Supplemental Materials and Methods

Antibodies, reagents and buffers
Antibodies used were: rabbit anti-CERT pAb A300-669A (Bethyl Laboratories), rabbit anti-phospho-T308-Akt, mouse anti-Akt 40D4 (pan), rabbit anti-phospho-Y1068-ErbB1 and mouse anti-phosphotyrosine P-Tyr-100 mAbs (Cell Signaling), mouse anti-ErbB1 Ab-13, mouse anti-ErbB2 Ab-17, mouse anti-ErbB3 clone 2F12 mAbs (Neomarkers), rabbit anti-GFP(FL), anti-paxillin H-114, anti-ErbB1 1005, anti-ErbB2 C-18, anti-ErbB3 C-17, anti-SMS1 H-130 and goat anti-SMS2 N-13 pAbs (Santa Cruz Biotechnology), mouse anti-phospho-Y397-FAK mAb (BD), mouse anti-talin, anti-tubulin and anti-Flag mAbs (Sigma), and mouse anti-transferrin receptor (Invitrogen). HRP-labeled secondary anti-mouse and anti-rabbit IgG antibodies were obtained from GE Healthcare. Alexa Fluor 488 and 546-labelled secondary anti-mouse and anti-rabbit antibodies were from Invitrogen. EGF, lysenin and filipin were from Sigma, HRG was from R&D, PD168393 was from Merck. Alexa Fluor 555-labeled EGF and transferrin, Vybrant Alexa Fluor 488 Lipid Raft Labeling Kit and C6-(7-nitro-2,1,3-benzoxadiazol-4-yl (NBD))-SM were purchased from Invitrogen. TEB buffer contained 50 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1 mM sodium orthovanadate, 10 mM sodium fluoride, and 20 mM β-glycerophosphate plus Complete protease inhibitors (Roche), RIPA buffer contained 50 mM Tris (pH 7.5), 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM sodium orthovanadate, 10 mM sodium fluoride, and 20 mM β-glycerophosphate plus Complete and PLD buffer contained 10 mM Tris (pH 7.5), 100 mM NaCl, 1 mM EDTA, 0.01% Triton X-100, 1 mM sodium orthovanadate, 0.5 mM PMSF plus Complete.

Plasmids and siRNAs
pcDNA3-Flag-CERT, pECFPN3-ErbB1 and pcDNA3-PLD2 were kindly provided by Juan Saus (Centro de Investigación Príncipe Felipe, Spain), Martin Offerdinger (Medical University Innsbruck, Austria) and Thomas Koch (Otto-von-Guericke-University Magdeburg, Germany),
respectively. The PLD2 cDNA was amplified by PCR using pcDNA3-PLD2 as a template and primers containing flanking EcoRI sites, followed by subeloning into the pEGFPC2 vector linearized with EcoRI and verification by sequencing. The siRNAs used were synthesized by MWG Biotech. The sequences are as follows: siLacZ (5′-GCGGCUGCCGAAUUUACC-3′), siCERT (5′-GAACAGAGGAAGCAUAUAA-3′) and siCERT#2 (5′- CCACAUGACUUACUAUA-3′) (Fugmann et al., 2007); siSMS1 (5′-CUACACUCCAGUACCUGG-3′) and siSMS2 (5′-GGCUCAAUUCCUUGCUGCU-3′) (Villani et al., 2008). SMARTpools for SMS1, SMS2 and PLD2 (and siRNAs contained within the pool) were obtained from Dharmacon. For RNA interference, cells were transfected with siRNAs using Oligofectamine (Invitrogen). For overexpression, cells were transfected by nucleofection (Amaxa) or using Lipofectamine 2000 (Invitrogen).

Clinical material and IHC evaluation

The study was conducted with approval of the institutional review board of the University Hospital of Tuebingen (ref. 397/2006). A database was constructed from 32 patients diagnosed with primary invasive ductal breast cancer and treated at the Breast Center of the Women’s Hospital, University of Tuebingen, Germany. For each patient, tumor size, hormone receptor status and HER2 expression was recorded. Immunostaining was evaluated using a semi-quantitative scoring system assessing the intensity of cytoplasmic staining in the majority of the tumor. No staining = score 0; weak staining, less than normal tissue = score 1; moderate staining of approximately equal intensity to normal breast tissue and endothelium in blood vessels = score 2; strong and intense staining = score 3. For statistical analysis, IHC results were analyzed in subgroups and compared to clinical parameters and breast cancer subtype as determined by immunohistochemical testing of ER, PR and HER2 status according to standard ASCO/CAP guidelines. Hormone receptors were considered positive, if there were more than 1% positive cells; HER2 was considered positive, if there was at least a strong membranous staining (score 3+ in more than 30% of the tumor cells) or FISH analysis showed gene amplification. The subtypes
were defined as published (Cheang et al., 2008): luminal A = ER and/or PR positive, HER2 negative; luminal B = ER and/or PR positive, HER2 positive; HER2 = ER and PR negative, HER2 positive; triple-negative = ER, PR and HER2 negative; basal-like confirmed subgroup of triple-negative cases = ER, PR and HER2 negative and either cytokeratin 5/6 or EGFR/ErbB1 positive. Correlation analysis was performed with Chi-Square test using SPSS software.

Confocal microscopy

Confocal analysis was performed with a laser scanning microscope (LSM710, Zeiss) using 405, 488 and 561 nm excitation and a Plan-Apochromat 63x/1.40 Oil DIC M27 objective. Images were processed with ZEN 2009 software (Zeiss). All images are stacks of several confocal sections.

Supplemental References
