Supplemental Material

Legends to Supplemental Figures

Supplemental Figure 1. Down-regulation of TACE or presenilin-1 suppresses proliferation of MCF-7 transfectants expressing ErbB4 JM-a CYT-2. Proliferation of MCF-7 transfectants in the presence and absence of chemical (A) or genetic (B) inhibitors of TACE or γ-secretase was determined by counting cells with hemocytometer. (A) MCF-7 transfectants were treated for the indicated time periods with or without a metalloprotease inhibitor ilomastat (GM-6001; 20 μM; Calbiochem), in the presence or absence of a γ-secretase inhibitor GSI IX (GSI; 10 μM; Calbiochem). (B) SureSilencing Human ADAM17 siRNA Kit (SuperArray) and Presenilin-1 Duplex (Dharmacon) were used to down-regulate TACE (ADAM17) and presenilin-1 (PS-1), respectively, in MCF-7 transfectants expressing ErbB4 JM-a CYT-2. Control siRNA and introduction of double-stranded siRNAs into cells are described in "Materials and methods".

Supplemental Figure 2. Selection of cell background for analysis of ErbB4 isoform function in the absence of endogenous ErbB expression. Cell lines previously used for ErbB transfection experiments were analyzed for ErbB expression by Western blotting (A) and real-time quantitative RT-PCR (B). (A) Mesenchymal CHO and NIH 3T3-7d lines had a background of endogenous ErbB2 expression (lanes 1 and 2). In addition, CHO cells also expressed some endogenous ErbB4 (lane 1), and NIH 3T3-7d cells very low levels of ErbB1 and ErbB3 (only detected after long exposure; data not shown). In

contrast, neither of the two hematopoietic cell lines, myeloid 32D or lymphoid Ba/F3 cells, had detectable endogenous ErbB protein expression (lanes 3 and 4). NIH 3T3-7d cells transfected with a plasmid encoding ErbB1 were analyzed as a positive control for anti-ErbB1, and T-47D breast cancer cells as a positive control for anti-ErbB2, -ErbB3, and -ErbB4 Western, respectively (lane 5). (B) The lack of mRNAs for any of the ErbB4 isoforms in 32D cells was also confirmed by real-time RT-PCR. Total RNA was extracted from cell lines and mouse tissues using RNAzol B reagent (Tel-Test Inc.) and subjected to real-time RT-PCR analysis, as described in "Materials and methods". Mouse ErbB4 JM isoform expression was analyzed using primers 5'-TTGCCATCCAAACTGCACC-3' and 5'-TCCAATGACTCCGGCTGC-3'; and probes 5'-6-FAM-ATGGACGGGCCATTCCACTTTACCA-TAMRA-3' (specific for JM-a) and 5'-6-FAM-TTCAAGCATTGAAGACTGCATCGGCCT-TAMRA-3' (specific for JM-b). Mouse ErbB4 CYT isoform expression was analyzed using primers 5'-TCCTCCCATCTACACATCCAGAA-3' and 5'-GGCATTCCTTGTTGTGTAGCAA-3'; and probes 5'-6-FAM-TGAAATTGGACACAGCCCTCCTCCTG-TAMRA-3' (specific for CYT-1) and 5'-6-FAM-AATTGACTCCAATAGGAATCAGTTTGTGTACCAAGATG-TAMRA-3' (specific for CYT-2). Mouse kidney and heart tissues were analyzed as positive controls for JM-a or JM-b isoform expression, respectively (Elenius et al., 1997).

Supplemental Figure 3. Identity of the 160 kD ErbB4 protein. Western blot analyses with an anti-ErbB4 (sc-283) antibody are shown. (A) 32D transfectants expressing ErbB4 JM-a CYT-1 were cultured for 6 h in the presence or absence of an inhibitor of N-

glycosylation, tunicamycin (50 μg/ml; Sigma). The two ErbB4 bands of 160 kD and 180 kD were both reduced into a single 140 kD ErbB4 protein upon treatment indicating that both forms were differentially glycosylated variants of a single 140 kD core protein. (B) 32D transfectants expressing ErbB4 JM-a CYT-2 were cultured for 0, 1 or 4 h in the presence or absence of an inhibitor of protein synthesis, cycloheximide (10 μg/ml; Sigma). The 160 kD protein was almost totally depleted at 1 h time point whereas a significant amount of the 180 kD protein was still present after a 4 h block in protein synthesis.

Supplemental Figure 4. Similar shedding of ErbB4 ectodomain from both cleavable ErbB4 isoforms. 32D transfectants expressing ErbB4 JM-a CYT-1 (lanes 2 and 3), or JM-a CYT-2 (lanes 4 and 5), or a control clone transfected with an empty vector (lane 1) were cultured for 6 h in serum- and IL-3-free RPMI. The media were concentrated 50 fold with Centricon centrifugal filter devices (YM-50; Millipore), and samples from concentrated media were analyzed for the presence of 120 kD ErbB4 ectodomain by Western blotting using a monoclonal antibody recognizing the N-terminal part of ErbB4 (Ab1479; kindly provided by Dr. M. Sliwkowski, Genentech, South San Francisco, CA). The levels of 120 kD ErbB4 ectodomain accumulation in media (lower panel) were similar to levels of 180 kD ErbB4 in cell lysates (upper panel; analyzed by Western using sc-283 antibody) of respective clones, suggesting similar ectodomain shedding for both cleavable ErbB4 isoforms.

Supplemental Figure 5. TACE-like metalloproteinase activity is necessary for generation of 80 kD ErbB4. Western blot analyses with an anti-ErbB4 (sc-283) antibody are shown. (A) 32D cells expressing ErbB4 JM-a CYT-1 or ErbB4 JM-a CYT-2 were pretreated for 30 min with TAPI-0 prior to addition of ALLN for a further 3 h. (B) 32D cells expressing ErbB4 JM-a CYT-1 or ErbB4 JM-a CYT-2 were pretreated for 30 min with TAPI-0 or GSI IX prior to addition of PMA for a further 4 h.