Supporting Materials

Ethics Statement.

All clinical investigation was conducted in accordance with the principles outlined in the Declaration of Helsinki. Blood was sampled from patients diagnosed with breast cancer after written consent was obtained from each individual. This study was approved by the institutional review board at the Centre Hospitalier Universitaire de Nantes.

Antibodies, plasmids and other reagents.

BTP2, LY294002, BAPTA-AM ([1,2-bis-(o-Aminophenoxy)ethane-N,N,N’,N’-tetraacetic Acid Tetra-(acetoxymethyl) Ester]) and zVAD-fmk (carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone) were obtained from Calbiochem (Merck Chemicals Ltd., Nottingham, UK). DPX mountant, anti-β-actin, anti-tubulin, DAPI, diphenyleneiodonium (DPI), apocynin, N-acetyl L-cysteine (NAC) and anti-c-yes were purchased from Sigma (L’Isle-d’Abeau-Chesnes, France). Fura-2-PE3/AM and Fluo8-AM were from Tefflabs (Mundolsheim, France). Anti-human CD31 and D2-40 and H2FDA were from Invitrogen (Saint Aubin, France). Erlotinib, anti-CD95, anti-c-yes, anti-p22Phox and anti-nox4 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Cetuximab was obtained from the Centre Hospitalier de Rennes. Anti-caspase-8 (C15) was purchased from Axxora (Coger S.A., Paris, France). Anti-human CD95 mAb (DX2) and CD95L mAb (G247-4) were from BD Biosciences (Le Pont de Claix, France). Anti-human Orai1, Anti-Akt, anti-phosphoS473 Akt (Akt-\textsuperscript{P473}), anti-p110α, anti-p110β, anti-EGF-R, anti-phosphoY845 EGFR (EGFR\textsuperscript{Y845}) and anti-phospho(Y416) Src antibodies were from Cell Signaling Technology (Boston, MA, USA). Anti-nox1, anti-nox2, anti-nox3 and anti-nox5 were purchased from Abcam (Abcam, Paris, France).
**Statistical analysis.**

Comparisons between groups were done by Pearson Chi² test (or Fisher exact test if necessary) for qualitative parameters and by ANOVA or Student t test (Kruskal-Wallis or Mann-Whitney test if necessary) for continuous parameters. Metastasis-free survival was calculated from the date of the diagnosis to the date of the first metastasis or last follow-up if no metastatic relapse. Survival data were available for 142 patients. Survival curves were calculated by means of Kaplan-Meier method and groups were compared by means of log rank test. All analyses were done with Stata10.1 (Statacorp, Texas USA). All tests were two-sided with p significant < 5%.

**Cell lines and shRNAmir lentiviral transduction.**

All cell lines came from ATCC (LGC Standards, Molsheim, France) and were authenticated by Short Tandem Repeat. Human breast adenocarcinoma cells MDA-MB-231, MDA-MB-468 and Hs578T were maintained in DMEM supplemented with 8% v/v heat-inactivated FCS and 2 mM L-glutamine at 37°C in a 5% CO2 incubator. Silencing experiments were performed by lentiviral transduction of breast cancer cells using shRNAmir-pGIPZ vectors (OpenBiosystems, Waltham, MA, USA) for c-yes (RHS4430-98843955, -99161516, -98843955), Orai1 (RHS4430-101067842), p110α (RHS4430-200225949 and RHS4430-200225687), p110β (RHS4430-101074610 and RHS4430-101519729) EGF-R (RHS4430-99291735, RHS4430-98895495 and RHS4430-101517905), p22phox (RHS4430-98520051, RHS4430-98842095 and RHS4430-101071465), Nox-2 (RHS4430-99157340), Nox-3 (RHS4430-101097818 and RHS4430-98911480) or a scrambled shRNAmir vector as a negative control.
**Immunohistochemistry.**

Breast cancers and benign breast diseases were embedded in paraffin and cut into 3 μm sections. Protein cross-links formed by formalin fixation were reversed (Target Retrieval Solution) and endogenous peroxidase was blocked using 3% w/v hydrogen peroxide in methanol for 15 min. Slides were incubated in 5% BSA for 30 min at RT and then stained overnight at 4°C for CD95L, CD31 or D2-40. Tissue sections were incubated with Envision+ system HRP-conjugated secondary antibodies for 30 min at RT and labeling was visualized by adding liquid DAB+. Consecutive sections of 3μm from TNBC breast samples were used to evaluate CD95L expression in CD31 or D2-40 positive endothelial cells. Sections were counterstained (hematoxylin) and mounted with DPX mounting medium.

**Mouse experiments.**

NOD/SCID/γc null mice (NSG) were obtained from Dr C. Rivers (UK). All experiments were performed in agreement with the French Guidelines for animal handling and approved by local ethics committee. Luciferase-expressing MDA-MB-231 cells resuspended with or without cl-CD95L (100 ng/ml) (5×10⁵ cells in 50μL PBS/Matrigel) were transplanted into mammary fat pads of mice (7 weeks old female). Next, caudal vein injections of cl-CD95L (10 μg/kg) or control medium were repeated 5 days a week until day 34. Bioluminescence analysis was performed using PhotonIMAGER (Biospace Lab), following intraperitoneal injection of endotoxin-free luciferin (30mg/kg). Tumor volume was calculated using the formula \( V = 0.52 \times (L \times W^2) \). After completion of the analysis, autopsy of mice was done, and organ luminescence was assessed.

**CD95L ELISA.**
Anti-CD95L ELISA (Diaclone, Besançon, France) was performed to accurately quantify cleaved-CD95L levels in sera.

Flow cytometry analysis.

Cells were pre-incubated for 30 min at 37°C with 3 μM of H2FDA resuspended in PBS. The cells were then pre-incubated for an additional 30 min with or without ROS inhibitors before stimulation with 100 ng/ml cl-CD95L for the indicated times. Cells were then washed and analyzed by FACScalibur (BD Bioscience).

Immunoblot analysis.

Cells were lysed for 30 min at 4°C in lysis buffer [25 mM HEPES (pH 7.4), 1% v/v Triton X-100, 150 mM NaCl, and 2 mM EGTA supplemented with a mix of protease inhibitors (Sigma)]. Protein concentration was determined by the bicinchoninic acid method (Pierce, Rockford, IL, USA). Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked for 15 min with TBST (50 mM Tris, 160 mM NaCl, 0.05% v/v Tween 20, pH 7.8) containing 5% w/v dried skimmed milk and then incubated overnight with primary antibody at 4°C in TBSTM. The membrane was washed (TBST) and secondary HRP-labeled antibody (SouthernBiotech, US) was added for 45 min. The proteins were visualized with the enhanced chemiluminescence substrate kit (Pierce).

C-yes activation.

TNBC cells (10^7 cells) were pre-incubated for 30 min with 1 μM of DPI or 100 μM of Apocynin and then stimulated with 100 ng/ml cl-CD95L for the indicated times, washed and lysed. C-yes was immunoprecipitated using 1 μg of anti-c-yes and protein G-sepharose beads.
After extensive washing, the immune complex was resolved by SDS-PAGE and the phosphorylation status of c-yes at tyrosine 426 was assessed by western blot using anti-PhosphoSrc.

**Immunofluorescence imaging.**

Adherent cells were fixed for 15 min in PBS containing 4% w/v paraformaldehyde. The aldehyde groups were quenched for 10 min in PBS supplemented with 5% FCS. Cells were then incubated with 5 μg/ml anti-CD95 mAb (DX2) for 30 min at 4°C and CD95 was visualized using Alexa488-conjugated GAM antibody (Invitrogen) for 30 min at 4°C. EGFR was stained by incubating the cells with 2 μg/ml Alexa555-conjugated anti-EGFR mAb (Millipore) for 30 min at 4°C. Nuclei were stained with DRAQ5™ (Cell Signaling Technology). Cells were washed with PBS, dried, and mounted with Fluorescent Mounting Media (Dako, Carpinteria, USA). Images were acquired using a TSC SP5 confocal microscope with a 63x objective (Leica, Wetzlar, Germany).

**Ca\textsuperscript{2+} monitoring.**

For experiments on parent cell lines, breast cancer cells were loaded with Fura2-PE3-AM (1 μM) at room temperature for 30 min in Hank’s Balanced Salt Solution (HBSS). After washing with HBSS, the cells were incubated for 15 min in the absence of Fura2-AM to complete de-esterification of the dye. Cells were placed at 37°C and fluorescence micrograph images were captured at 510 nm with an inverted epifluorescence microscope (Olympus IX70) equipped with an x40 UApO/340–1.15W objective. To minimize UV light exposure, a 4×4 binning function was used. Fura2-AM was alternately excited at 340 and 380 nm, and the ratios of the resulting images (emission filter at 520 nm) were produced at constant intervals (10 seconds). The Fura-2 ratio (F\textsubscript{ratio} 340/380) images were analyzed offline with Metafluor.
and Metamorph. \( F_{\text{ratio}} \) reflects the intracellular \( \text{Ca}^{2+} \) concentration changes. Each experiment was repeated 3 times, and for each experimental condition, the average of more than 20 single-cell traces was used.

For experiments on GFP-expressing cell lines, Fluo8-AM was used, because GFP fluorescence disturbs \( \text{Ca}^{2+} \) measurement with Fura2-PE3. ShRNA-transduced cells (GFP expressing cells) were located by their emission of fluorescence at 530 nm for a light excitation at 485 nm. \( \text{Ca}^{2+} \) changes were evaluated by exciting Fluo8-AM-loaded cells at 535 nm. The values of the emitted fluorescence (605 nm) for each cell (F) were normalized to the starting fluorescence (\( F_0 \)) and reported as \( F/F_0 \) (relative \( \text{Ca}^{2+}_{\text{[CYT]}} \)). Only GFP-positive cells were considered. As for Fura2-PE3-AM, mammary cells were loaded with Fluo8-AM (1 \( \mu \text{M} \)) for 30 min in HBSS and then incubated for 15 min in the Fluo8-AM free HBSS to complete de-esterification of the dye.