Expanded View Figures

Figure EV1. *NTN1* and *DAPK1* are concomitantly altered in human breast cancers.

A Bisulfite PCR sequencing (4 CpGs analyzed, region: light gray boxes in Fig 1A) indicated that DNA methylation of the *NTN1* CpG island (CGI) was inversely correlated with its expression. Pearson correlation, \( P = 0.008, r = -0.55, n = 18 \).

B Bisulfite PCR sequencing (3 CpGs analyzed, region: light gray boxes in Fig 2A) indicated that DNA methylation of the *DAPK1* CGI was inversely correlated with its expression. Pearson correlation, \( P = 0.003, r = -0.66, n = 22 \).

C Tissue microarrays (70 paraffin embedded sections) from human breast carcinomas were immuno-stained with antibodies against *DAPK1*, UNC5B, and netrin-1. Samples were classified in quartiles according to the level of netrin-1 expression. The levels of *DAPK1* and UNC5B expression (index constructed from the percentage of sections exhibiting a positive staining) in the first and fourth quartiles of netrin-1 expressing groups were compared using a chi-squared test.

D Representative staining corresponding to low and high levels of expression is shown for each antibody, and expression levels were determined from the percentage of sections exhibiting a positive staining. As a control, staining of the samples using a non-related isotype antibody was performed.

E Quantification of the presence or absence of alterations in gene A was associated with the presence or absence of alterations in gene B in human breast tumors which was determined from cBioPortal web site, z-score threshold ± 2.2.

F Individual methylation plots. Red rectangles represent methylated CpG and blue rectangles unmethylated CpG.

G Mean DNA methylation inhibition of *DAPK1* and *NTN1* in MDA-MB-231 and HMLER cells upon DAC treatment.
**DNA methylation (%)**

**Gene** A  |  **Gene** B  |  Odds Ratio  |  P value  |  Association
---|---|---|---|---
**DAPK1**  |  **NTN1**  |  4.71  |  0.003  |  Tendency towards co-occurrence
**DAPK1**  |  **UNC5B**  |  < -3  |  0.351  |  Tendency towards mutual exclusivity
**UNC5B**  |  **NTN1**  |  < -3  |  0.606  |  Tendency towards mutual exclusivity

**Figure EV1.**
Figure EV2. The NTN1 DR apoptotic pathway, mechanism, and specificity.

A, B Other NTN1 DR gene expression in breast cell lines, and impact of DAPK1 and NTN1 on the induction of apoptotic cell death in vitro. Gene expression was measured by qRT–PCR after 72 h in MDA-MB-231 (A) and HMLER (B) cells treated daily with 10 μM decitabine (DAC). The level of PBGD expression was used as an internal control. Data are represented as mean ± s.e.m. for 3 independent experiments. **p < 0.0001, two-tailed unpaired Student’s t-test.

C Schematic representation of the cellular effect on HMLER cells of DAPK1 overexpression and/or recombinant netrin-1 treatment.

D Caspase-3 activation upon DAPK1 overexpression in HMLER and its reversion by the addition of netrin-1. ***p < 0.0001, one-way ANOVA.

E Schematic representation of DAC and effect of NTN1 siRNA on HMLER cells.

F Effect of NTN1 siRNA (siNTN1) on the expression of NTN2 by qRT–PCR. The level of PBGD expression was used as an internal control. ***p < 0.0001, one-way ANOVA.

G, H siNTN1 triggers apoptosis and cell death in hypomethylated HMLER cells. Cells were treated with DAC (10 μM, 72 h), and/or siNTN1 (30 pmol, 48 h), and caspase-3 activity (G) and cellular mortality (H) were measured. As a control, a scramble siRNA was used. Data (G, H) are expressed as mean ± s.e.m. of at least 3 independent experiments. ****p < 0.0001, one-way ANOVA.

I, J Representative images of TUNEL experiments shown in Fig 3, in MDA-MB-231 and HMLER cells, respectively. Scale bars = 50 μm.
Figure EV3. Netrin-1 neutralizing antibody net1-mAb triggers apoptosis in DAC-treated breast cancer cell lines.

A–E Gene expression was measured by qRT–PCR after 72 h in human breast cancer cell lines: MDA-MB-231 (A), HMLER (B) and SKBR3, MDA-MB-157, AU565, T47D, and BT20 (C–E) treated daily with DAC at final concentrations of 0.3, 1, 2, 5, and 10 μM. The level of PBGD expression was used as an internal control. Data are expressed as mean ± s.e.m. of at least 3 independent experiments. ****P < 0.0001, one-way ANOVA. Colors of the stars correspond to the gene analyzed (A and B) or to the cell lines analyzed (C–F).

F Comparison of cellular mortality in DAC-treated cells versus DAC + net1-mAb treated cells assessed using the caspase cleavage assay. Data are expressed as mean ± s.e.m. of at least 3 independent. ****P < 0.0001, two-way ANOVA and post hoc Tukey test. ns = not significant.
Figure EV4. Response of MDA-MB-231 cell lines stably transfected with shRNA targeting DAPK1, UNC5B, and NTN1 to treatments combining DAC and net1-mAb.

A Gene expression was measured by qRT–PCR after 72 h in cell lines stably transfected with shRNA. Cells were treated daily with 10 μM DAC. The level of PBGD expression was used as an internal control. Data are expressed as mean ± s.e.m. of at least 3 independent experiments. **** P < 0.0001, one-way ANOVA.

B–D Levels of DAPK1, UNC5B, and netrin-1 were measured by immunohistochemistry in paraffin-embedded xenografts of MDA-MB-231 stably transfected with shRNA. Left panels: levels of DAPK1 (B), UNC5B (C), and netrin-1 (D) in xenografts of MDA-MB-231 shDAPK1, shUNC5B, and shNTN1, respectively. Protein levels are expressed as the percentage of total tumor surface; P-values from at least 3 tumors per group. Middle panels: median number of apoptotic cells per mm²; values from at least 3 tumors per group. Error bars = s.e.m. Mann–Whitney test. ns = not significant. Right panels: representative sections of in vivo protein levels and induction of apoptosis in MDA-MB-231 tumor cells depleted of key genes from the netrin-1 dependence receptor pathway. Scale bars = 50 μm.