexa555 is bound to the cell surface EGFR in Sil as well as in Scr and C. After 30 min at 37°C, the distribution of EGF-Alexa555 inside the cell is noticeably different. Apparently, both in C and Scr the fluorescent EGF is concentrated in sickle-shaped structures, while in most of Sil it is distributed in a punctate manner near the cell surface. As a control of the ERp57 depletion, Sil, Scr and C samples remained in the 6-well plate after the removal of coverslips, were collected and lysed. Figure 3B reports the Western blotting analysis of the distribution of EGFR and ERp57 in the six samples. ERp57 is decreased in Sil, while the EGFR total amount is unaffected.

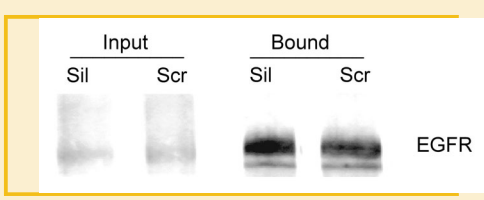


**Fig. 2.** Sil and Scr cells were labeled with Sulfo-NHS-SS-biotin and lysed. An equal protein amount from Sil and Scr samples was incubated with a streptavidin resin and biotinylated proteins were eluted. SDS–PAGE and Western blotting with anti-EGFR was performed on Input and Eluate (Bound) fractions from Sil and Scr samples.

## EGFR AND ERp57 INTERNALIZATION

In double immunostaining experiments, cells grown on glass microscope coverslips and treated with the specific siRNA or scrambled RNA as previously described, were stimulated with 100 ng/ml EGF for 50 min at 4°C to allow the binding to the surface receptor or left untreated as a control. To visualize EGFR on the cell membrane, the cells were immediately fixed with formaldehyde, or alternatively endocytosis was stimulated by shifting the temperature to 37°C, and then the cells were fixed. After incubation with anti-EGFR and anti-ERp57, and with FITC- and TRITC-conjugated secondary antibodies, untreated cells and cells incubated at 37°C were analyzed by means of immunofluorescence microscopy to observe the distribution of both proteins in the cells (Fig. 4). In the upper panel, the images of untreated Scr or Sil are shown. After incubation with EGF at 37°C, in Sil cells, EGFR seems to reside into small vesicles located in proximity to the cell membrane, whereas in Scr incubated at 37°C the fluorescence is located inside the cell, referring to EGFR internalization.

The involvement of ERp57 in EGF pathway was also analyzed by means of a biotinylation-based internalization assay. MDA-MB-468 cells were incubated with the biotinylation reagent Sulfo-NHS-SS-biotin to label covalently the cell membrane proteins. Then, the cell suspension was split in two equal aliquots, which were either stimulated with EGF for 30 min or left unstimulated. Therefore, the internalization of external membrane proteins gave rise to a pool of biotin-labeled proteins inside the cell. To distinguish between cell surface and internalized biotinylated molecules, the cells were incubated with the reducing agent glutathione, which could remove the label only from the membrane proteins, by reducing the disulfide bridge present in the spacer arm of the biotinylation reagent. On the contrary, internalized proteins were inaccessible to its reducing activity. The cells were then subjected to lysis with SDS and the homogenate was applied to a monomeric avidin resin for the specific binding of biotin-labeled proteins. The eluted proteins were electrophoretically separated and analyzed by Western blotting. As shown in Figure 5, EGFR is internalized following EGF treatment, as

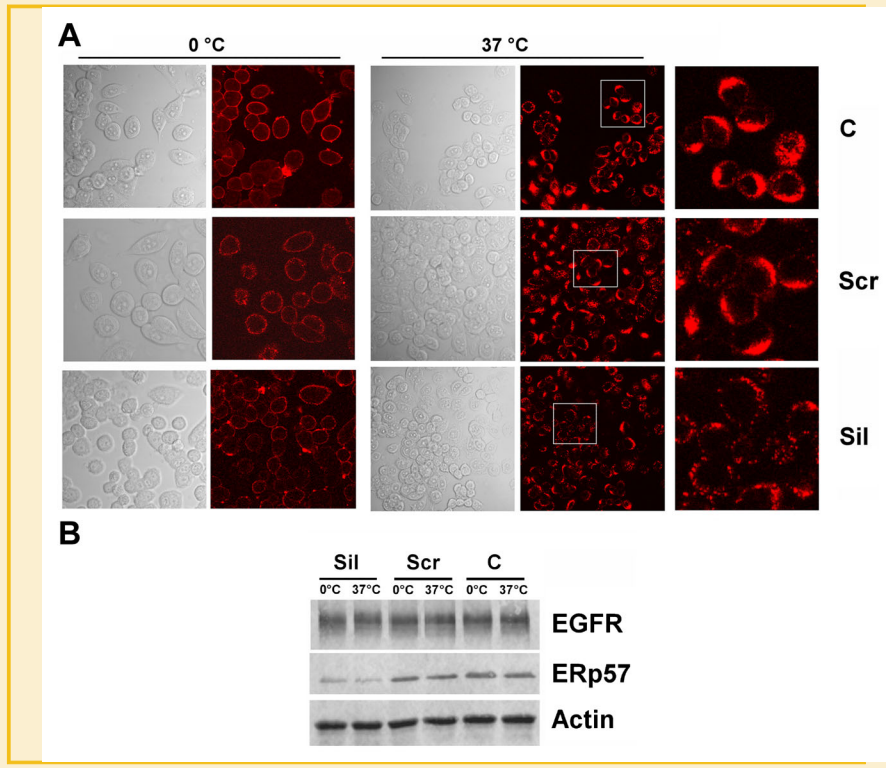


Fig. 3. A: Visualization of EGF-Alexa555 in control (C), Sil and Scr cells. The cells had been either stimulated for 50 min at 0°C and immediately fixed, or incubated for additional 30 min at 37°C and then fixed with 4% formaldehyde. B: Western blotting of the same cell samples used for confocal microscopy, performed with anti-EGFR, anti-ERp57 and anti-Actin.

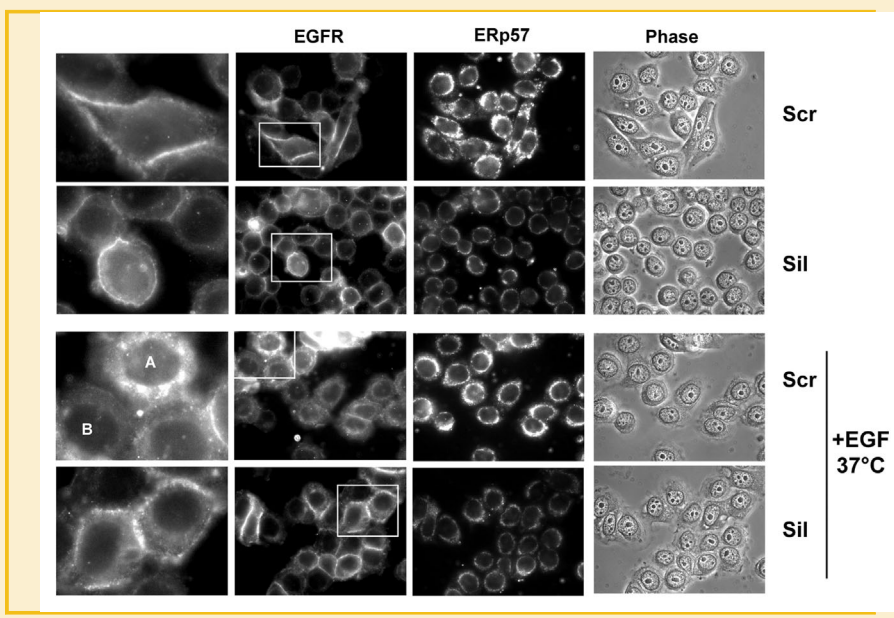


Fig. 4. Sil or Scr cells on glass coverslips were untreated or treated with EGF for 40 min at 37°C, after a pre-binding step of 50 min at 4°C, and then fixed with 4% formaldehyde. Double immunostaining was performed with anti-EGFR and anti-ERp57 as primary antibodies and TRITC or FITC-conjugated (respectively) secondary antibodies.

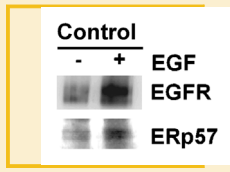


Fig. 5. The cells were labeled with Sulfo-NHS-SS-biotin and stimulated with 100 ng/ml EGF for 30 min at 37°C. Cell surface biotin was cleaved off with glutathione, then the cells were lysed and the lysate was applied to an avidin resin. SDS–PAGE and Western blotting were performed with anti-ERp57 and EGFR on the biotinylated bound (i.e., internalized) fractions of EGF-stimulated or unstimulated samples.

expected. Of note, the treatment with EGF induces also the ERp57 endocytosis.

The internalization assay has then been performed on Scr and Sil cells. As reported in Figure 6A, the band intensity of internalized EGFR in Scr cells following stimulation with EGF is in lower amount than in unstimulated cells. It can be hypothesized that the stimulation with EGF induces the downregulation of the receptor. To confirm such hypothesis, the biotinylation-internalization assay has been also performed at r.t. halving the incubation time with EGF (Fig. 6B). In this case, it is possible to note the increase of internalized EGFR after EGF stimulation. Significantly, EGFR is only negligibly present in the bound (internalized) fractions of silenced cells.

## EGFR PHOSPHORYLATION AND ACTIVATION OF DOWNSTREAM SIGNALING PATHWAYS

The effect of ERp57 on the activation of EGFR, which consists in the phosphorylation of several tyrosine residues, was subsequently investigated.

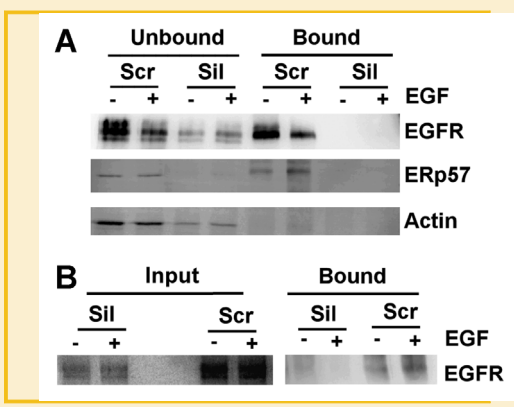


Fig. 6. A: The biotinylation-internalization assay was performed on Sil and Scr cells. Unbound and bound fractions of EGF-stimulated or unstimulated Sil or Scr cells were subjected to Western blotting for EGFR and ERp57. Actin was used as a reference to normalize the amount of protein loaded. B: The stimulation with EGF was conducted for 15 min at r.t. Input and bound fractions of EGF-stimulated or unstimulated Sil or Scr cells were subjected to Western blotting with anti-EGFR.

Immunofluorescence experiments were performed on Sil or Scr MDA-MB-468 cells, stimulated with EGF at 4°C, a condition non permissive for internalization, or at 37°C for 40 min, to induce the ligand-receptor complex endocytosis. In Figure 7 the double immunofluorescence images of the cells, treated as just described, are shown to visualize both ERp57 and phospho-EGFR. An antibody against the intracellular activated EGFR was used. At 4°C, the treatment with EGF induces the EGFR phosphorylation, both in scrambled and in silenced cells. After a 40-min incubation at 37°C, phospho-EGFR is completely internalized in Scr, as expected, while in Sil the fluorescence is drastically diminished.

Subsequently, we focused on the phosphorylation status of two specific EGFR tyrosine residues, which are implicated in the activation of downstream signaling pathways: tyrosine 1173 and tyrosine 1068. Sil or Scr cells were stimulated with EGF for 5 or 30 min or left unstimulated. The cells were then harvested in a buffer containing the phosphatase inhibitor sodium orthovanadate and subjected to lysis. The cell lysates were then electrophoretically separated and analyzed by Western blotting for their amount of phospho-Y1173 or phospho-Y1068, as shown in Figure 8. In both cases the most noticeable difference is between the silenced and scrambled unstimulated samples, since the constitutive phosphorylation of the receptor is only visible in Scr. Furthermore, a difference in the time course of Y1068 phosphorylation may be noticed, as it occurs in 5 min in Scr but in 30 min in Sil. ERp57 knockdown in the silenced cells was also checked and resulted almost complete.

To investigate the downstream signaling pathways activated by EGFR, a Western blotting analysis was also performed with specific antibodies. As shown in Figure 8, in the case of Sil, the phosphorylation of ERK and Akt is increased after 5 min of

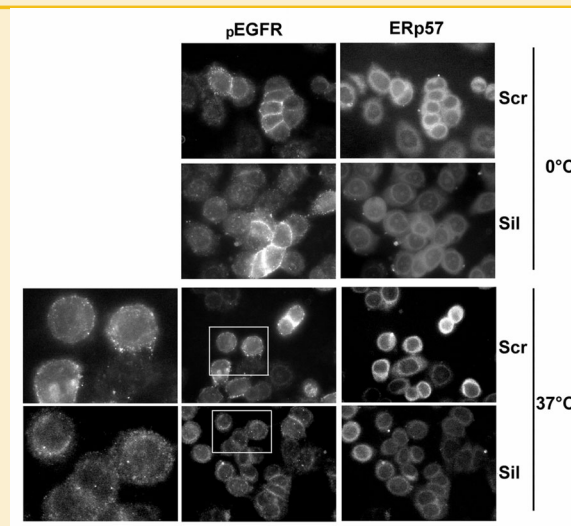


Fig. 7. Sil and Scr cells on glass coverslips were incubated with 100 ng/ml EGF for 50 min at 0°C and immediately fixed, or incubated for 40 min at 37°C after the pre-binding step and then fixed with 4% formaldehyde. Double immunostaining was performed with anti-intracellular (activated) EGFR and anti-ERp57 as primary antibodies and FITC or TRITC-conjugated (respectively) secondary antibodies.

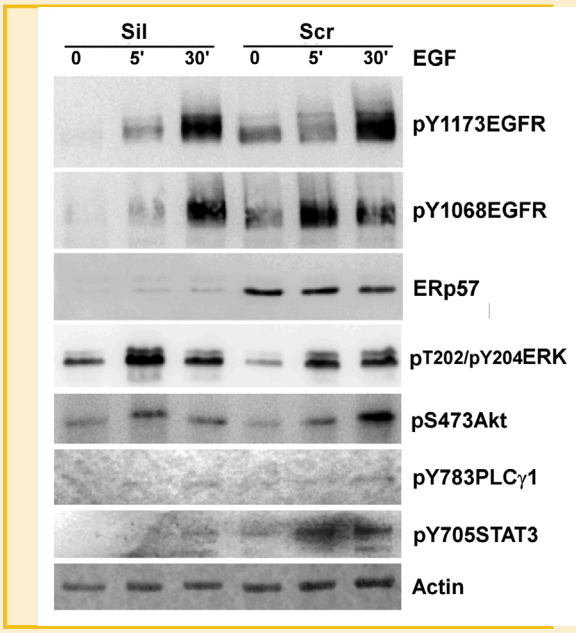


**Fig. 8.** Sil or Scr cells were stimulated with 100 ng/ml EGF for 5 or 30 min, or unstimulated, lysed and subjected to SDS–PAGE and Western blotting for the detection of phosphoTyr1068-EGFR (pY1068), phosphoTyr1173-EGFR (pY1173), phosphoThr202/phosphoTyr204-ERK (pT202/pY204ERK), phosphoSer473-Akt (pS473), phosphoTyr783-PLCγ1 (pY783PLCγ1), phosphoTyr705-STAT3 (pY705STAT3), ERp57 and Actin as loading control.

stimulation, while it decreases after 30 min. In Scr the phosphorylation of Akt increases after 30 min of incubation with EGF, while ERK is phosphorylated after 5 min and is unchanged after 30 min. On the contrary, STAT3 and PLCγ1 are less phosphorylated in Sil than in Scr.

## DISCUSSION

The functions of the protein ERp57 inside its canonical compartment, the ER, are well established. It is involved in the folding and quality control of neo-synthesized glycoproteins [Oliver et al., 1997] and in the assembly of MHC class I [Lindquist et al., 1998].

Recently, additional unconventional activities have come out, in different cell compartments [reviewed in Turano et al., 2011], and a considerable bunch of evidences outline its involvement in signal transduction. Early works described its association with vasopressin [Aiyar et al., 1989] and angiotensin receptors [Mah et al., 1992]. Particularly relevant is the participation in the pathway involving STAT3, which is a member of the STAT family of signal transducers and transcription factors. At first, cytosolic multiprotein complexes containing both STAT3 and ERp57 were found [Ndubuisi et al., 1999]. Subsequently, the association has been found also in plasma membrane rafts [Guo et al., 2002; Sehgal et al., 2002] as well as in the nucleus, where the two proteins bind to the same DNA sequence [Eufemi et al., 2004]. In addition, ERp57 has been demonstrated to participate in the phosphorylation reaction and in the DNA binding activity of STAT3 [Chichiarelli et al., 2010]. Furthermore, in an article by Ramírez-Rangel et al. [2011], ERp57 has been demonstrated to positively regulate the activity of the mammalian target of rapamycin complex 1 (mTORC1), being involved in the assembly of cytosolic or ER-associated multiprotein complexes.

ERp57 itself has been identified as the membrane receptor of 1,25-dihydroxyvitamin $D_3$ [Nemere et al., 2004] and has been also demonstrated to activate protein kinase C alpha and beta [Tunsophon and Nemere, 2010]. In this context, ERp57 has been found to mediate the growth inhibitory effect of vitamin D in breast cancer cells, influencing the hormone induced apoptosis and differentiation [Richard et al., 2010].

In the present work, we aimed to investigate the role of ERp57 in the EGFR signaling pathway in MDA-MB-468 cells, a human basal-like breast cancer cell line, also defined as triple negative, due to the lack of oestrogen receptor, progesterone receptor and ErbB2 [Oliveras-Ferraros et al., 2008]. The ERp57 expression has been knocked down by means of specific siRNA. The ERp57 protein and mRNA levels after the treatment, checked by Western blotting or RT-PCR, were found to be very low after 96 h (Fig. 1). Then, the effect of silencing on the activity of EGFR has been studied.

First of all, considering that in the ER ERp57 is involved in the quality control of glycoproteins destined to the cell membrane or to be secreted, the EGFR expression and exposure on the plasma membrane of silenced cells compared to control cells have been evaluated, to check the possible impairment of EGFR expression following treatment with siRNA, as well as its proper membrane exposure (Fig. 2). By means of a membrane impermeable biotin reagent, only the plasma membrane proteins of silenced or scrambled MDA-MB-468 cells were labeled and recovered with a streptavidin resin. The Western blotting analysis of total and membrane proteins has demonstrated that the ERp57 knockdown does not negatively influence the EGFR expression or exposure on the cell membrane.

For what concerns the ligand binding, silenced, scrambled and control cells were incubated with a fluorophore-conjugated EGF and examined by confocal microscopy (Fig. 3A). In all three samples, at 0° C, a condition which impairs endocytosis, the fluorescence is located around the cells, corresponding to EGF bound to EGFR on the cell surface. Increasing the temperature at 37°C for 20 min, in scrambled and control cells EGF accumulates in internal perinuclear structures resembling sickles, while in the majority of silenced cells this localization is not detectable.

In the double immunofluorescence (Fig. 4), EGFR has been taken into account, instead of the ligand. In untreated cells the receptor is located at the plasma membrane level. After stimulation with EGF, in scrambled cells the receptor is internalized and accumulates in perinuclear vesicles or is partially degraded (compare "A" and "B" in the higher magnification images in Fig. 4). In silenced cells, EGFR remains confined near the plasma membrane. Figures 5 and 6 report the biotinylation internalization assays. In scrambled cells, intracellular EGFR, which is present in the avidin-bound fraction, increases after stimulation with EGF, while in silenced cells it does not (Fig. 6A). This effect can be explained by the prevention of EGFR internalization, as well as with the recycling of the activated receptor back on the cell membrane, where the biotin label would be removed by treatment with glutathione (see Material and Methods). Surprisingly, ERp57 itself is internalized in response to EGF stimulation (Fig. 5). It can be speculated that the two proteins are sorted into different endosomal

compartments, because in scrambled cells, after treatment with EGF, intracellular EGFR is partially degraded, while the increase in internalized ERp57 is still marked (Fig. 6A).

Cell fractionation studies have also been conducted to follow the redistribution of EGFR inside the cell. By means of a subcellular protein fractionation kit (see Supplementary data), cytoplasmic and membrane extracts have been prepared from Sil and Scr cells. In Scr cells (Fig. 1S, Supplementary data), EGF treatment induces the decrease of EGFR in the cytoplasmic content and the increase in the membranes fraction, which includes plasma membrane, mitochondria and ER/Golgi. Such redistribution does not occur in Sil cells.

The effect of ERp57 silencing on EGFR redistribution was also studied in HeLa cells, a human cervical adenocarcinoma cell line, not overexpressing EGFR. The same pattern observed in MDA-MB-468 cells occurs also in HeLa cells, even more evidently (Fig. 2S, Supplementary data).

The influence of the ERp57 downregulation on the autophosphorylation reaction of EGFR was analyzed by a double immunofluorescence experiment, where both ERp57 and phospho-EGFR were visualized. An antibody specific for the intracellular activated EGFR was used, which recognizes several internal phosphorylation sites, in the phosphorylated form, characteristic of the activated receptor. After stimulation with EGF at 0°C and at 37°C, the EGFR phosphorylation is decreased in silenced cells compared to scrambled cells (Fig. 7). A Western blotting analysis has been also performed to check the phosphorylation status of two specific sites: Tyr 1068 and Tyr 1173, which are known to be essential for the activation of downstream molecules. It is interesting to notice that the constitutive phosphorylation appears particularly affected (Fig. 8). In the case of Tyr1068-EGFR after EGF stimulation, also a delay in the phosphorylation reaction is evident. The impairment of EGFR phosphorylation after ERp57 silencing occurs also in HeLa cells, as shown in Figure 3S (Supplementary data). In this context, the work by Mathea et al. [2011] has described the inhibitory activity of the peptidyl prolyl isomerase FKBP12 in the EGFR autophosphorylation process. According to our experiments, instead, ERp57 should have an activating role. The main EGFR downstream pathways were also investigated. The four proteins analyzed can be divided in two groups: PLCγ1 and STAT3 are less activated while ERK and Akt are more activated in silenced cells compared to scrambled cells after stimulation with EGF for 5 min (Fig. 8).

Therefore, the ERp57 knockdown negatively influences the EGFR trafficking and autophosphorylation, whereas the EGFR expression, exposure on the plasma membrane and ligand binding are not significantly affected. A hypothesis can be made about the implication of ERp57 in the proper folding and consequently in the activity of EGFR. A work by Jung et al. [2011] has demonstrated that ERp57 is required for the activity of a disulfide bond-containing myelin glycoprotein, expressed on the cell surface, and may have a role in the pathogenesis of neuropathies. Even more intriguing is the finding that ERp57 associates with seven-transmembrane receptors, participating in the formation of homodimers of angiotensin receptor 1 (AT1R) or heterodimers with the β2 adrenergic receptor (b2AR/AT1R) [Hammad and Dupré, 2010]. Therefore, this would be the first evidence of the involvement of ERp57 in the function of a receptor tyrosine kinase.

The effect of ERp57 silencing on EGFR trafficking and autophosphorylation suggests that ERp57 could be necessary for the accomplishment of a conformation suitable for such two activities, while it seems to be disposable for the ligand binding. Considering that the ligand-receptor complex endocytosis and endosomal sorting are composite mechanisms, which require numerous accessory proteins, an indirect involvement of ERp57 cannot be excluded. On the other hand, the effect on EGFR autophosphorylation necessarily implies that the molecule directly affected is EGFR. At present, no information about a direct interaction between the two proteins or the location of such hypothetical interaction is available.

An impairment of endosomal trafficking of EGFR could be hypothesized. In fact, as shown in Figure 3A, EGF-containing vesicles appear more dispersed in Sil cells, compared to Scr cells, meaning that the clustering of early endosomes could be involved. Intriguingly, very recently an interaction of ERp57 with the small GTPase RalA, which is involved in the regulation of vesicle trafficking, has come out [Brymora et al., 2012]. The process, anyway, remains to be fully characterized.

It has been demonstrated that EGFR degradation, which occurs via sorting into late endosomes and their fusion with lysosomes, is reduced after impairment of receptor endocytosis [Brankatschk et al., 2012] and that a Rab protein, key molecules for recycling, increases the internalization of EGFR and consequently its degradation [Yang et al., 2012]. The increased EGFR stability could potentially prolong receptor signaling. A recent work by Sousa et al. [2012] has shown that the Akt and MAPK pathways are more stimulated after the suppression of EGFR endocytosis, leading to the conclusion that in these cases the signaling proceeds from EGFR located in the cell membrane. In Figure 8 the over-activation of ERK and Akt in Sil cells after 5 min of stimulation with EGF could be explained by an increased number of EGFR molecules in the plasma membrane or early endosomes at the steady state compared to scrambled, as suggested by Figures 3 and 4.

Currently, targeted therapies in breast cancer include monoclonal antibodies or inhibitors against growth factors receptors or the downstream activated signaling molecules [Alvarez et al., 2010]. Chaperones are as well appealing targets, as described in a review [McLaughlin and Vandenbroeck, 2011]. For instance, the heat shock protein Hsp90 has been found to regulate the activity of many oncoproteins, such as EGFR or ErbB2, and specific inhibitors are now in trials [Porter et al., 2010]. ERp57, participating in the unfolded protein response, has been reported to be over-expressed in many conditions of cellular stress and in neoplastic transformation [Lee, 2001]. Recently, a multimeric protein complex associated with ERp57 has been characterized in the nucleus of ovarian cancer cells, connected to the resistance to the chemotherapeutic paclitaxel [Cicchillitti et al., 2010]. Considering the involvement in the EGFR signaling activity herein described, both in MDA-MB-468 cells and in HeLa cells, ERp57 could become a promising target in combination therapy.

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## REFERENCES

Aiyar N, Bennett CF, Nambi P, Valinski W, Angioli M, Minnich M, Crooke ST. 1989. Solubilization of rat liver vasopressin receptors as a complex with a guanine-nucleotide-binding protein and phosphoinositide-specific phospholipase C. Biochem J 261:63–70.

Altieri F, Maras B, Eufemi M, Ferraro A, Turano C. 1993. Purification of a 57 kDa nuclear matrix protein associated with thiol: Protein disulfide oxidoreductase and phospholipase C activities. Biochem Biophys Res Commun 194:992–1000.

Alvarez RH, Valero V, Hortobagyi GN. 2010. Emerging targeted therapies for breast cancer. J Clin Oncol 28:3366–3379.

Bild A, Turkson J, Jove R. 2002. Cytoplasmic transport of Stat3 by receptor-mediated endocytosis. EMBO J 21:3255–3263.

Brankatschk B, Wichert S, Johnson S, Schaad O, Rossner M, Gruenberg J. 2012. Regulation of the EGF transcriptional response by endocytic sorting. Sci Signal 5(215):ra21.[doi: 10.1126/scisignal.2002351]

Brymora A, Duggin IG, Berven LA, van Dam EM, Roufogalis BD, Robinson PJ. 2012. Identification and characterisation of the RalA-ERp57 interaction: Evidence for GDI activity of ERp57. PLoS ONE 7(11):e50879.doi: 10.1371/journal.pone.0050879

Chichiarelli S, Gaucci E, Ferraro A, Grillo C, Altieri F, Cocchiola R, Arcangeli V, Turano C, Eufemi M. 2010. Role of ERp57 in the signaling and transcriptional activity of STAT3 in a melanoma cell line. Arch Biochem Biophys 494:178–183.

Cicchillitti L, Della Corte A, Di Michele M, Donati MB, Rotilio D, Scambia G. 2010. Characterisation of a multimeric protein complex associated with ERp57 within the nucleus in paclitaxel-sensitive and -resistant epithelial ovarian cancer cells: The involvement of specific conformational states of beta-actin. Int J Oncol 37:445–454.

Coppari S, Altieri F, Ferraro A, Chichiarelli S, Eufemi M, Turano C. 2002. Nuclear localization and DNA interaction of protein disulfide isomerase ERp57 in mammalian cells. J Cell Biochem 85:325–333.

Ellerman D, Myles D, Primakoff P. 2006. A role for sperm surface protein disulfide isomerase activity in gamete fusion: Evidence for the participation of ERp57. Dev Cell 10:831–837.

Eufemi M, Coppari S, Altieri F, Grillo C, Ferraro A, Turano C. 2004. ERp57 is present in STAT3-DNA complexes. Biochem Biophys Res Commun 323:1306–1312.

Ferguson K, Berger M, Mendrola J, Cho H, Leahy D, Lemmon M. 2003. EGF activates its receptor by removing interactions that autoinhibit ectodomain dimerization. Mol Cell 11:507–517.

Gáborik Z, Hunyady L. 2004. Intracellular trafficking of hormone receptors. Trends Endocrinol Metab 15(6):286–293.

Grampp T, Sauter K, Markovic B, Benke D. 2007. γ-aminobutyric acid type B receptors are constitutively internalized via the clathrin-dependent pathway and targeted to lysosomes for degradation. J Biol Chem 282:24157–24165.

Grindel BJ, Rohe B, Safford SE, Bennett JJ, Farach-Carson MC. 2011. Tumor necrosis factor α treatment of HepG2 cells mobilizes a cytoplasmic pool of ERp57/1,2 5D(3)-MARRS to the nucleus. J Cell Biochem 112:2606–2615.

Guo GG, Patel K, Kumar V, Shah M, Fried VA, Etlinger JD, Sehgal PB. 2002. Association of the chaperone glucose-regulated protein 58 (GRP58/ER-60/ERp57) with Stat3 in cytosol and plasma membrane complexes. J Interferon Cytokine Res 22:555–563.

Hammad MM, Dupré DJ. 2010. Chaperones contribute to G protein coupled receptor oligomerization, but do not participate in assembly of the G protein with the receptor signaling complex. J Mol Signal 5:16–29.

Jiang X, Sorkin A. 2002. Coordinated traffic of Grb2 and Ras during epidermal growth factor receptor endocytosis visualized in living cells. Mol Biol Cell 13:1522–1535.

Jorissen RN, Walker F, Pouliot N, Garrett TP, Ward CW, Burgess AW. 2003. Epidermal growth factor receptor: Mechanisms of activation and signaling. Exp Cell Res 284:31–53.

Jung J, Coe H, Michalak M. 2011. Specialization of endoplasmic reticulum chaperones for the folding and function of myelin glycoproteins P0 and PMP22. FASEB J 25:3929–3937.

Kharchenko M, Aksyonov A, Melikova M, Kornilova E. 2007. Epidermal growth factor (EGF) receptor endocytosis is accompanied by reorganization of microtubule system in HeLa cells. Cell Biol Int 31:349–359.

Lee A. 2001. The glucose-regulated proteins: Stress induction and clinical applications. Trends Biochem Sci 26:504–510.

Lindquist JA, Jensen ON, Mann M, Hämmerling GJ. 1998. ER-60, a chaperone with thiol-dependent reductase activity involved in MHC class I assembly. EMBO J 17:2186–2195.

Lo H, Xia W, Wei Y, Ali-Seyed M, Huang S, Hung M. 2005. Novel prognostic value of nuclear EGF receptor in breast cancer. Cancer Res 65:338–348.

Mah SJ, Ades AM, Mir R, Siemens IR, Williamson JR, Fluharty SJ. 1992. Association of solubilized angiotensin II receptors with phospholipase C-alpha in murine neuroblastoma NIE-115 cells. Mol Pharmacol 42:217–226.

Mathea S, Li S, Schierhorn A, Jahreis G, Schiene-Fisher C. 2011. Suppression of EGFR autophosphorylation by FKBP12. Biochemistry 50:10844–10850.

McLaughlin M, Vandenbroeck K. 2011. The endoplasmic reticulum protein folding factory and its chaperones: New targets for drug discovery? Br J Pharmacol 162:328–345.

Ndubuisi M, Guo G, Fried V, Etlinger J, Sehgal P. 1999. Cellular physiology of STAT3: Where′s the cytoplasmic monomer? J Biol Chem 274:25499–25509.

Nemere I, Safford S, Rohe B, De Souza M, Farach-Carson M. 2004. Identification and characterization of 1,25D3 membrane-associated rapid response, steroid (1,25D3-MARRS) binding protein. J Steroid Biochem Mol Biol 89–90:281–285.

Obeid M, Tesniere A, Ghirindelli F, Fimia G, Apetoh L, Perfettini J, Castedo M, Mignon G, Panaretakis T, Casares N, Meivier D, Larochette N, Van Endert P, Ciccosanti F, Piacentini M, Zitvogel L, Kroemer G. 2007. Calreticulin exposure dictates the immunogenicity of cancer cell death. Nat Med 13:54–61.

Oliver JD, van der Wal FJ, Bulleid NJ, High S. 1997. Interaction of the thiol-dependent reductase ERp57 with nascent glycoproteins. Science 275:86–88.

Oliveras-Ferraros C, Vazquez-Martin A, Lopez-Bonet E, Martin-Castillo B, Del Barco S, Brunet J, Menendez JA. 2008. Growth and molecular interactions of the anti-EGFR antibody Cetuximab and the DNA cross-linking agent cisplatin in gefitinib-resistant MDA-MB-468 cells: New prospects in the treatment of triple-negative/basal-like breast cancer. Int J Oncol 33:1165–1176.

Park OK, Schaefer TS, Nathans D. 1996. In vitro activation of Stat3 by epidermal growth factor receptor kinase. Proc Natl Acad Sci USA 93:13704–13708.

Pol A, Calvo M, Enrich C. 1998. Isolated endosomes from quiescent rat liver contain the signal transduction machinery. Differential distribution of activated Raf-1 and Mek in the endocytic compartment. FEBS Lett 441:34–38.

Polo S, Di Fiore P. 2006. Endocytosis conducts the cell signaling orchestra. Cell 124:897–900.

Porter JR, Fritz CC, Depew KM. 2010. Discovery and development of Hsp90 inhibitors: A promising pathway for cancer therapy. Curr Opin Chem Biol 14:412–420.

Ramírez-Rangel I, Bracho-Valdés I, Vázquez-Macías A, Carretero-Ortega J, Reyes-Cruz G, Vázquez-Prado J. 2011. Regulation of mTORC1 complex assembly and signaling by GRp58/ERp57. Mol Cell Biol 31:1657–1671.

Richard CL, Farach-Carson MC, Rohe B, Nemere I, Meckling KA. 2010. Involvement of 1,25D3-MARRS (membrane-associated, rapid response steroid

binding), a novel vitamin D receptor, in growth inhibition of breast cancer cells. Exp Cell Res 316:695–703.

Sanderson MP, Dempsey PJ, Dunbar AJ. 2006. Control of ErbB signaling through metalloprotease mediated ectodomain shedding of EGF-like factors. Growth Factors 24:121–136.

Schelhaas M, Malmström J, Pelkmans L, Haugstetter J, Ellgaard L, Grünewald K, Helenius A. 2007. Simian Virus 40 depends on ER protein folding and quality control factors for entry into host cells. Cell 131:516–529.

Sehgal PB, Guo GG, Shah M, Kumar V, Patel K. 2002. Cytokine signaling: STATs in plasma membrane rafts. J Biol Chem 277:12067–12074.

Sigismund S, Argenzio E, Tosoni D, Cavallaio E, Polo S, Di Fiore P. 2008. Clathrin-mediated internalization is essential for sustained EGFR signaling but dispensable for degradation. Dev Cell 15:209–219.

Sousa L, Lax I, Shen H, Ferguson S, Camilli P, Schlessinger J. 2012. Suppression of EGFR endocytosis by dynamin depletion reveals that EGFR signaling occurs primarily at the plasma membrane. Proc Natl Acad Sci USA 109:4419–4424.

Tunsophon S, Nemere I. 2010. Protein kinase C isotypes in signal transduction for the 1,25D3-MARRS receptor (ERp57/PDIA3) in steroid-hormone-stimulated phosphate uptake. Steroids 75:307–313.

Turano C, Gaucci E, Grillo C, Chichiarelli S. 2011. ERp57/GRP58: A protein with multiple functions. Cell Mol Biol Lett 16:539–563.

Walczak CP, Tsai B. 2011. A PDI family network acts distinctly and coordinately with ERp29 to facilitate polyomavirus infection. J Virol 85:2386–2396.

Wilson KJ, Gilmore JL, Foley J, Lemmon MA, Riese DJ. 2009. Functional selectivity of EGF family peptide growth factors: Implications for cancer. Pharmacol Ther 122:1–8.

Xiao G, Chung T, Pyun H, Fine R, Johnson R. 1999. KDEL proteins are found on the surface of NG108-15 cells. Mol Brain Res 72:121–128.

Yang X, Zhang Y, Li S, Liu C, Jin Z, Wang Y, Ren F, Chang Z. 2012. Rab21 attenuates EGF-mediated MAPK signaling through enhancing EGFR internalization and degradation. Biochem Biophys Res Commun 421: 651–657.

Zandi R, Larsen A, Andersen P, Stockhausen M, Poulsen H. 2007. Mechanisms for oncogenic acivation of the epidermal growth factor receptor. Cell Signal 19:2013–2023.

## SUPPORTING INFORMATION

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**Fig. 1S.** Silenced (Sil) and scrambled (Scr) MDA-MB-468 cells, stimulated with EGF or untreated, were subjected to fractionation. The membranes and the cytosolic extracts were analyzed by SDSPAGE and Western blotting with anti-EGFR and anti-ERp57.

**Fig. 2S.** Silenced (Sil) and scrambled (Scr) HeLa cells, stimulated with EGF or untreated, were subjected to fractionation. The membranes and the cytosolic extracts were analyzed by SDS–PAGE and Western blotting with anti-EGFR and anti-ERp57.

**Fig. 3S.** Sil or Scr HeLa cells were stimulated with 100 ng/ml EGF for 5 or 30 min, or unstimulated, lysed and subjected to SDS–PAGE and Western blotting for the detection of phosphoTyr1068-EGFR (pY1068), phosphoTyr1173-EGFR (pY1173), ERp57 and Actin as loading control.