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Supplemental Material to:

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Engineering multivalent antibodies to target heregulin-induced HER3 signaling in breast cancer cells

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SUPPLEMENTARY MATERIALS

Labeling of antibodies

Antibodies were labeled with either Alexa Fluor® 647 carboxylic acid, succinimidyl ester (Life Technologies, Catalog # A-20173) or Alexa Fluor® 488 carboxylic acid, succinimidyl ester (Life Technologies, Catalog # A-10235) using methods recommended by the manufacturer. Following the labeling reaction, antibodies were extensively dialyzed against PBS to remove unincorporated Alexa dye.

Flow cytometry

Cells were seeded at a density of 100,000 cells per well in 24 well plates, incubated overnight, and subsequently treated with either 1 μM lapatinib or vehicle control (DMSO) for 24 hours at 37°C in a CO_2 incubator. Treated cells were incubated with Alexa 647-labeled Ab6 and Alexa 488-labeled trastuzumab (50 nM each) for 15 minutes at 37°C in a CO_2 incubator. Following incubation, cells were trypsinized, washed, and suspended in PBS. Stained cells were analyzed using a BD FACScalibur and data processed using FlowJo (Tree Star).

Size exclusion chromatography

Following protein G-Sepharose purification, antibodies were analyzed by size exclusion chromatography using HPLC and a Hiload 16/600 Superdex 200pg column (GE Healthcare, catalog no. 28989335) or a Yarra 3U SEC-3000 (catalog no. 00H-4513-E0) column. TAb6, Ab6tet and Ab6 were also purified as homodimeric, bispecific antibodies using the Hiload 16/600 Superdex 200pg column. We observed that the purified,

homodimeric proteins had analogous activities to that of the material purified only on protein G-Sepharose in both MTS/proliferation and immunoblotting experiments (data not shown).

Serum stability studies

Antibodies were diluted into mouse serum (Thermo Scientific, catalog no. 31881) to a concentration of 40 μg/mL and incubated for 3 or 6 days at 37^{o}C. Samples were diluted in PBS and analyzed by sandwich ELISA. 96 well plates were coated with goat ~~species~~ anti-human Cκ antibody (Sigma-Aldrich, catalog no. K3502), blocked with 1% BSA/PBS and dilutions of serum samples in PBS added. Bound antibodies were detected using a goat anti-human IgG (Fc-specific) antibody conjugated to horseradish peroxidase (Sigma-Aldrich, catalog no. A0170).

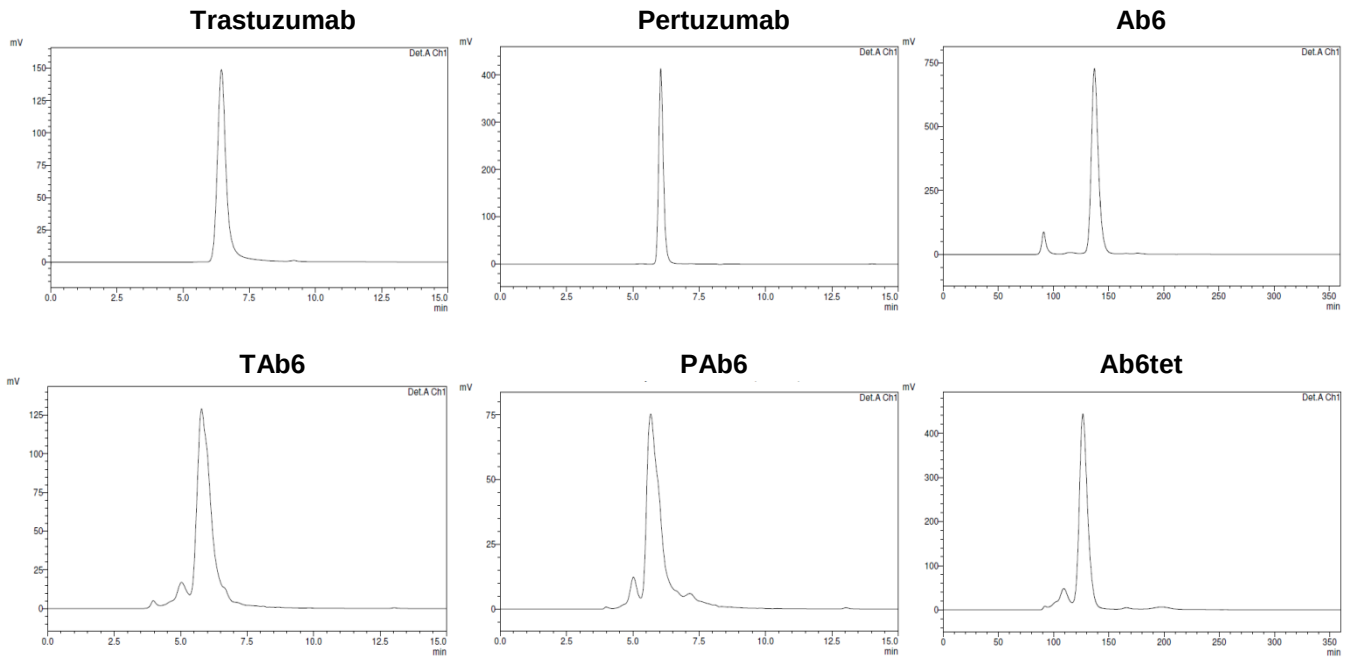

Figure S1: Analysis of trastuzumab, pertuzumab, Ab6, bispecific trastuzumab with anti-HER3 Ab6 scFv (TAb6), bispecific pertuzumab with anti-HER3 Ab6 scFv (PAb6), and tetrameric anti-HER3 (Ab6tet) using HPLC and size exclusion chromatography. Trastuzumab, pertuzumab, TAb6, and PAb6 were analyzed using a Yarra 3U SEC-3000 column, whereas a Hiload 16/600 Superdex 200pg column was used for Ab6 and Ab6tet. The major peak runs at the expected size for either an antibody homodimer (150 kDa) or bispecific homodimer (200 kDa).

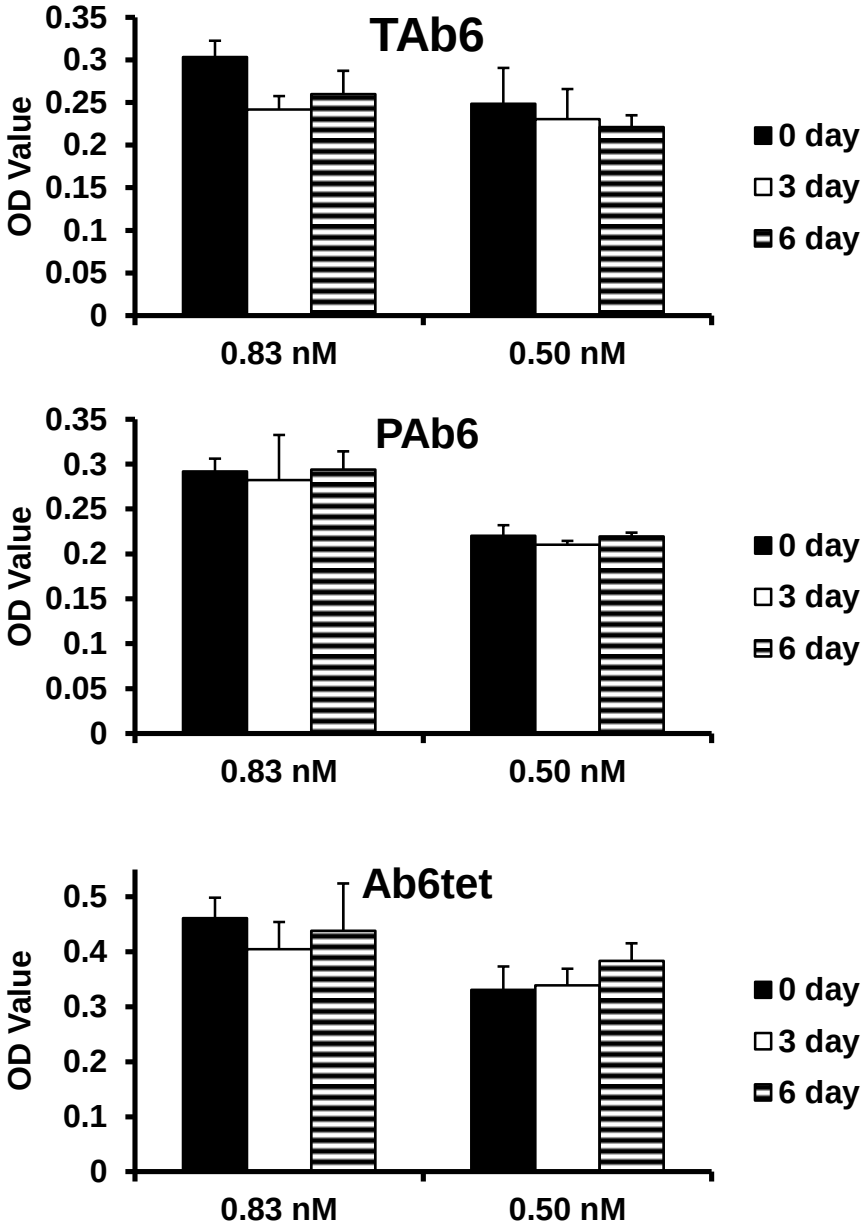

Figure S2: Serum stability analyses of the bispecific trastuzumab with anti-HER3 Ab6 scFv (TAb6), bispecific pertuzumab with anti-HER3 scFv (PAb6), and tetrameric anti-HER3 (Ab6tet). Antibodies were incubated in mouse serum for 0, 3, or 6 days at 37ºC, diluted into PBS to concentrations of 0.83 or 0.50 nM, and analyzed by sandwich ELISA. Differences were not significant for comparisons of 0 and 6 day samples (p value > 0.05; Student's *t*-test)

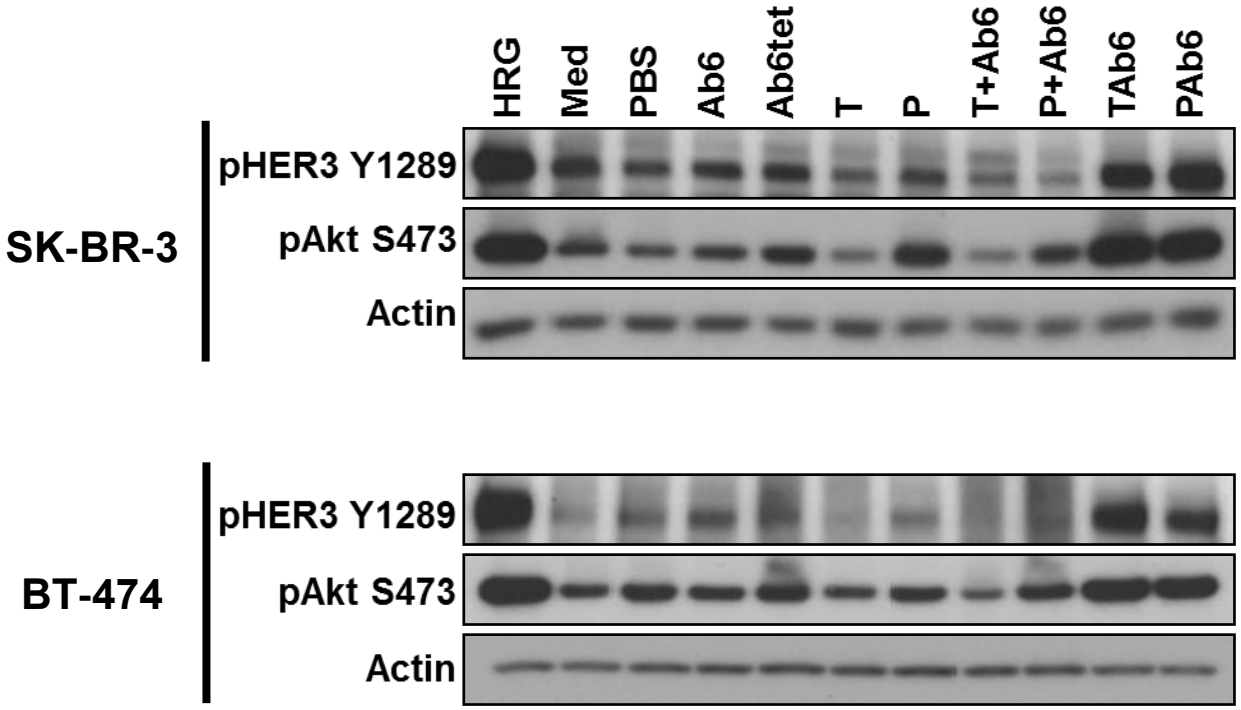

Figure S3: The bispecific anti-HER2/HER3 antibodies, TAb6 and PAb6, induce phosphorylation of Akt and HER3 within 15 minutes. SK-BR-3 and BT-474 cells were treated with 50 nM anti-HER3 (Ab6), tetrameric anti-HER3 (Ab6tet), trastuzumab (T), pertuzumab (P), trastuzumab and Ab6 (T + Ab6), pertuzumab and Ab6 (P + Ab6), bispecific trastuzumab with anti-HER3 Ab6 scFv (TAb6) or bispecific pertuzumab with anti-HER3 scFv (PAb6) for 15 minutes. Cell lysates were analyzed by immunoblotting.

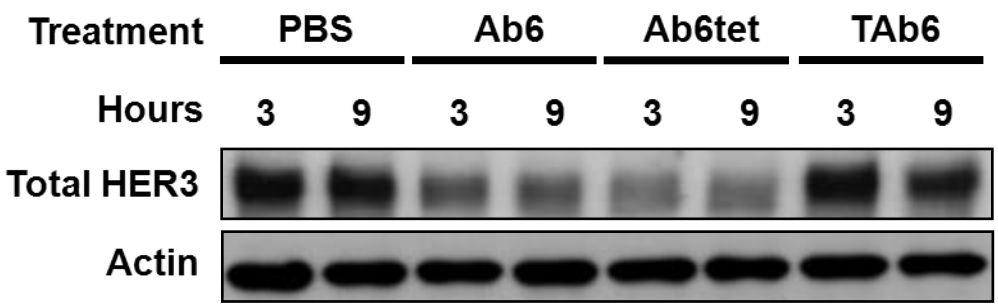

Figure S4: The multivalent anti-HER3 antibody, Ab6tet, induces higher levels of HER3 degradation compared with the bivalent counterpart, Ab6, and bispecific TAb6. SK-BR-3 cells were treated with 50 nM anti-HER3 antibody (Ab6), Ab6tet, bispecific trastuzumab with anti-HER3 Ab6 scFv (TAb6), or PBS vehicle for 3 or 9 hours. Cell lysates were analyzed for total HER3 using immunoblotting.

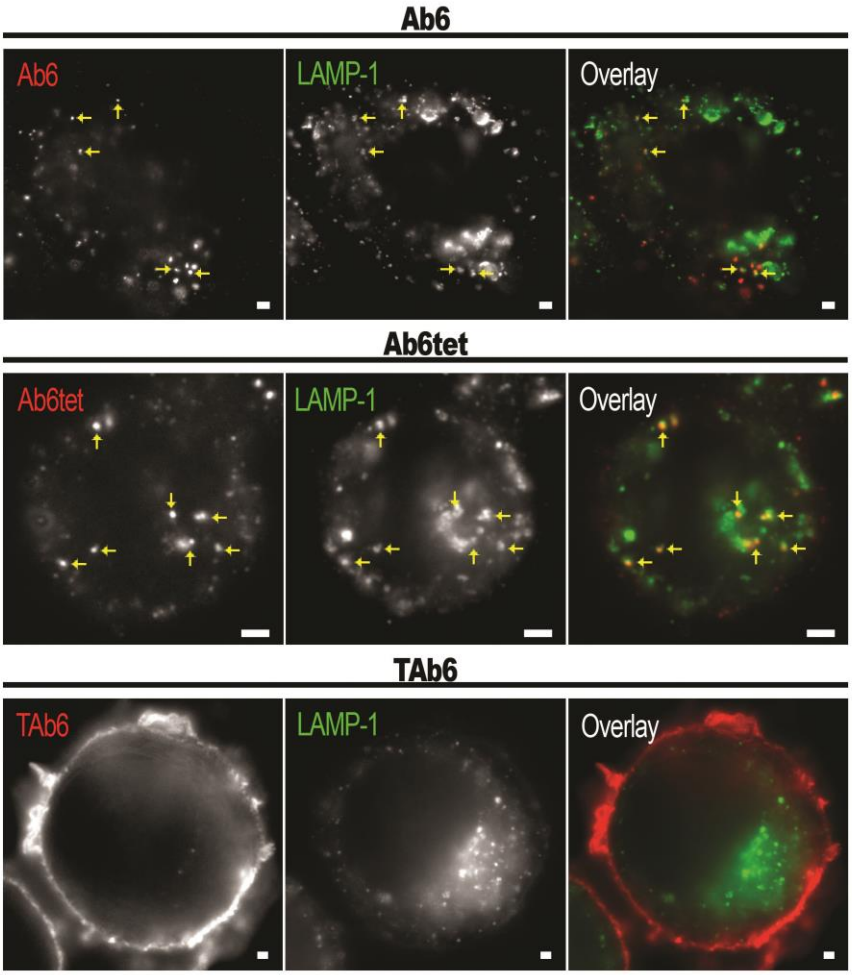

Figure S5: The bivalent anti-HER3 antibody, Ab6, and its multivalent counterpart, Ab6tet, traffic into LAMP-1 positive endosomal structures within one hour of treatment. SK-BR-3 cells were treated with 50 nM Ab6, Ab6tet, or bispecific trastuzumab with anti-HER3 scFv Ab6 (TAb6) for 15 minutes at 37°C and chased in medium for 45 minutes at 37°C. Cells were fixed, permeabilized and stained for LAMP-1. Anti-HER3 or HER2/HER3 antibodies were detected with Alexa 555-labeled secondary antibody (pseudocolored red in overlay) and anti-LAMP-1 antibody with Alexa 488-labeled secondary antibody (pseudocolored green in overlay). Examples of co-localization of antibody and LAMP-1+ compartments are indicated by yellow arrows. Scale bars = 2 μm.

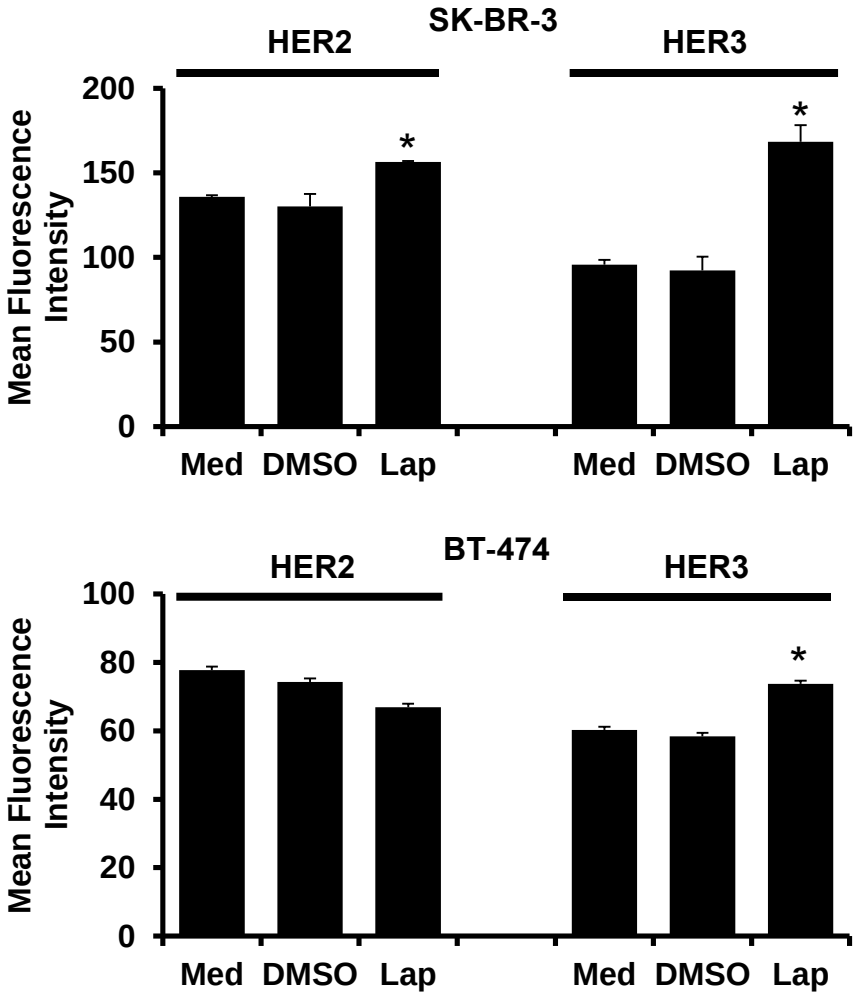

Figure S6: Lapatinib treatment induces upregulation of HER2 and HER3 (SK-BR-3) or HER3 (BT-474) levels on the plasma membrane of breast cancer cells. Cells were treated with 1 μM lapatinib (Lap), medium (Med) or vehicle control (DMSO) for 24 hours. Cell surface HER2 or HER3 was detected by incubation with 50 nM Alexa 488-labeled trastuzumab or Alexa 647-labeled Ab6 followed by flow cytometric analyses. Data shown represent means of mean fluorescence intensities ± standard deviation of triplicate samples following subtraction of background fluorescence intensities. * indicates statistically significant differences between lapatinib and DMSO (vehicle) treated cells ($p < 0.005$; Student's *t*-test). Data shown are representative of at least two independent experiments.

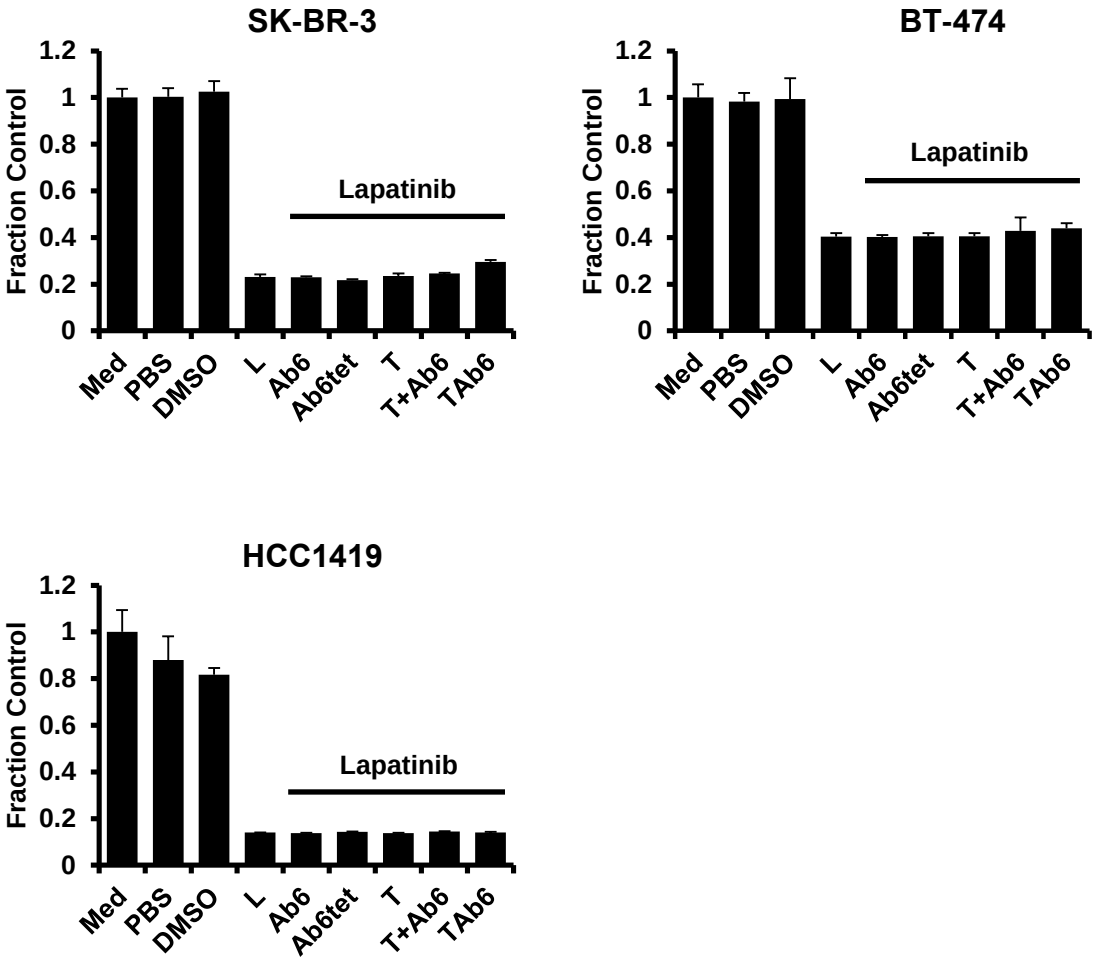

Figure S7: Antibodies specific for HER2 and/or HER3 do not increase the anti-proliferative effect of lapatinib. SK-BR-3, BT-474, and HCC1419 cells were treated 1 μM lapatinib (L) alone or lapatinib in combination with 50 nM anti-HER3 (Ab6), tetrameric anti-HER3 (Ab6tet), trastuzumab (T), trastuzumab and Ab6 (T + Ab6) or bispecific trastuzumab with anti-HER3 Ab6 scFv (TAb6) for 5 days. Proliferative responses were assessed using the MTS reagent and were normalized against the proliferation of cells incubated in medium (Med) only. Data shown are means of triplicates ± standard deviation and are representative of at least two independent experiments.

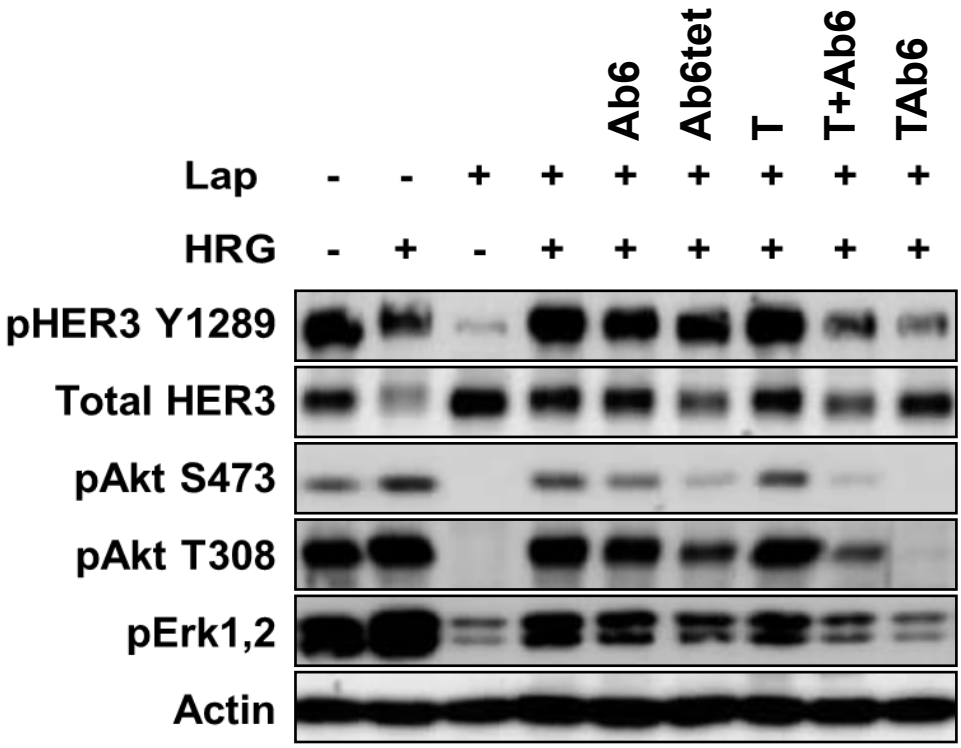

Figure S8: The bispecific anti-HER2/HER3 antibody, TAb6, has the highest activity in reducing PI3K/Akt signaling in HCC1419 cells in the presence of heregulin and lapatinib. Cells were treated for 24 hours with 1 μM lapatinib (Lap), 6.25 nM heregulin (HRG) and 50 nM anti-HER3 (Ab6), tetrameric anti-HER3 (Ab6tet), trastuzumab (T), trastuzumab and Ab6 (T + Ab6) or bispecific trastuzumab with anti-HER3 Ab6 scFv (TAb6) as indicated. Cell lysates were analyzed by immunoblotting.

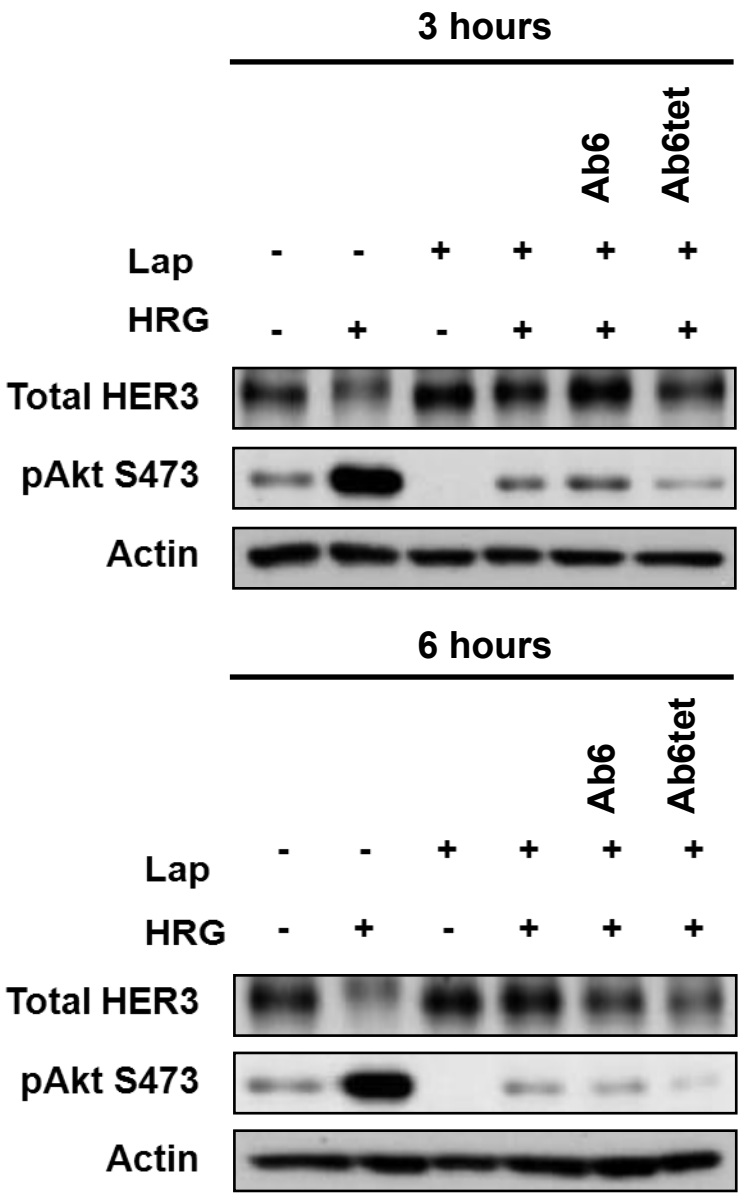

Figure S9: The multivalent anti-HER3 antibody, Ab6tet, induces higher levels of HER3 degradation compared with the bivalent counterpart, Ab6, in the presence of heregulin (HRG) and lapatinib (Lap). SK-BR-3 cells were treated as indicated with combinations of 1 μM lapatinib, 6.25 nM heregulin, 50 nM anti-HER3 (Ab6) or tetrameric anti-HER3 (Ab6tet) for 3 or 6 hours. Cell lysates were analyzed for total HER3 and pAkt (S473) by immunoblotting.