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ORIGINAL ARTICLE

Flotillins as regulators of ErbB2 levels in breast cancer

S Pust[1,2], TI Klokk[1,2], N Musa[1,2], M Jenstad[2,3], B Risberg[2,3,4], B Erikstein[5], L Tcatchoff[1,2], K Liestøl[2], HE Danielsen[2,3], B van Deurs[6] and K Sandvig[1,2,7]

Amplification and overexpression of the receptor tyrosine kinase ErbB2 occur in up to 30% of human breast cancers, and high ErbB2 levels are correlated with poor prognosis for breast cancer patients. In contrast to the epithelial growth factor receptor (ErbB1), ErbB2 is not downregulated by ligand-induced mechanisms. Here we show that flotillins are involved in the stabilization of ErbB2 at the plasma membrane. In SKBR3 breast cancer cells and breast cancer tissue, a positive correlation between flotillin and ErbB2 expression levels could be demonstrated. Moreover, the tissue microarray analyses of biopsies from 194 patients diagnosed with carcinomas of the breast showed that flotillin-2 emerged as a potential predictor of prognosis in breast cancer. Depletion of flotillin-1 and flotillin-2 leads to internalization and degradation of ErbB2. Furthermore, flotillin-1 and -2 were found to be in a molecular complex with ErbB2 and Hsp90. The depletion of one of these proteins results in disruption of this complex, followed by destabilization of ErbB2 at the membrane, and its internalization and degradation. As a consequence, ErbB2-triggered downstream signalling is inhibited. Our data demonstrate a novel mechanism for interfering with ErbB2 signalling, which potentially can have clinical impact.

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Keywords: ErbB2 receptor tyrosine kinase; flotillin; breast cancer; endocytosis; downregulation

INDRODUCTION

The ligand-less receptor tyrosine kinase ErbB2 is a preferred dimerization partner for the other members (EGFR/ErbB1, ErbB3 and ErbB4) of the EGFR (epithelial growth factor receptor) family.[1] ErbB2 has been demonstrated to enable strong and constitutive signalling through different pathways that induce cell transformation, migration and proliferation.[2] In contrast to EGFR/ErbB1, ErbB2 is endocytosed at a very slow rate and continuously recycled back to the cell surface.[3–5] The precise activation and deactivation of signalling cascades is a crucial cellular process and dysregulation can induce cancer development or progression. Thus, one of the challenges in cancer treatment is to promote silencing of signalling cascades. ErbB2 has been shown to be involved in the progression and development of a variety of different human tumours. Remarkably, in up to 20–30% of all human breast cancers, amplification and overexpression of ErbB2 can be found and high expression of ErbB2 has been demonstrated to be correlated with poor prognosis in breast cancer patients.[6–9] The inhibition of oncogenic signalling triggered by ErbB2 can be achieved by different strategies, and administration of monoclonal antibodies (trastuzumab/Herceptin) became the first-line treatment in patients with ErbB2-overexpressing metastatic breast cancer.[6,10] In breast cancer cells, ErbB2 is strongly concentrated at the plasma membrane and its stabilization at the membrane is mediated by Hsp90. Thus, another approach to abrogate oncogenic signalling is the induction of ErbB2 internalization and degradation by inhibition of Hsp90. Treatment with the Hsp90 inhibitor geldanamycin (GA) has been shown to induce ErbB2 downregulation.[11–13] Hsp90 is a known target in cancer therapy and GA analogues (for example, 17-AAG/tanespimycin) are used in the treatment and therapy of ErbB2-positive cancers in clinic.[14–16] To understand in greater detail how ErbB2 stabilization at the plasma membrane is mediated, we have investigated the involvement of the lipid raft-associated flotillins (flotillin-1 and -2) in this process. Flotillins, which are also termed reggies,[17] have been described to be localized mainly at the plasma membrane and in endosomal/lysosomal compartments.[18–20] It has been demonstrated that flotillin proteins, among other functions, are involved in endocytic mechanisms and cellular trafficking processes.[21–23]

In this study, we describe how flotillin-1 and -2 contribute to the stabilization of ErbB2 at the cell surface, and we show that ErbB2 is internalized and degraded by a GA-independent mechanism upon flotillin depletion. Importantly, flotillin and ErbB2 expression levels are positively correlated on a cellular level as well as in breast cancer tissue.

RESULTS

Association of flotillins with ErbB2

First, we analysed the cellular localization of ErbB2 and flotillins in SKBR3 breast cancer cells. We could show by confocal microscopy that flotillin-1 and -2 partially co-localize with ErbB2 at the plasma membrane (Figure 1a). In addition, interaction between flotillins and ErbB2 could be demonstrated by co-immunoprecipitation experiments, as immunoprecipitation of endogenous flotillin-1 or -2 resulted in pull down of ErbB2 (Figure 1b, quantification of pull down see Supplementary Figure S1a). We next analysed the impact of flotillins on the internalization and subsequent degradation of ErbB2. As described above, ErbB2 is stably

[1]Department of Biochemistry, Institute for Cancer Research, Oslo University Hospital, Oslo, Norway; [2]Centre for Cancer Biomedicine, Faculty of Medicine, University of Oslo, Oslo, Norway; [3]Institute for Medical Informatics, Oslo University Hospital, Oslo, Norway; [4]Department of Pathology, Oslo University Hospital, Oslo, Norway; [5]Oslo University Hospital, Oslo, Norway; [6]Department of Cellular and Molecular Medicine, The Panum Building, Faculty of Health Sciences, University of Copenhagen, Copenhagen, Denmark and [7]Department of Molecular Biosciences, Faculty of Mathematics and Natural Sciences, University of Oslo, Oslo, Norway. Correspondence: Professor K Sandvig, Department of Biochemistry, Institute for Cancer Research, Oslo University Hospital, Oslo 0379, Norway.
E-mail: ksandvig@radium.uio.no
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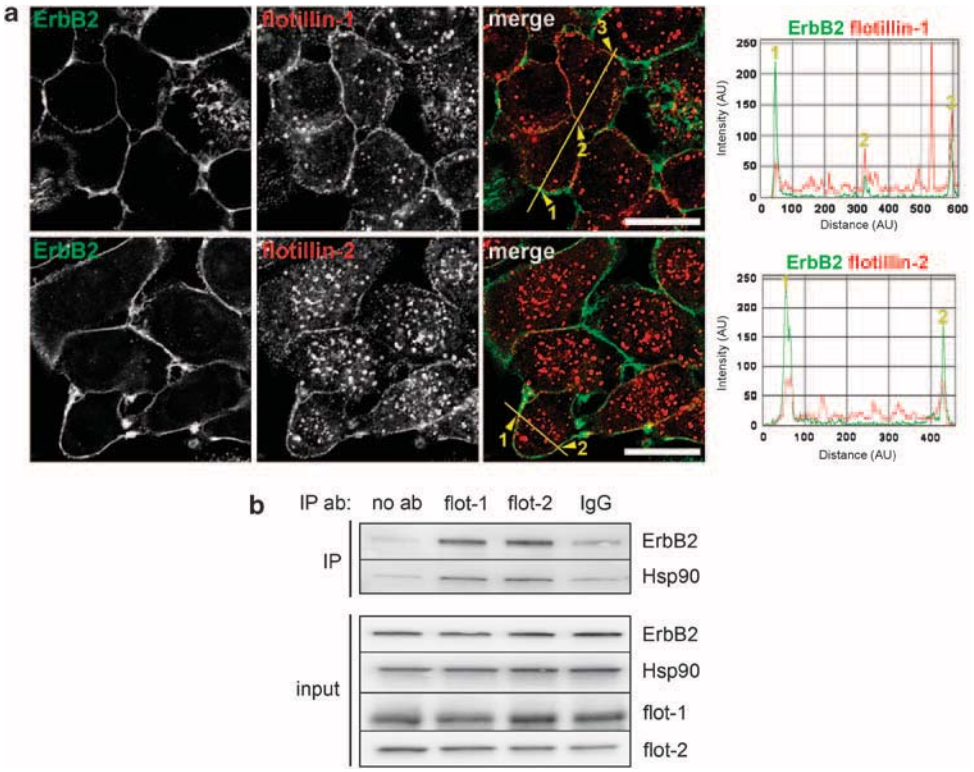

Figure 1. ErbB2 receptors are co-localizing and interacting with flotillin proteins. (**a**) ErbB2 is partially co-localized with flotillin-1 and -2 at the cell surface. SKBR3 breast cancer cells were immunostained for endogenous ErbB2, flotillin-1 or -2, nuclei were stained by DAPI. The samples were visualized by confocal microscopy. Yellow lines in the merged pictures indicate the regions highlighted as fluorescence intensity profiles on the right. Scale bars, 20 μm. (**b**) Interaction of ErbB2 and Hsp90 with flotillin-1 and -2. SKBR3 cells were lysed, flotillin-1 or -2 were immunoprecipitated and ErbB2 or Hsp90 were subsequently detected by western blot analysis. The two upper panels show the detection of immunocomplexes consisting of flotillin-1 or -2 and Hsp90 or ErbB2, respectively. The lower panels show the input of ErbB2, Hsp90, flotillin-1 and -2.

associated with the plasma membrane, but its internalization and degradation can be triggered by administration of the Hsp90 inhibitor GA.[11] The internalization process can easily be monitored by confocal microscopy, and visualized as a dotted staining of intracellular ErbB2 (Supplementary Figure S1b, lower panel). Western blot analysis of ErbB2 degradation and treatment of SKBR3 cells with 3 μM GA showed, as expected, a rapid decrease of ErbB2 levels within 2–4 h. However, protein levels of flotillin-1 or -2 and their intracellular localization were not affected by GA treatment (Supplementary Figures S1c and d). Importantly, internalization of ErbB2 could be observed by confocal microscopy when cells were depleted for flotillin-1 or -2, in the absence of GA (Figures 2a and b). The percentage of cells with internalized ErbB2 significantly increases after depletion of flotillins (Supplementary Figure S1e). Interestingly, after flotillin depletion, ErbB2 is found to be localized in Lamp-1-positive vesicles (Supplementary Figure S1f), supporting the data showing reduced levels of ErbB2 after flotillin knockdown. To investigate whether there is a correlation between the levels of membrane-associated ErbB2 and flotillin, we performed confocal microscopy studies in flotillin knockdown cells. Analysis of the fluorescence intensities of extracellular ErbB2 and total flotillin-1 or -2 showed approximately linear relationships (Figures 2c and d) with good correlations, especially for flotillin-2 (flotillin-1: $R = 0.647$, flotillin-2: $R = 0.855$). To measure the direct uptake of ErbB2 after flotillin depletion, we labelled ErbB2 with specific antibodies on ice and incubated the cells at 37 °C for 30 min. Afterwards, cells were fixed and stained for ErbB2 and ErbB2 internalization was visualized by confocal microscopy (Figure 2e). As expected, control cells showed no uptake of ErbB2 within 30 min, whereas in GA-treated cells as well as in flotillin-depleted cells (Figure 2e, arrowheads), intracellular ErbB2 could be detected.

These data suggest that flotillins stabilize ErbB2 at the plasma membrane and thereby inhibit its degradation. Consequently, we examined by western blot analysis whether flotillin knockdown triggers ErbB2 degradation. For this purpose, SKBR3 were depleted of either flotillin-1 or -2 and subsequently incubated with or without 3 μM GA for 2 h (Figures 3a and b). The results revealed that knockdown of flotillin-2 resulted in a stronger reduction of ErbB2 (~40%) than depletion of flotillin-1 (~20%), and the reduction after flotillin-2 depletion is comparable to the effect obtained upon treatment with GA alone.

To analyse in more detail the interaction of flotillins with ErbB2, we studied the association of ErbB2 with flotillin-1 or -2 in response to GA treatment. To achieve this, we performed an *in situ* proximity ligation assay (PLA) with the DuolinkII kit (Olink Bioscience, Uppsala, Sweden). This established method relies on antibody-based analysis and quantification of protein proximity by fluorescence microscopy.[24,25] Briefly, after an amplification reaction, proteins of interest with a distance of less than 40 nm from each other can be visualized by fluorescence microscopy as distinct fluorescent dots, suggesting an association or complex of these proteins. By this assay, we could demonstrate a close proximity between flotillins and ErbB2 receptors (Figure 3c). This proximity was disrupted upon GA treatment, as a significantly reduced level of flotillin/ErbB2 signals was observed in GA-treated cells (Figure 3d). Importantly, the specific association of ErbB2 and flotillins is supported by a control experiment analysing proximity of ErbB2 with the membrane protein Par-2 (Supplementary Figure S2a).

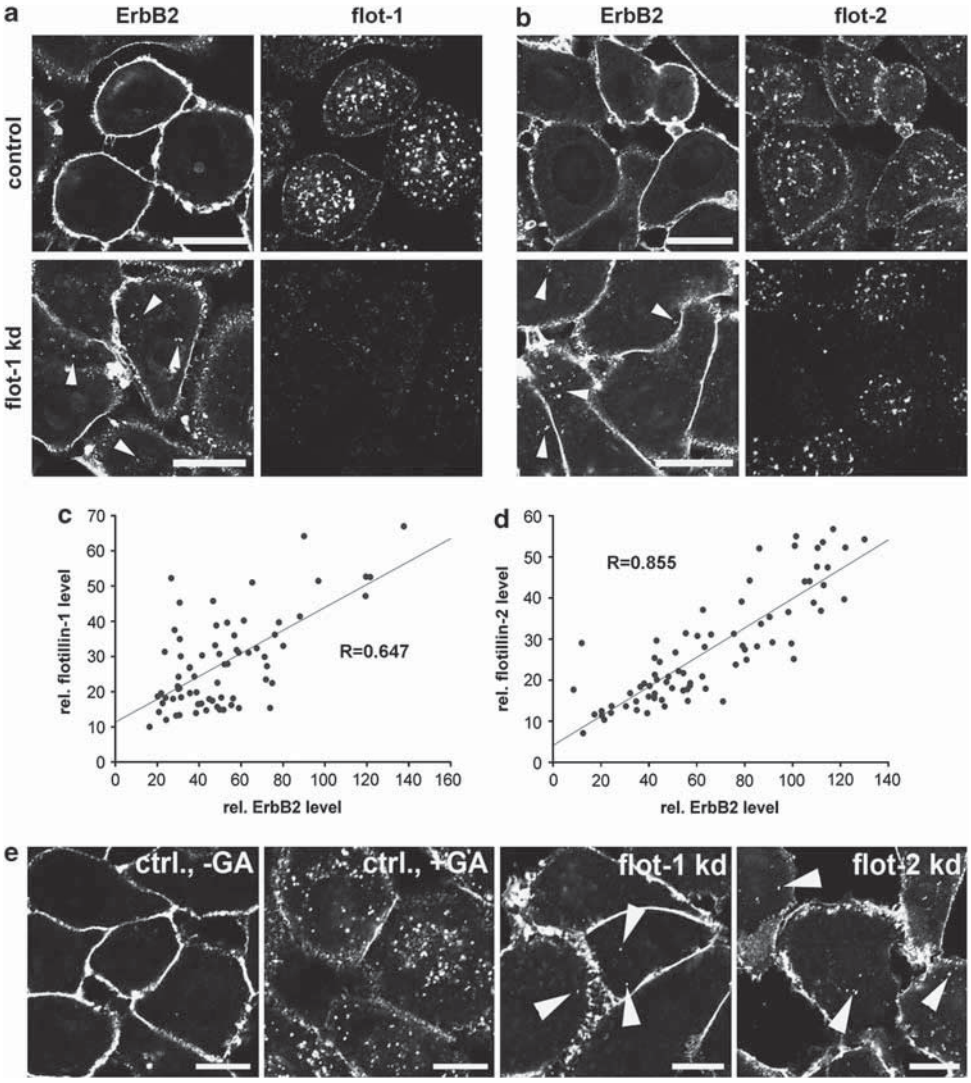

Figure 2. GA treatment and flotillin depletion results in internalization and reduced levels of ErbB2. (**a**) Flotillin-1 depletion triggers internalization of ErbB2. SKBR3 cells were transfected with control or flotillin-1-targeting siRNA oligonucleotides, proteins were stained by specific antibodies and visualized by confocal fluorescence microscopy. After flotillin-1 knockdown, intracellular ErbB2 could be detected as dotted structures (arrowheads). Scale bar, 20 μm. (**b**) ErbB2 internalization in flotillin-2-depleted cells. SKBR3 cells were transfected with control or flotillin-2-targeting siRNA oligonucleotides and treated as described above. Similar to after flotillin-1 knockdown, intracellular ErbB2 appeared as dotted structures after depletion of flotillin-2 (arrowheads). Scale bar, 20 μm. (**c**) Representative correlation of extracellular ErbB2 and flotillin-1 levels. SKBR3 cells depleted of flotillin-1 were fixed and non-permeabilized cells were stained for extracellular ErbB2, followed by permeabilization and staining for flotillin-1. For further analysis the fluorescence intensities of ErbB2 and flotillin-1 were measured. The calculated *R*-value was 0.647 ($n = 71$; $P < 0.05$). (**d**) Representative correlation of extracellular ErbB2 and flotillin-2 levels. Cells were depleted for flotillin-2 and treated and analysed as described in **c**. The calculated *R*-value was 0.855 ($n = 77$; $P < 0.05$). (**e**) Internalization of antibody-labelled ErbB2. Cells were transfected with control or flotillin-1 or -2-targeting siRNA oligonucleotides and incubated for additional 3 days. Afterwards, cells were labelled on ice with an ErbB2-specific antibody followed by 30 min incubation at 37 °C, methanol fixation and detection of ErbB2 via fluorescently labelled secondary antibodies. Analysis by confocal microscopy showed internalized ErbB2 after GA treatment and flotillin depletion (indicated by arrowheads), but no ErbB2 internalization in control cells. Scale bars, 10 μm.

No interaction of these proteins has been reported so far. However, by using PLA, a significant number of ErbB2/Par-2 signals per cell can be detected but the number of signals is not changed by GA treatment. This indicates that the ErbB2/Par-2 signals are probably due to the close spatial distribution of the two highly expressed membrane proteins rather than specific association of ErbB2 with Par-2. On the other hand, ErbB2/flotillin proximity is sensitive to GA treatment, indicating a specific association of ErbB2 and flotillins. Moreover, the number of flotillin-2/Par-2 proximity signals are 70% reduced compared with the number of ErbB2/Par-2 signals (− GA).

Flotillins associate with Hsp90 in an ErbB2-dependent manner

As already mentioned, Hsp90 is an essential component for the stabilization of ErbB2 at the plasma membrane.[26] Thus, we tested whether flotillins interact with Hsp90 or regulate Hsp90 protein levels. By co-immunoprecipitation, we could show that flotillins interact with Hsp90 (Figure 1b). These findings were in agreement with a partial co-localization of flotillins and Hsp90 at the plasma membrane, visualized by confocal microscopy (Figure 4a). Based on these results, we investigated whether flotillins regulate Hsp90 protein levels and thereby stabilize ErbB2 receptors at the plasma membrane. However, depletion of flotillin-1 or -2 or flotillin double

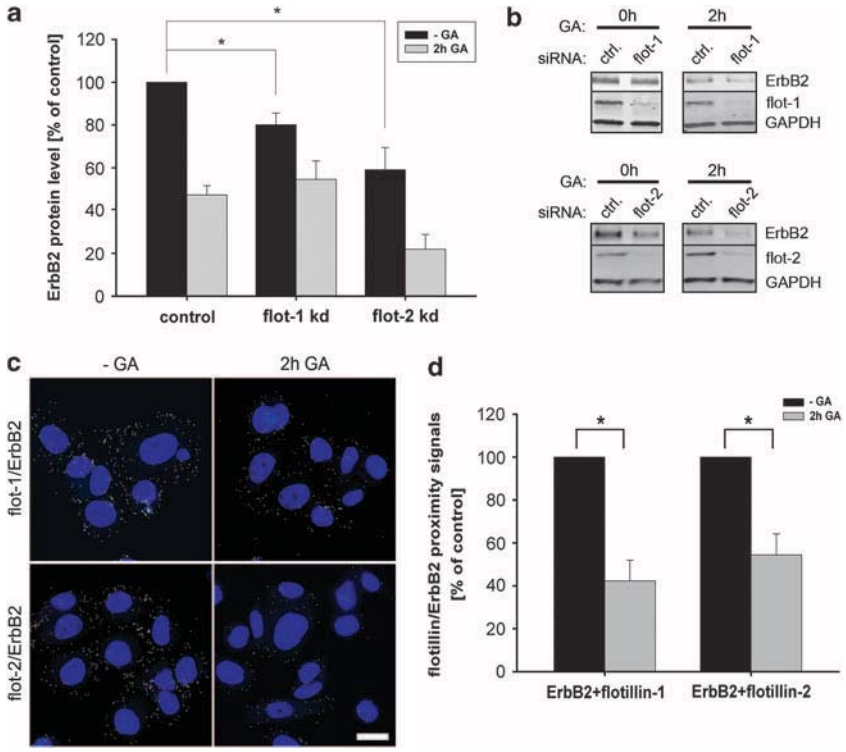

Figure 3. Depletion of flotillins leads to reduced ErbB2 levels and association of flotillins with ErbB2 is dependent on Hsp90. (**a**) Quantification of ErbB2 levels. Control, flotillin-1 or -2 knockdown cells were treated with 3 μM GA for 2 h or left untreated, lysed and analysed by SDS–PAGE. ErbB2 levels were quantified and showed to be significantly reduced upon flotillin depletion and GA treatment. Significances for GA-free conditions are indicated (*$P<0.05$). Number of independent experiments: flotillin-1 knockdown $n=4$ and flotillin-2 knockdown $n=5$, mean ± s.e.m. (**b**) Depletion of flotillin-1 (upper panel) or flotillin-2 (lower panel) leads to reduced ErbB2 protein levels, as detected by western blot analysis. GA treatment upon flotillin depletion results in increased ErbB2 degradation. GAPDH levels were detected as loading controls. (**c**) Detection of flotillin/ErbB2 proximity by fluorescent microscopy using *in situ* PLA. SKBR3 cells were pretreated for 2 h with 3 μM GA, or left untreated, incubated with primary antibodies against ErbB2 and flotillin-1 or -2 and processed according to the manufacturer's instructions. Projected confocal z-stacks were analysed. Fluorescent dots represent flotillin-1 or flotillin-2 associated with ErbB2 receptors, nuclei were stained with DAPI in blue. Scale bar, 20 μm. (**d**) Quantification of ErbB2-associated flotillin-1 or -2. Flotillin/ErbB2 proximity signals are significantly reduced after GA treatment (*$P<0.05$, Mann–Whitney Rank Sum Test). Analysis of >70 cells/condition in $n=4$ (flot-1/ErbB2, $P=0.029$) and $n=3$ (flot-2/ErbB2, $P=0.012$) independent experiments, mean ± s.e.m.

knockdown did not significantly alter total Hsp90 levels (Figure 4b). Next, we analysed the role of ErbB2 with respect to the association of flotillin with Hsp90. In agreement with our data obtained by immunoprecipitation, Hsp90 is in close proximity to flotillin-1 and -2, as determined by PLA, and the level of detectable Hsp90/flotillin proximity signals were strongly reduced by administration of the Hsp90 inhibitor GA (Figures 4c and d; control cells). Interestingly, also ErbB2 depletion (~70% decreased protein levels, Supplementary Figure S2a) reduced the level of Hsp90/flotillin proximity signals (Figures 4c and d), and this reduction was comparable to the effect induced by GA in control cells. However, depletion of ErbB2 had no effect on flotillin or Hsp90 protein levels (Figure 4e). Altogether, our data indicate that flotillins interact, directly or indirectly, with ErbB2 and Hsp90 at the plasma membrane.

Flotillin depletion leads to reduced ErbB2/Hsp90 interaction, ErbB2 phosphorylation and Akt signalling

Next, we tested by PLA how depletion of flotillin proteins affects the proximity of ErbB2 and Hsp90. In control cells, GA treatment for 2 h led to significantly reduced ErbB2/Hsp90 proximity signals (Figure 5a and Supplementary Figure S2b). Interestingly, also depletion of flotillin-1 or -2 resulted in a reduced level of ErbB2/Hsp90 proximity signals. Noteworthy, GA treatment in combination with flotillin knockdown did not significantly change ErbB2/Hsp90 proximity signals compared with flotillin knockdown alone. In accordance with this finding, rescue of flotillin protein levels, by transfection of siRNA-resistant genes, resulted in a recovery of the ErbB2/Hsp90 interaction (Supplementary Figures S2c and d).

After demonstrating that flotillin depletion triggers internalization and downregulation of ErbB2, we analysed whether flotillin depletion impairs the activation of ErbB2-induced signalling pathways. ErbB2 has been described as the only member of the ErbB tyrosine kinase receptor family without any known ligand. However, ErbB2 is a preferred dimerization partner for all of the other ErbB proteins. Dimer formation results in phosphorylation of the dimer partners and triggers the activation of signalling pathways.[27] Among several others, the two major pathways induced by ErbB dimers are the Ras/MAPK and the PI3K/Akt pathway, both involved in stimulating cell proliferation and tumour cell survival.[1,2] First, we analysed whether depletion of flotillin-1 or -2 changed the phosphorylation status of ErbB2 receptors. The activation status of ErbB2 was determined by quantification of p-ErbB2$^{(Tyr1248)}$ levels by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) (Figure 5b). The residue Tyr^{1248} is the major phosphorylation site of ErbB2 and p-ErbB2$^{(Tyr1248)}$ has been described as a predictor of poor prognosis in breast cancer.[28] Analysis of our data indicated that the levels of p-ErbB2$^{(Tyr1248)}$ were significantly reduced after depletion of flotillins. Knockdown of flotillin-1 led to a 40% reduction, knockdown of flotillin-2 led to a 65% reduction in p-ErbB2 levels (Figures 5b and c), and

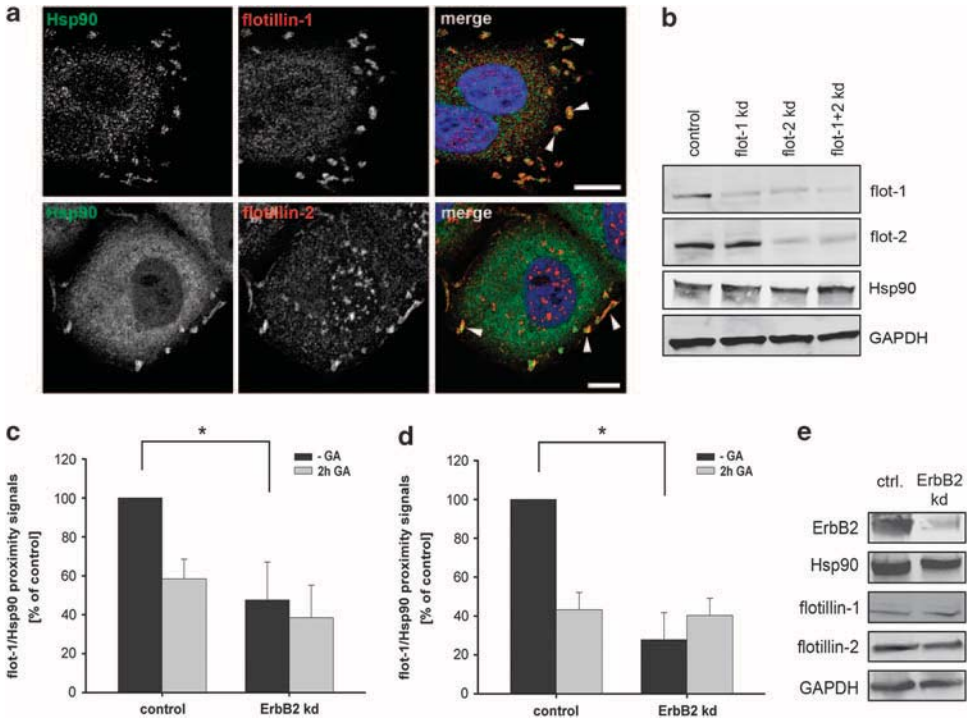

Figure 4. Analysis of flotillins/Hsp90 proximity and role of flotillins in the maintenance of ErbB2/Hsp90 interaction. (**a**) Co-localization of Hsp90 and flotillins. SKBR3 cells were immunostained for Hsp90 and flotillin-1 or -2. Partial co-localization of Hsp90 and flotillin-1 and -2 can be found at the plasma membrane (arrowheads). Scale bars, 10 μm. (**b**) Representative western blots of flotillin and Hsp90 proteins levels in control and flotillin knockdown cells. SKBR3 cells were depleted for flotillin-1 or -2 by siRNA treatment, cells were lysed and protein levels determined by western blot analysis. Knockdown of flotillin-1 or -2 does not affect Hsp90 levels. GAPDH levels were analysed as loading controls. (**c**) Quantification of flotillin-1/Hsp90 proximity by PLA. The level of flotillin-1/Hsp90 proximity signals is reduced by GA, but is also significantly reduced by ErbB2 knockdown ($P = 0.036$, $n = 3$, mean ± s.e.m.). (**d**) Quantification of flotillin-2/Hsp90 proximity by PLA. Knockdown of ErbB2 results in significantly reduced level of flotillin-2/Hsp90 proximity signal ($P = 0.029$, $n = 4$, mean ± s.e.m.). (**e**) Protein expression levels in ErbB2-depleted cells. SKBR3 cells were treated with siRNA oligonucleotides targeting ErbB2 and incubated for 3 days, cells were lysed and analysed by SDS–PAGE. ErbB2 levels were significantly reduced (~70% reduction compared with control cells), but protein levels of flotillin-1 or -2 and Hsp90 were not influenced. GAPDH levels were determined as loading controls.

administration of GA for 2 h resulted in ~90% reduction of the pErbB2 levels. Interestingly, treatment of cells with GA or depletion of flotillins resulted in a significantly stronger reduction of p-ErbB2 levels compared with the reduction of total ErbB2.

Furthermore, we investigated the influence of flotillin-induced ErbB2 downregulation on Akt signalling. ErbB2 is able to bind to PI3K, inducing phosphorylation of PI(4,5)P2 and the subsequent recruitment of Akt kinase, the main effector of PI3K. Upon activation, Akt phosphorylates several downstream targets that lead to tumour cell survival and anti-apoptotic signalling.[29,30] By measuring the phosphorylation of Akt upon GA treatment, a ~90% reduction in p Akt levels was observed, whereas total Akt levels were not significantly changed. Cells depleted of flotillin-2 showed a 40% reduced phosphorylation of Akt (Figures 5d and e). In contrast to the data obtained with flotillin-2, only a slight but not significant reduction in the p-Akt levels was observed upon flotillin-1 depletion.

High expression of flotillin-2 correlates with poor prognosis in breast cancer

To verify the physiological role of flotillins as regulators of the receptor tyrosine kinase ErbB2, and to determine whether flotillin levels are clinically correlated with ErbB2 expression, we examined how flotillin levels correlate with ErbB2 levels in breast cancer tissues. To this end, we used immunohistochemistry to analyse flotillin and ErbB2 levels in tissues from 194 patients diagnosed with carcinomas of the breast (stage I/II). The samples were scored and a correlation coefficient was calculated as described in the Materials and Methods. The analysis (Figures 6a–c, Supplementary Figure S3 and Supplementary Table S1) showed a correlation of flotillin-1 and flotillin-2 levels ($R = 0.746$, $P < 0.001$, $n = 130$), of flotillin-1 and ErbB2 levels ($R = 0.431$, $P < 0.001$, $n = 140$) and of flotillin-2 and ErbB2 levels ($R = 0.307$, $P < 0.001$, $n = 133$). Moreover, our study demonstrate that the expression of flotillin-2 is correlated with survival time (log-rank, $P = 0.0349$; Figure 6d), but that is not the case for flotillin-1 ($P = 0.427$; Figure 6e). Thus, high expression of flotillin-2 was associated with poor prognosis and reduced survival time, and it emerges as a potential predictor of relapse in breast cancer.

DISCUSSION

The most important finding in the present article is that stabilization of ErbB2 at the cell surface is dependent on functional flotillin proteins. This stabilization is mediated by a complex containing ErbB2, Hsp90 and flotillin proteins. Depletion of or functional interference with one of these components disrupts this complex, and results in destabilization, internalization and degradation of ErbB2, thereby interfering with ErbB2 signalling. This is reflected by reduced phosphorylation of ErbB2 and reduced activation of the ErbB2 effector Akt.

We have here demonstrated that depletion of flotillins induced the internalization and degradation of ErbB2, and depletion of flotillin-2 resulted in a stronger reduction of ErbB2 levels than seen

after depletion of flotillin-1. In agreement with this observation is the finding that Akt phosphorylation was reduced after knockdown of flotillin-2 but not after flotillin-1 depletion, and that only flotillin-2 can serve as a prognostic marker for breast cancer at early stages. The differences seen with flotillin-1 and flotillin-2 may reflect the slightly different nature, different membrane-anchoring properties, intracellular localization and functions of flotillin-1 and -2, as described earlier by us and other groups.[22,31,32] Moreover, a stronger reduction of ErbB2 levels was obtained when GA treatment was combined with depletion of flotillin-2. This indicates that flotillin-2 depletion and GA treatment can have synergistic effects that might be of therapeutical interest. In line with a potential role of flotillins as stabilizers of proteins at the membrane, flotillin-1 has been reported to stabilize the level of caveolin-1 in intestinal epithelial cells,[33] and caveolins by themselves are involved in membrane organization. As the ErbB2/Hsp90 interaction is impaired by flotillin depletion, and also the association between flotillin and Hsp90 is affected upon depletion of ErbB2, the data suggest that there is a molecular complex containing ErbB2, Hsp90 and flotillin proteins. However, future studies are required to characterize other proteins in this complex that may participate in the regulation and stabilization of ErbB2 receptors.

Inhibition of Hsp90 has been shown to induce rapid ubiquitination of ErbB2 followed by receptor downregulation.[34,35] Indeed, in our experiments, GA treatment results in strong receptor ubiquitination (Supplementary Figure S2e). However, no or very little ErbB2 ubiquitination can be observed after flotillin depletion. In contrast to the short-term effect of GA, depletion of flotillins occurs over a period of 3 days. Thus, it seems likely that most of the internalized ErbB2 is already degraded at that time. This is supported by our data showing reduced ErbB2 levels after flotillin depletion and less internalized ErbB2 compared with GA treatment. On the other hand, we cannot exclude the possibility that flotillin depletion leads to ErbB2 internalization by a mechanism independent of ubiquitination. However, further studies are necessary to elucidate this process.

Data from two very recent publications indicate that in HeLa cells flotillin-1 has a functional role in receptor tyrosine kinase signalling, especially in the activation of MAP kinase signalling and the regulation of FGF signalling.[36,37] However, in the SKBR3 cells studied here, we found that Akt phosphorylation is reduced upon depletion of flotillin-2, but not after flotillin-1 knockdown. Thus, receptor tyrosine kinases or receptor tyrosine kinase-mediated signalling pathways might be regulated by flotillins in different ways and/or in a cell type-specific manner. Moreover, it is possible that the different signalling pathways triggered by ErbB2 are regulated by either flotillin-1 or flotillin-2. Nevertheless, the reduced p-ErbB2 and p-Akt levels triggered by flotillin

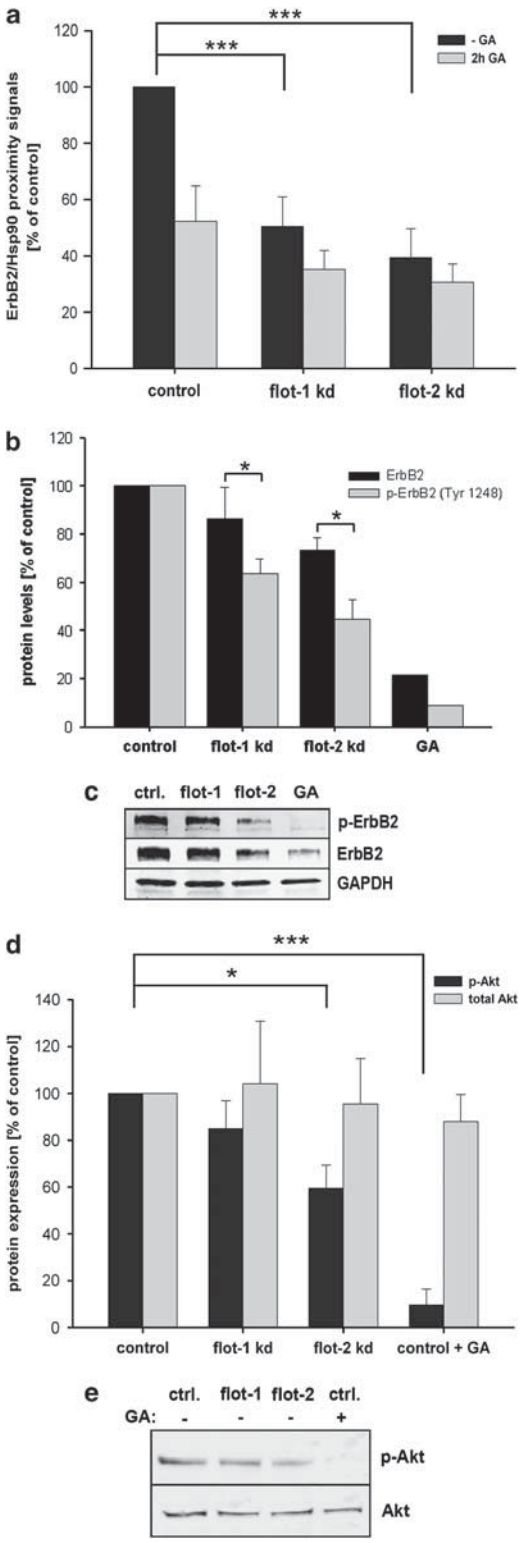

Figure 5. Flotillin knockdown interferes with ErbB2/Hsp90 interaction and reduces the level of p-ErbB2^{Tyr1248} and p-Akt. (**a**) Relevance of flotillins in the maintenance of ErbB2/Hsp90 complexes. Quantification of ErbB2/Hsp90 proximity by PLA experiments (flotillin-1 kd: $n=4$, flotillin-2 kd: $n=3$, mean + s.e.m.). Knockdown of flotillin-1 or -2 significantly reduces the level of ErbB2/Hsp90 proximity signals (***$P<0.005$, Mann–Whitney Rank Sum Test), as indicated for GA-free conditions. Analysis of >70 cells per condition and experiment. (**b**) Quantification of total ErbB2 and p-ErbB2^{Tyr1248} levels by western blot analysis. Depletion of flotillins or treatment with GA (3 μM, 2 h) leads to a stronger reduction of p-ErbB2 levels compared with total ErbB2 levels (*$P<0.05$, Mann–Whitney Rank Sum Test; flotillin-1/-2 kd: $n=3$, GA treatment: $n=1$, mean + s.e.m.). (**c**) Representative blots showing total ErbB2 and p-ErbB2$^{Tyr\ 1248}$ levels after GA treatment and flotillin depletion. GAPDH levels are shown as loading control. (**d**) Western blot analysis of p-Akt^{Ser473} levels after flotillin knockdown. The depletion of flotillin-2 results in a ~40% reduced level of p-Akt compared with control cells (*$P=0.015$, $n=3$). The depletion of flotillin-1 led only to a slight but not significant reduction of p-Akt ($P=0.274$, $n=3$). GA treatment results in a ~90% reduction of p-Akt levels (***$P<0.001$, $n=3$). Mean + s.e.m. (**e**) Decrease of Akt phosphorylation after flotillin knockdown. Representative western blot of p-Akt protein levels, quantification of pooled data shown in **d**. Depletion of flotillin-1 or -2 reduces the level of p-Akt^{Ser473}.

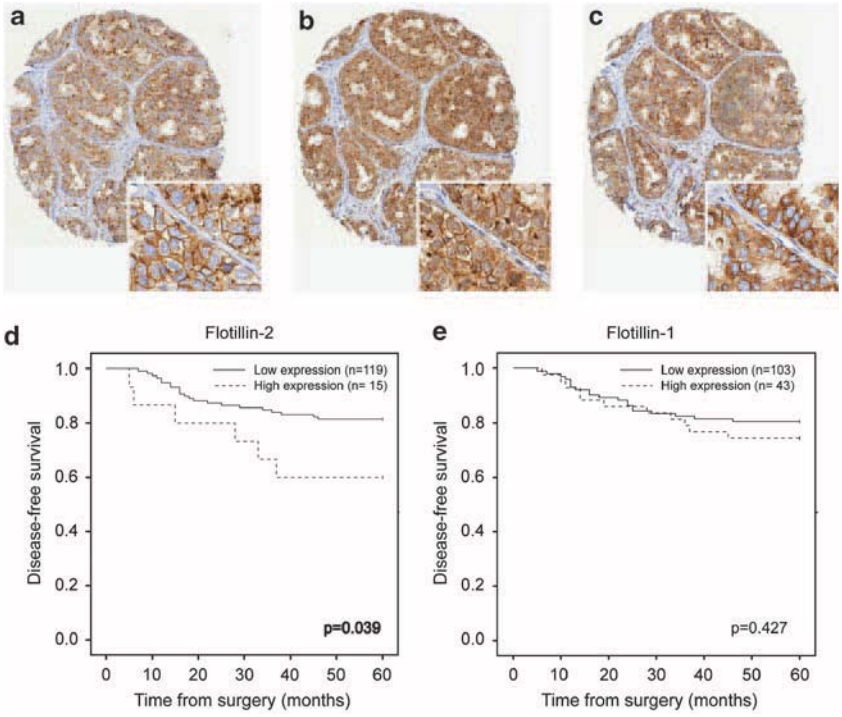

Figure 6. Correlation between flotillin and ErbB2 expression in breast cancer tissues. (**a**–**c**) Representative protein expression of flotillin-1 (**a**), flotillin-2 (**b**) and ErbB2 (**c**) in a tissue microarray core taken from a breast carcinoma. Inlays show detailed view. (**d**) Kaplan–Meier-like survival curves with univariate analysis (log-rank, $P = 0.039$) for patients with tumours expressing low (with a total score of 0–6, $n = 119$) versus high (total score 7–8, $n = 15$) protein levels of flotillin-2. High flotillin-2 expression is significantly correlated with poor patient outcome. (**e**) Kaplan–Meier-like survival curves with univariate analysis (log-rank, $P = 0.43$) for patients with tumours expressing low ($n = 103$) versus high ($n = 43$) protein levels of flotillin-1. High flotillin-1 expression is not significantly correlated with poor patient outcome.

knockdown support our hypothesis of a flotillin-mediated stabilization of ErbB2.

ErbB2 is known to be associated with poor prognosis in breast cancer, but also flotillins have been described to be associated with tumorigenesis.[38,39] However, this is the first study determining the functional role of both flotillin proteins on the regulation of ErbB2. We demonstrated that the expression of flotillin-2 is correlated with survival time. Thus, high expression of flotillin-2 is associated with poor prognosis and reduced survival time, and it emerges as a potential predictor of relapse in breast cancer. Up to 70% of all breast tumours are oestrogen receptor-positive, and oestrogen receptor expression is an important prognostic factor. Interestingly, it has been reported that flotillin-2 also interacts with oestrogen receptors,[40] indicating a significant impact of flotillin-2 function on breast cancer development. On the other hand, based on our tissue microarray studies, flotillin-1 did not emerge as a prognostic marker for relapse in breast cancer, which did not confirm the study of Lin *et al*.[39] However, in their study, more than 40% of the analysed breast cancer tissue was stage III/IV; whereas in our study, only material of early stages (stage I/II) was used to get a high prognostic relevance. Therefore, it might be possible that flotillin-1 affects tumour progression only at later stages. Further studies are needed to get a detailed picture of the function of flotillins in tumour development.

In conclusion, this is the first demonstration of a positive correlation between flotillin and ErbB2 levels in cultured breast cancer cells as well as in patient material. Moreover, flotillin-2 expression levels seem to correlate with poor prognosis in breast cancer, indicating that flotillin-2 might be a prognostic biomarker for breast cancer. As it has been found that ErbB2-overexpressing tumours are more likely to be resistant to treatment with tamoxifen and standard chemotherapy, an antibody-based (trastuzumab/Herceptin) therapy has become the clinical first-line treatment in patients with ErbB2-overexpressing metastatic breast cancer.[6,10] However, even in combination with other chemotherapeutical medications, more than 70% of patients with ErbB2-overexpression show no response to the treatment.[41] Thus, by targeting flotillin expression, new strategies in cancer treatment can be developed.

MATERIALS AND METHODS

Antibodies and other reagents

HEPES, bovine serum albumin, *n*-octylglucopyranoside and rabbit anti-flotillin-2 antibody (F1680) were purchased from Sigma-Aldrich (St Louis, MO, USA). For western blot analysis, anti-flotillin-1/-2 antibodies were from BD Bioscience (St Jose, CA, USA), the rabbit anti-flotillin-1 antibody was a gift from Dr G van der Goot (EPFL, Lausanne, Switzerland), anti-ErbB2 (29D8), anti-Akt and anti-p-Akt^{Ser473} was from Cell Signaling Technologies (Boston, MA, USA), anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase, ab9484) was from Abcam (Cambridge, UK), anti-p-ErbB2^{Tyr1248} (Ab-18) and anti-ErbB2 (Ab-17) was purchased from Thermo Scientific (Waltham, MA, USA) and fluorescently labelled secondary antibodies from Li-Cor Biosciences (Lincoln, NE, USA) (IRDyes) were used for western blot analysis. Anti-ErbB2 (Santa Cruz Biotechnology (Santa Cruz, CA, USA), 9G6), anti-Hsp90 (Sigma-Aldrich, H-114), anti-flotillin-1 (van der Goot) and anti-flotillin-2 (Sigma-Aldrich, F1680) and secondary antibodies from Jackson ImmunoResearch (Suffolk, UK) were used for confocal fluorescence microscopy. Protein concentrations in the lysates were determined by the BCA protein assay (Pierce, Rockford, IL, USA) or with the use of bovine serum albumin as the standard by using the NanoDrop 2000 spectrophotometer (Thermo Scientific)

Cell culture and transfections

SKBR3 cells were grown under 5% CO_2 in Dulbecco's modified Eagle's medium (Invitrogen, Paisley, UK) supplemented with 10% foetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. For

siRNA transfection, cells were seeded out in six-well plates at a density of 2×10^5 cells/well, 24 h before transfection. siRNAs targeting non-overlapping parts of the mRNA sequence were used for flotillin-1 and -2, as described before.[22] For the depletion of ErbB2, a pool of four different siRNA oligonucleotides with the sequences 5′-UGGAAGAGAUCACAGG UUA-3′, 5′-GAGACCCGCUGAACAAUAC-3′, 5′-GGAGGAAUGCCGAGUACUG-3′ and 5′-GCUCAUCGCUCACAACCAA-3′ (Dharmacon, RNAi Technologies, Lafayette, CO, USA) has been used. To reduce unspecific off-target effects, the constructs were ordered as ON-TARGETplus oligos from Dharmacon RNAi Technologies, as negative controls non-targeting oligos were used. For siRNA transfection, the cells were seeded out without antibiotics, grown for 24 h and transfected by using Lipofectamine RNAiMax transfection reagent (Invitrogen) according to the manufacturer's procedure. After 4 h of transfection, the medium was changed to complete growth medium containing serum and antibiotics, and the cells were grown for 3 days before experiments were started. For rescue experiments, siRNA-resistant flotillin-1 and -2 (DNA 2.0, Menlo Park, CA, USA) were cloned into the mammalian expression vector pcDNA3 (Invitrogen). SKBR3 cells were seeded and transfected and incubated for 2 days with siRNA oligos (as described above) and subsequently transfected and incubated for 24 h with 1 μg of siRNA-resistant flotillin-1 or -2 or empty pcDNA3 vector by using FuGene6 (Roche Applied Science, Basel, Switzerland), as described elsewhere.[22]

Protein extraction and western blot

The cells were washed in cold phosphate-buffered saline (PBS) and lysed in lysis buffer containing 0.1 M NaCl, 10 mM Na_2HPO_4, 1 mM EDTA, 1% Triton X-100, 60 mM *n*-octylglucopyranoside, pH 7.4 and supplemented with Complete protease inhibitors and PhosSTOP (Roche Diagnostics, Basel, Switzerland). The lysate was cleared by centrifugation (8000 r.p.m., 10 min) and proteins were separated by SDS–PAGE under reducing conditions. After blotting onto a PVDF membrane (Immobilon-FL, Millipore, Billerica, MA, USA), the membrane was blocked by air drying. Thereafter, the membrane was re-wet in PBS with 0.5% Tween-20 (PBS-T) (or in Tris-buffered saline (TBS) with 0.5% Tween-20 for phospho-specific antibodies) and incubated with the primary antibody in 1% BSA in PBS-T overnight at 4 °C. After extensive washing in PBS-T (or TBS-T), LI-CORE infrared dye secondary antibodies (Li-Cor Biosciences) were applied, and the bands were detected and quantified using the Odyssey imaging system (Li-Cor Biosciences).

Confocal fluorescence microscopy and imaging

Cells were grown on glass coverslips and transfected with siRNA oligonucleotides targeting flotillin-1 or flotillin-2 or non-targeting control oligos and incubated for 3 days. Subsequently, either the cells were fixed for 15 min with 4% paraformaldehyde and permeabilized for 2 min with 0.1% Triton X-100/PBS or fixed and permeabilized for 7 min with ice-cold methanol, before immunostained with appropriate antibodies. Fluorophore-labelled secondary antibodies used for confocal studies were obtained from Jackson ImmunoResearch Laboratories. The cells were mounted in Mowiol or Prolong Gold containing DAPI for nuclear staining (Molecular Probes, Invitrogen, Paisley, UK) and examined by a laser-scanning confocal microscope LSM 710 or LSM 780 (Carl Zeiss, Jena, Germany) and a PlanApo $63 \times /1.4$ NA oil-immersion objective. Images were prepared and analysed with the LSM Image Browser software (Carl Zeiss) or ImageJ software.

In situ PLA

The *in situ* PLA (Olink Bioscience) was used to detect flotillin/ErbB2, flotillin/Hsp90 and ErbB2/Hsp90 associations. The assay is based on oligonucleotide-conjugated PLA probes, containing secondary antibodies directed against primary antibodies for either flotillin-1/-2, ErbB2 or Hsp90. Annealing of the probes occurs when the target proteins are in close proximity, which then initiates the amplification. The amplicons can be detected by fluorescence microscopy in a quantifiable manner. For this assay, SKBR3 cells were seeded in six-well plates on coverslips and either transfected with siRNA oligos and/or pretreated with 3 μM GA for 2 h, as indicated in the figure legends. Cells were washed, fixed and permeabilized as described in the section above. Antibodies against ErbB2 (1:200, Santa Cruz Biotechnology, 9G6), Hsp90 (1:100, Santa Cruz Biotechnology, H-114), flotillin-1 (1:2000, van der Goot) and flotillin-2 (1:200, Sigma-Aldrich, F1680) were used as primary antibodies and the assays were performed as described in the manufacturer's manual. Slides were mounted with Duolink Mounting Medium (Olink Bioscience) and samples were observed by fluorescence microscopy with a LSM 780 confocal microscope (Carl Zeiss). The number of dots were calculated by ImageJ software and normalized to control conditions.

Immunoprecipitation

Cell lysates from non-treated SKBR3 cells were made in RIPA buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1% NP40, 60 mM *n*-octylglucopyranoside) supplemented with Complete protease inhibitors and PhosSTOP (Roche Diagnostics), and cleared by centrifugation (8000 r.p.m., 10 min). Protein G-sepharose beads (Amersham Biosciences, Piscataway, NJ, USA) in PBS were incubated with the desired antibody for 2 h before washing with PBS. Beads pre-bound to antibody were mixed with the lysate and incubated at 4 °C overnight. After extensive washing, associated proteins were eluted by boiling at 95 °C for 5 min, separated by SDS–PAGE, and blotted onto a PVDF membrane (Immobilon-FL, Millipore). The membrane was processed and the bands detected as described for western blot analysis.

Immunohistochemistry and scoring of tissue samples

The patient material consists of 194 patients diagnosed with carcinomas of the breast (Stage I/II) at the Norwegian Radium Hospital in the period 1978–1994. To obtain a material well suited to test potential prognostic markers, the patients either had relapse or cancer-related death within 5 years after prognosis, or disease-free survival for at least 10 years. From each patient, two cylindrical cores from formalin-fixed and paraffin-embedded tumour tissue were taken, and all the cores were collected in three tissue microarrays as described elsewhere.[42]

The use of the material has been approved by the South East Region Committee for Research Ethics in Norway (REK 6.2008.1000). For immunohistochemistry analysis, antibodies were purchased from Sigma (anti-flotillin-1, HPA1392, 1:1500 and anti-flotillin-2, HPA 1396, 1:4000) and from Novocastra (anti-c-erbB-2, NCL-CB11, 1:700). Sections (3 μm) of the tissue microarrays were stained with an AutostainerLink48 (DAKO) with high pH antibody retrieval, peroxidase block, 30 min antibody incubation, EnVision FLEX + mouse linker and HRP/DAB visualization.

The membrane *in situ* expression of the three proteins was scored by a pathologist according to the proportion and intensity categories proposed by Allred *et al.*[43] Following this system, the intensity was scored as 0: no staining; 1: weak staining; 2: intermediate staining; 3: strong staining. The proportion was scored as 0:0% tumour cells with membrane staining; $1{:}0 \leqslant 1/100$; $2{:}1/100 \leqslant 1/10$; $3{:}1/10 \leqslant 1/3$; $4{:}1/3 \leqslant 2/3$; $5{:}2/3 \leqslant 100\%$. For the correlation analysis, a total score (TS) was calculated by adding the values for proportion and intensity. For the survival analysis, the total scores were further categorized in two different ways: total score = 0–6, low expression; total score = 7–8, high expression. In the analysis, some cores were excluded owing to loss of the core during processing, too poor quality to be scored, only benign tissue left in the core or lack of correspondence between the score for intensity and proportion (for example, proportion = 0 and intensity = 2).

Statistics

Values of three or more experiments were given as mean ± s.e.m. A *P*-value of 0.05 or less was considered to be statistically significant and determined by the Student's *t*-test or Mann–Whitney Rank Sum test, as indicated in the figure legends. For all statistical measurements, a minimum of 50 cells per condition and experiments has been analysed. For immunohistochemistry analysis, Pearson correlation coefficients were calculated between the total scores of the three different proteins. We have made Kaplan–Meier-like curves on categorized total scores to illustrate differences between groups, although the study design imply that this will not be proper survival curves for Stage I/II breast cancer patients. The Mantel–Cox log-rank test was used for testing equality of survival distributions. An event was defined as relapse or cancer-related death. The statistical analyses were performed in SPSS 18.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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REFERENCES

1 Olayioye MA, Neve RM, Lane HA, Hynes NE. The ErbB signaling network: receptor heterodimerization in development and cancer. *EMBO J* 2000; **19**: 3159–3167.
2 Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. *Nat Rev Mol Cell Biol* 2001; **2**: 127–137.
3 Arias-Romero LE, Saha S, Villamar-Cruz O, Yip SC, Ethier SP, Zhang ZY *et al.* Activation of Src by protein tyrosine phosphatase 1B Is required for ErbB2 transformation of human breast epithelial cells. *Cancer Res* 2009; **69**: 4582–4588.
4 Baulida J, Kraus MH, Alimandi M, Di Fiore PP, Carpenter G. All ErbB receptors other than the epidermal growth factor receptor are endocytosis impaired. *J Biol Chem* 1996; **271**: 5251–5257.
5 Jones RB, Gordus A, Krall JA, MacBeath G. A quantitative protein interaction network for the ErbB receptors using protein microarrays. *Nature* 2006; **439**: 168–174.
6 Baselga J, Swain SM. Novel anticancer targets: revisiting ERBB2 and discovering ERBB3. *Nat Rev Cancer* 2009; **9**: 463–475.
7 Pauletti G, Dandekar S, Rong H, Ramos L, Peng H, Seshadri R *et al.* Assessment of methods for tissue-based detection of the HER-2/neu alteration in human breast cancer: a direct comparison of fluorescence in situ hybridization and immunohistochemistry. *J Clin Oncol* 2000; **18**: 3651–3664.
8 Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* 1987; **235**: 177–182.
9 Wu Y, Khan H, Chillar R, Vadgama JV. Prognostic value of plasma HER-2/neu in African American and Hispanic women with breast cancer. *Int J Oncol* 1999; **14**: 1021–1037.
10 Slamon DJ, Leyland-Jones B, Shak S, Fuchs H, Paton V, Bajamonde A *et al.* Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N Engl J Med* 2001; **344**: 783–792.
11 Lerdrup M, Hommelgaard AM, Grandal M, van Deurs B. Geldanamycin stimulates internalization of ErbB2 in a proteasome-dependent way. *J Cell Sci* 2006; **119**: 85–95.
12 Mimnaugh EG, Chavany C, Neckers L. Polyubiquitination and proteasomal degradation of the p185c-erbB-2 receptor protein-tyrosine kinase induced by geldanamycin. *J Biol Chem* 1996; **271**: 22796–22801.
13 Xu W, Mimnaugh E, Rosser MF, Nicchitta C, Marcu M, Yarden Y *et al.* Sensitivity of mature Erbb2 to geldanamycin is conferred by its kinase domain and is mediated by the chaperone protein Hsp90. *J Biol Chem* 2001; **276**: 3702–3708.
14 Jones KL, Buzdar AU. Evolving novel anti-HER2 strategies. *Lancet Oncol* 2009; **10**: 1179–1187.
15 Modi S, D'Andrea G, Norton L, Yao TJ, Caravelli J, Rosen PP *et al.* A phase I study of cetuximab/paclitaxel in patients with advanced-stage breast cancer. *Clin Breast Cancer* 2006; **7**: 270–277.
16 Modi S, Stopeck AT, Gordon MS, Mendelson D, Solit DB, Bagatell R *et al.* Combination of trastuzumab and tanespimycin (17-AAG, KOS-953) is safe and active in trastuzumab-refractory HER-2 overexpressing breast cancer: a phase I dose-escalation study. *J Clin Oncol* 2007; **25**: 5410–5417.
17 Schulte T, Paschke KA, Laessing U, Lottspeich F, Stuermer CA. Reggie-1 and reggie-2, two cell surface proteins expressed by retinal ganglion cells during axon regeneration. *Development* 1997; **124**: 577–587.
18 Langhorst MF, Reuter A, Stuermer CA. Scaffolding microdomains and beyond: the function of reggie/flotillin proteins. *Cell Mol Life Sci* 2005; **62**: 2228–2240.
19 Neumann-Giesen C, Falkenbach B, Beicht P, Claasen S, Luers G, Stuermer CA *et al.* Membrane and raft association of reggie-1/flotillin-2: role of myristoylation, palmitoylation and oligomerization and induction of filopodia by overexpression. *Biochem J* 2004; **378**: 509–518.
20 Stuermer CA, Lang DM, Kirsch F, Wiechers M, Deininger SO, Plattner H. Glycosylphosphatidyl inositol-anchored proteins and fyn kinase assemble in noncaveolar plasma membrane microdomains defined by reggie-1 and -2. *Mol Biol Cell* 2001; **12**: 3031–3045.
21 Glebov OO, Bright NA, Nichols BJ. Flotillin-1 defines a clathrin-independent endocytic pathway in mammalian cells. *Nat Cell Biol* 2006; **8**: 46–54.
22 Pust S, Dyve AB, Torgersen ML, van Deurs B, Sandvig K. Interplay between toxin transport and flotillin localization. *PLoS One* 2010; **5**: e8844.
23 Saslowsky DE, Cho JA, Chinnapen H, Massol RH, Chinnapen DJ, Wagner JS *et al.* Intoxication of zebrafish and mammalian cells by cholera toxin depends on the flotillin/reggie proteins but not Derlin-1 or -2. *J Clin Invest* 2010; **120**: 4399–4409.
24 Soderberg O, Gullberg M, Jarvius M, Ridderstrale K, Leuchowius KJ, Jarvius J *et al.* Direct observation of individual endogenous protein complexes in situ by proximity ligation. *Nat Methods* 2006; **3**: 995–1000.
25 Spears M, Taylor KJ, Munro AF, Cunningham CA, Mallon EA, Twelves CJ *et al.* In situ detection of HER2:HER2 and HER2:HER3 protein-protein interactions demonstrates prognostic significance in early breast cancer. *Breast Cancer Res Treat* 2012; **132**: 463–470.
26 Citri A, Kochupurakkal BS, Yarden Y. The achilles heel of ErbB-2/HER2: regulation by the Hsp90 chaperone machine and potential for pharmacological intervention. *Cell Cycle* 2004; **3**: 51–60.
27 Garrett TP, McKern NM, Lou M, Elleman TC, Adams TE, Lovrecz GO *et al.* The crystal structure of a truncated ErbB2 ectodomain reveals an active conformation, poised to interact with other ErbB receptors. *Mol Cell* 2003; **11**: 495–505.
28 Cicenas J, Urban P, Kung W, Vuaroqueaux V, Labuhn M, Wight E *et al.* Phosphorylation of tyrosine 1248-ERBB2 measured by chemiluminescence-linked immunoassay is an independent predictor of poor prognosis in primary breast cancer patients. *Eur J Cancer* 2006; **42**: 636–645.
29 Engelman JA, Luo J, Cantley LC. The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism. *Nat Rev Genet* 2006; **7**: 606–619.
30 Yuan TL, Cantley LC. PI3K pathway alterations in cancer: variations on a theme. *Oncogene* 2008; **27**: 5497–5510.
31 Gomez V, Sese M, Santamaria A, Martinez JD, Castellanos E, Soler M *et al.* Regulation of aurora B kinase by the lipid raft protein flotillin-1. *J Biol Chem* 2010; **285**: 20683–20690.
32 Zhao F, Zhang J, Liu YS, Li L, He YL. Research advances on flotillins. *Virol J* 2011; **8**: 479.
33 Vassilieva EV, Ivanov AI, Nusrat A. Flotillin-1 stabilizes caveolin-1 in intestinal epithelial cells. *Biochem Biophys Res Commun* 2009; **379**: 460–465.
34 Xu W, Marcu M, Yuan X, Mimnaugh E, Patterson C, Neckers L. Chaperone-dependent E3 ubiquitin ligase CHIP mediates a degradative pathway for c-ErbB2/Neu. *Proc Natl Acad Sci USA* 2002; **99**: 12847–12852.
35 Zhou P, Fernandes N, Dodge IL, Reddi AL, Rao N, Safran H *et al.* ErbB2 degradation mediated by the co-chaperone protein CHIP. *J Biol Chem* 2003; **278**: 13829–13837.
36 Amaddii M, Meister M, Banning A, Tomasovic A, Mooz J, Rajalingam K *et al.* Flotillin-1/reggie-2 plays a dual role in the activation of receptor tyrosine kinase/map kinase signaling. *J Biol Chem* 2012; **287**: 7265–7278.
37 Tomasovic A, Traub S, Tikkanen R. Molecular networks in FGF signaling: Flotillin-1 and Cbl-associated protein compete for the binding to fibroblast growth factor receptor substrate 2. *PLoS One* 2012; **7**: e29739.
38 Hazarika P, McCarty MF, Prieto VG, George S, Babu D, Koul D *et al.* Up-regulation of Flotillin-2 is associated with melanoma progression and modulates expression of the thrombin receptor protease activated receptor 1. *Cancer Res* 2004; **64**: 7361–7369.
39 Lin C, Wu Z, Lin X, Yu C, Shi T, Zeng Y *et al.* Knockdown of FLOT1 impairs cell proliferation and tumorigenicity in breast cancer through upregulation of FOXO3a. *Clin Cancer Res* 2011; **17**: 3089–3099.
40 Marquez DC, Chen HW, Curran EM, Welshons WV, Pietras RJ. Estrogen receptors in membrane lipid rafts and signal transduction in breast cancer. *Mol Cell Endocrinol* 2006; **246**: 91–100.
41 Wu Y, Shang X, Sarkissyan M, Slamon D, Vadgama JV. FOXO1A is a target for HER2-overexpressing breast tumors. *Cancer Res* 2010; **70**: 5475–5485.
42 Kononen J, Bubendorf L, Kallioniemi A, Barlund M, Schraml P, Leighton S *et al.* Tissue microarrays for high-throughput molecular profiling of tumor specimens. *Nat Med* 1998; **4**: 844–847.
43 Allred DC, Harvey JM, Berardo M, Clark GM. Prognostic and predictive factors in breast cancer by immunohistochemical analysis. *Mod Pathol* 1998; **11**: 155–168.

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