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Supplemental Material to:

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The level of HER2 expression is a predictor of antibody-HER2 trafficking behavior in cancer cells

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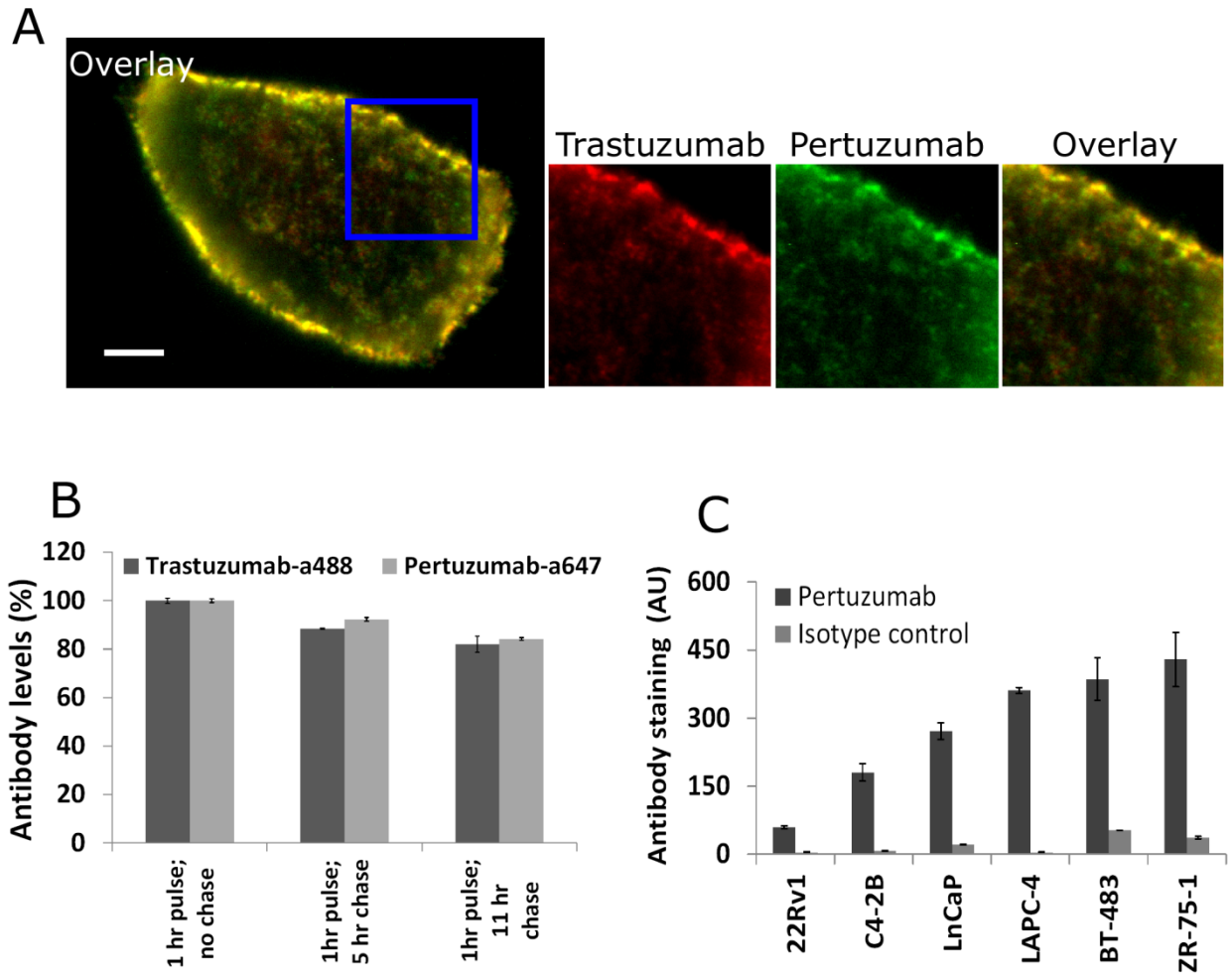
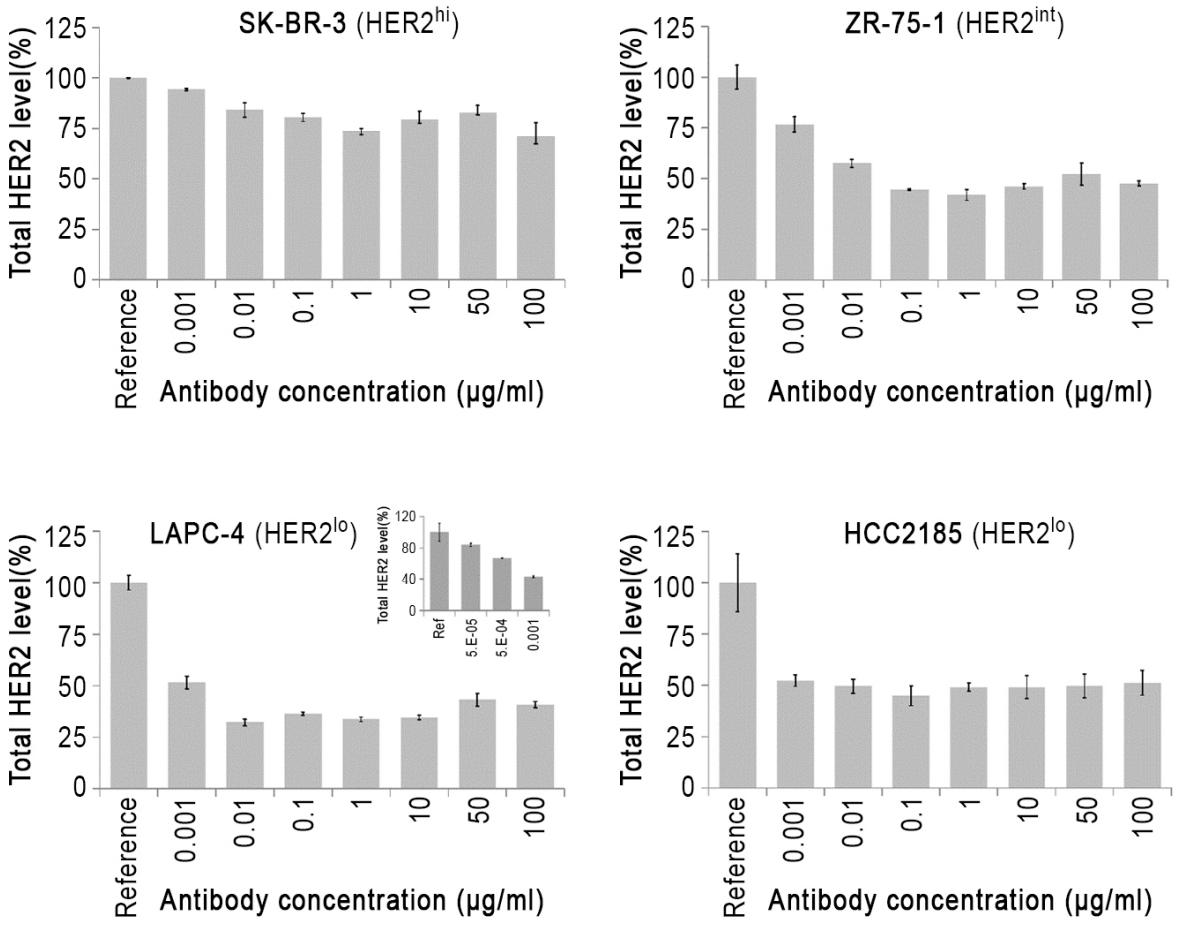


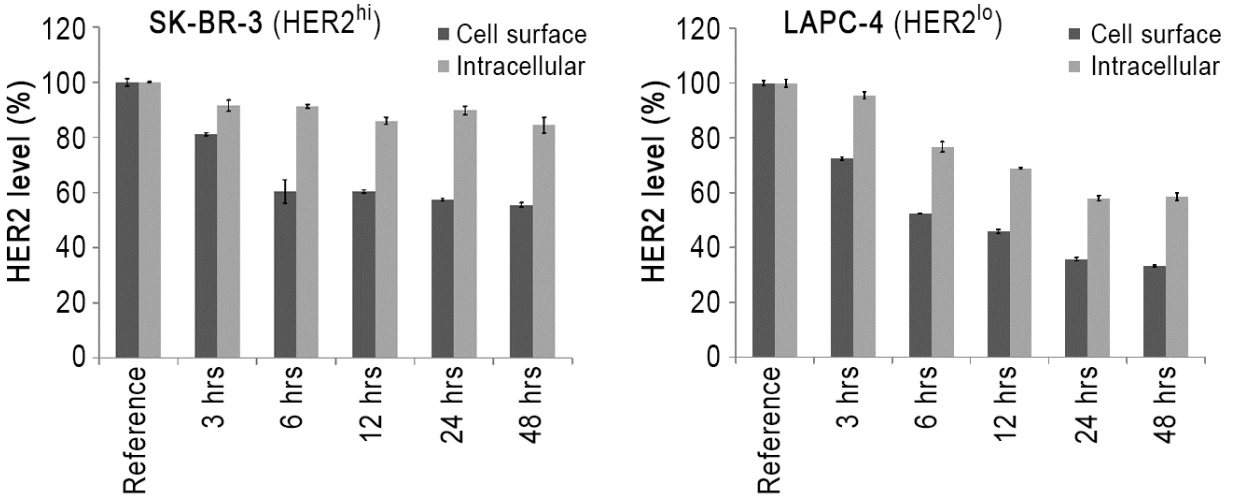
Supplementary Figure 1: Validation of the use of trastuzumab and pertuzumab for staining HER2 in cells. **A,** ZR-75-1 cells were pulsed with 10 μg/ml Alexa 555-labeled trastuzumab for 15 minutes at 37°C. The cells were then washed, fixed with 1.7% PFA for 15 minutes at 37°C, permeabilized with 0.025% saponin for 10 minutes at room temperature and stained with 10 μg/ml Alexa 647-labeled pertuzumab for 25 minutes at 37°C. Similar results were also obtained when cells were pulsed with Alexa 488-labeled pertuzumab and then stained with Alexa 647-labeled trastuzumab-Alexa647 (data not

shown). Scale bar = 10 μm. **B,** HCC1954 cells were pulsed with 10 μg/ml Alexa 488-labeled trastuzumab (trastuzumab-a488) for 1 hour and chased for different times. The cells were harvested, fixed, permeabilized and total HER2 levels detected by staining with Alexa 647-labeled pertuzumab (pertuzumab-a647). The simultaneous reduction in Alexa 647-labeled pertuzumab and Alexa 488-labeled trastuzumab signals indicate that the change in trastuzumab levels is also observed for pertuzumab levels. This provides additional support for the use of pertuzumab staining to detect HER2 levels in cells pretreated with trastuzumab. Error bars indicate S.D. The experiments are representative of at least two independent experiments. **C,** Cells were fixed, and stained with 5 μg/ml pertuzumab or 5 μg/ml human anti-HEL antibody (isotype control), then counter-stained with Alexa 647-labeled anti-human IgG and analyzed using flow cytometry. The consistently low signal observed with isotype control when compared to pertuzumab, especially in the $HER2^{lo}$ cell lines, demonstrates that pertuzumab staining is HER2-specific.

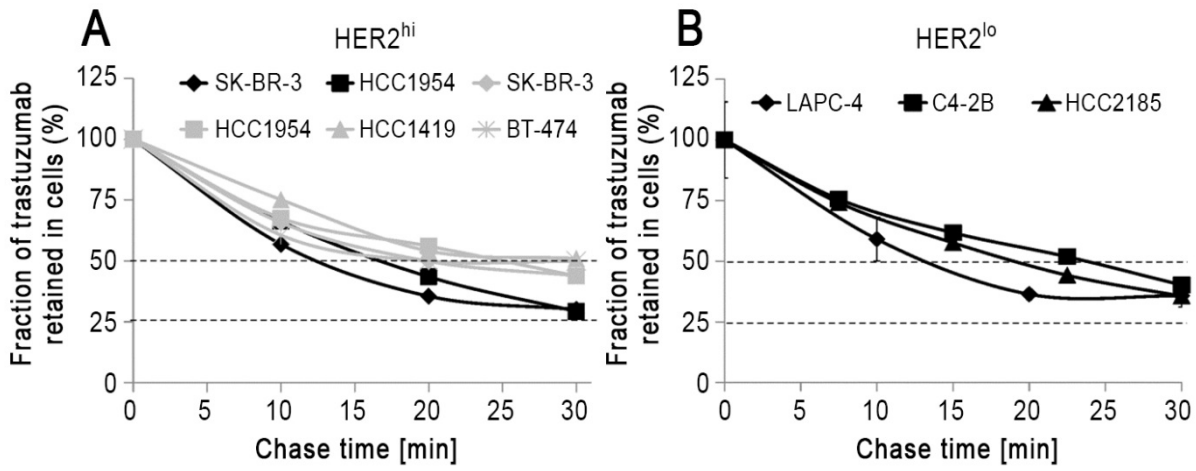


Supplementary Figure 2: **Effect of trastuzumab concentration on HER2 levels in cells**. Cells were treated with different concentrations of trastuzumab for 48 hours, harvested and total HER2 levels determined by flow cytometry. For each cell line, the HER2 level in untreated cells is labeled as reference. The HER2 level for all of the time points is normalized with respect to reference and is expressed as a percentage. Error bars indicate S.D. The experiments are representative of at least two independent experiments. The percentage reductions in HER2 levels are relatively constant for a wide range of antibody concentrations. For instance, for the SK-BR-3 cell line, 1-100

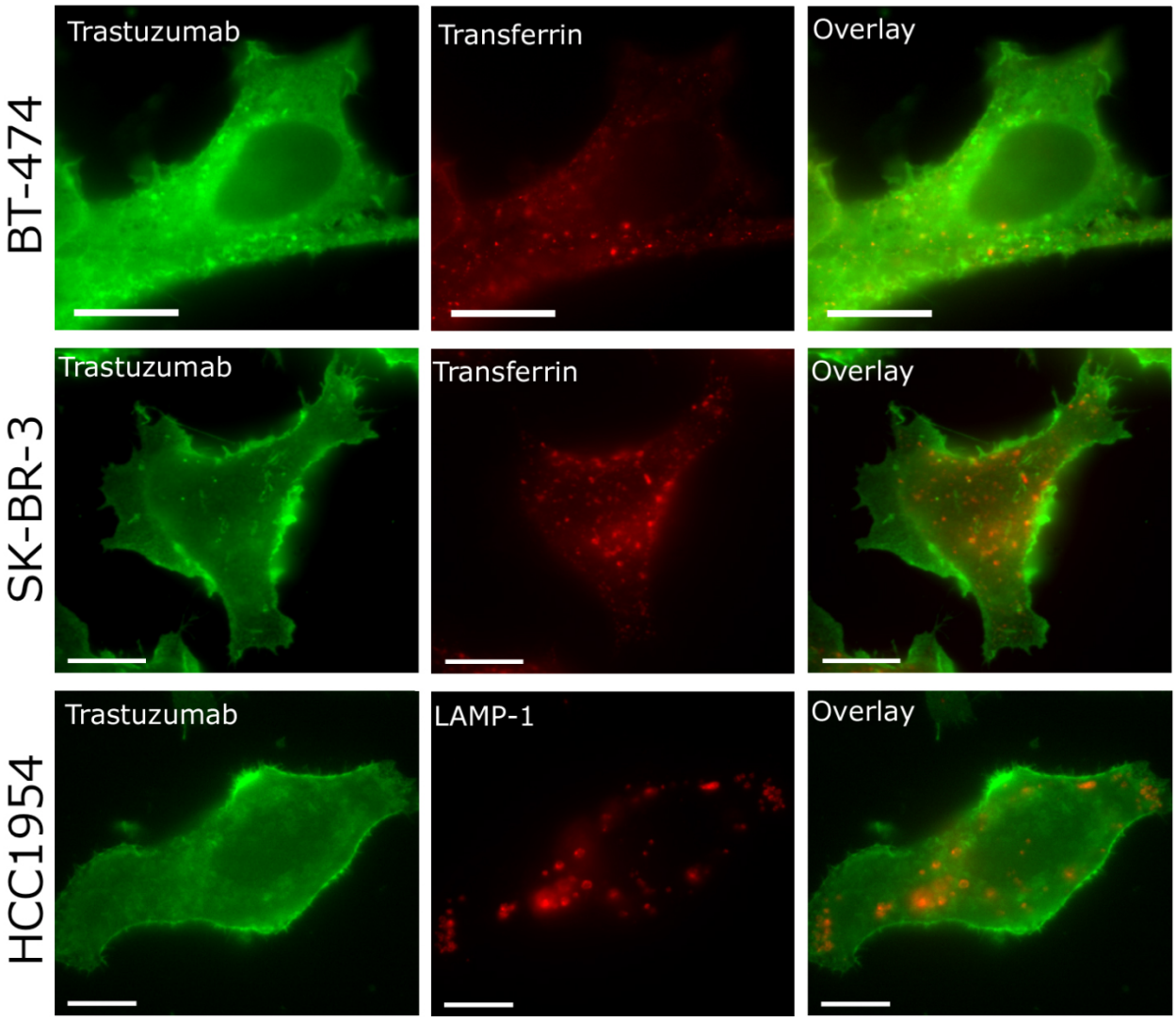
μg/ml trastuzumab results in similar reductions in HER2 levels. A concentration of 1 μg/ml of trastuzumab will pertain to a total of ~$4x10^{12}$ IgG molecules or ~$8x10^{12}$ HER2 binding sites (for 1 ml of antibody). In SK-BR-3 cells, this could result in saturable binding of all the HER2 receptors during continuous incubation with antibody given estimates of ~$2x10^{6}$ HER2 molecules per SK-BR-3 cell[1-3] and 10^{5} cells per ml of incubation medium. In the case of ZR-75-1, the trastuzumab dose response curve plateaus at 0.1 μg/ml, consistent with the ~10 fold lower level of HER2 expression in this cell line compared with SK-BR-3. In the $HER2^{lo}$ cell lines, the maximal reduction of HER2 levels occurs at an even lower antibody concentration, consistent with their correspondingly lower HER2 expression levels compared with ZR-75-1 cells.



Supplementary Figure 3: Effect of trastuzumab treatment on cell surface and intracellular HER2 levels. Cells were treated with 15 μg/ml trastuzumab for the indicated times. At the end of each time point the cells were harvested, and cell surface and intracellular HER2 levels were determined by flow cytometry. For each cell line, the HER2 level in untreated cells is labeled as reference. The HER2 level for all of the time points is normalized with respect to the reference and expressed as a percentage. Error bars indicate S.D. The experiments are representative of at least two independent experiments.

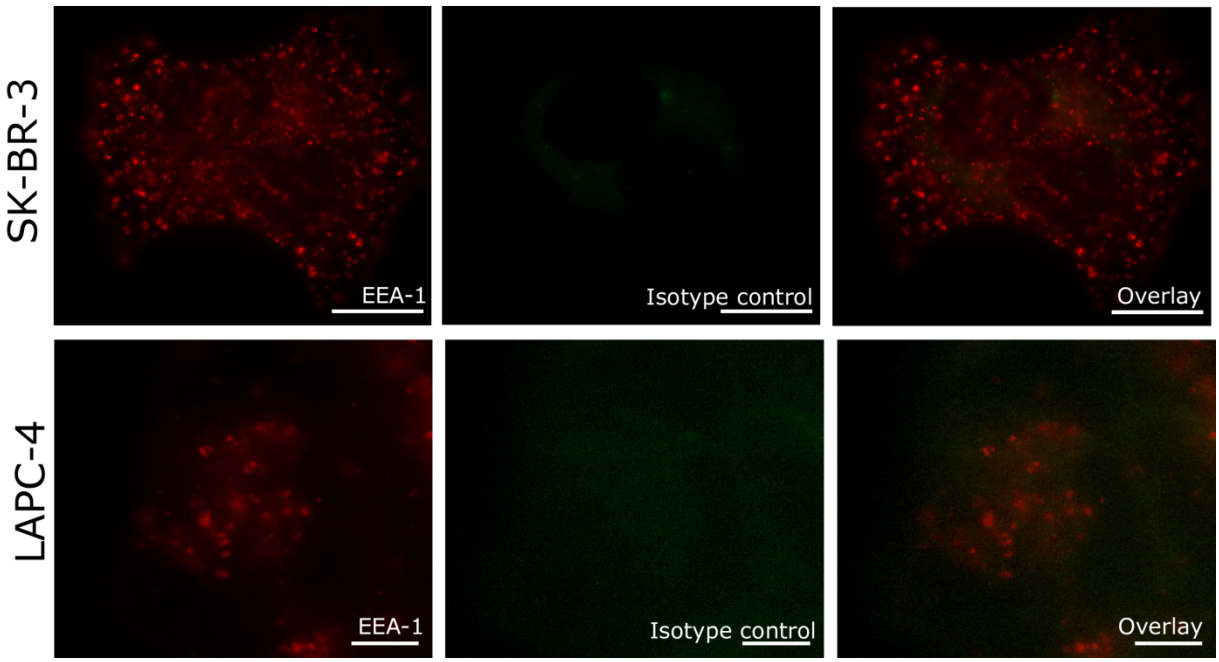


Supplementary Figure 4: Recycling behavior of transferrin. **A**, transferrin recycling in $HER2^{hi}$ cells in the presence (black) or absence (grey) of unlabeled transferrin during the chase phase. A higher fraction of transferrin is retained in cells in the absence of unlabeled transferrin. **B**, transferrin recycling in $HER2^{lo}$ cells in the presence of unlabeled transferrin during the chase phase. A and B correspond to Figures 2A and 2B, respectively. Error bars indicate S.D. The experiments are representative of at least two independent experiments.

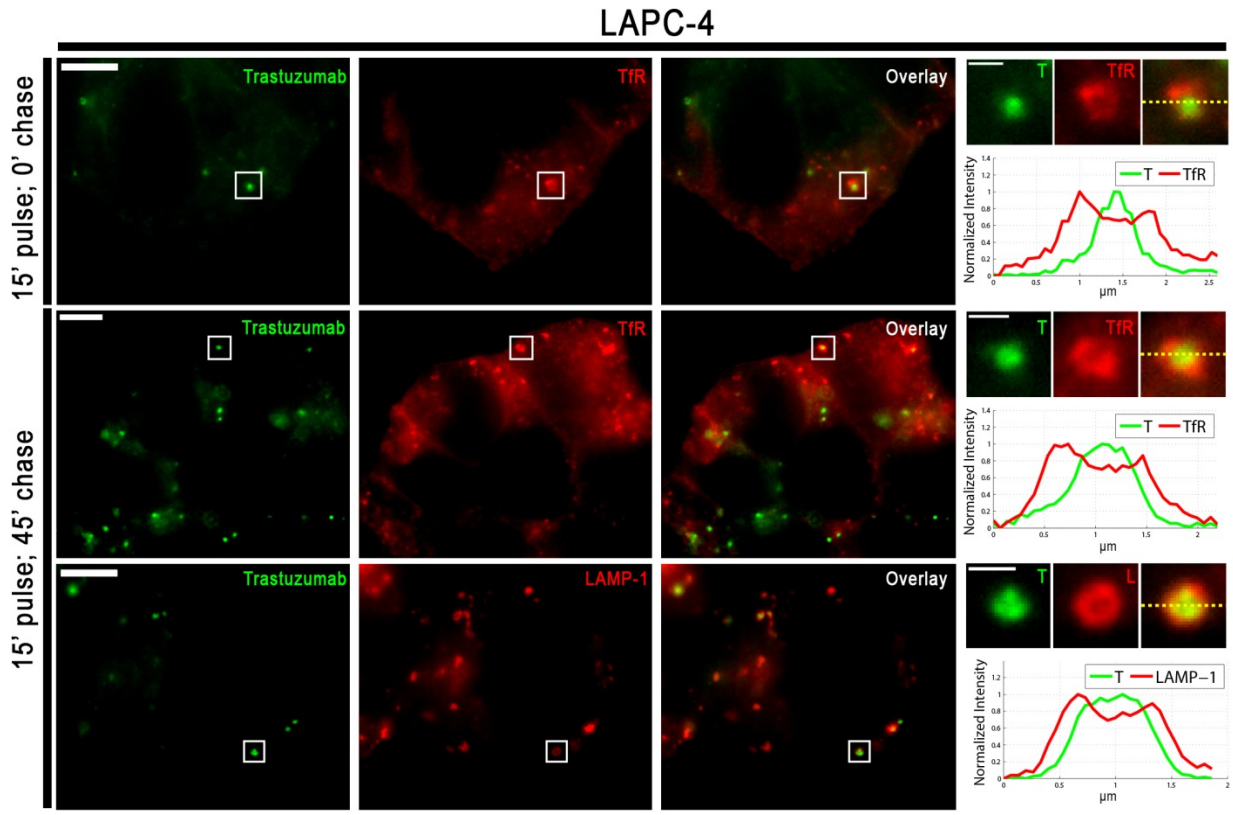


Supplementary Figure 5: Effect of HER2 overexpression on trastuzumab signal. A robust trastuzumab signal from the cell surface of $HER2^{hi}$ cells arises due to HER2 overexpression. Cells were pulsed with 2-10 μg/ml Alexa 488-labeled labeled trastuzumab either alone (HCC1954) or in combination with 5 μg/ml Alexa 555-labeled labeled transferrin (SK-BR-3 and BT-474) for 15-30 minutes at 37°C and then fixed. HCC1954 cells were permeabilized and stained for LAMP-1 as described in the Materials and Methods. Bound anti-LAMP-1 antibody was detected using an Alexa 555-

labeled secondary antibody. The trastuzumab on the plasma membrane dominates the total signal in the Alexa 488 channel and obscures the trastuzumab signal within intracellular compartments. In all panels, trastuzumab is pseudocolored in green and transferrin or LAMP-1 is pseudocolored in red. Scale bar = 10 μm.



Supplementary Figure 6. Non-specific uptake of Alexa 488-labeled human IgG1. Cells were pulsed with 5 μg/ml Alexa 488-labeled (SK-BR-3) or 10 μg/ml Alexa 555-labeled (LAPC-4) human anti-hen egg lysozyme antibody (human IgG1 isotype control) for 15 minutes at 37°C, fixed, permeabilized and stained for EEA-1. Cells were imaged using identical imaging conditions to those shown in Figures 4 and 5. The undetectable signal level in the isotype control channel indicates that the non-specific uptake of human IgG is immeasurably low under the conditions of the experiment. Scale bars = 10 μm.



Supplementary Figure 7. Impact of anti-Alexa 488 quenching antibody on trastuzumab localization in LAPC-4 cells. Cells were treated as in Figure 5, except that anti-Alexa 488 antibody was used to quench the signal from Alexa 488-labeled trastuzumab on the plasma membrane. Boxed regions for each set of images are presented on the right hand side of each row as expanded images of individual endosomes/lysosomes. These individual endosomes/lysosomes are shown for trastuzumab (T), transferrin receptor (TfR) or LAMP-1 (L). Normalized fluorescence intensities along the dotted lines in the overlays are presented in the fluorescence intensity plots. In all panels, trastuzumab is pseudocolored green and endosomal staining (TfR or LAMP-1) is pseudocolored red. Scale bars represent 5 μm (large panels) or 1 μm (expanded regions of interest).

References

1. Yang S, Raymond-Stintz MA, Ying W, Zhang J, Lidke DS, Steinberg SL, Williams L, Oliver JM, Wilson BS. Mapping ErbB receptors on breast cancer cell membranes during signal transduction. J Cell Sci 2007; 120:2763–73.

2. DeFazio-Eli L, Strommen K, Dao-Pick T, Parry G, Goodman L, Winslow J. Quantitative assays for the measurement of HER1-HER2 heterodimerization and phosphorylation in cell lines and breast tumors: applications for diagnostics and targeted drug mechanism of action. Breast Cancer Res 2011; 13:R44.

3. Costantini DL, Bateman K, McLarty K, Vallis KA, Reilly RM. Trastuzumab-resistant breast cancer cells remain sensitive to the auger electron-emitting radiotherapeutic agent 111In-NLS-trastuzumab and are radiosensitized by methotrexate. J Nucl Med 2008 ; 49:1498–505.