Supporting Information

Gaborit et al. 10.1073/pnas.1423645112

SI Materials and Methods

Cell Lines. The human pancreatic carcinoma (BXPC3), ovarian carcinoma (OVCAR-5), breast cancer (T47D, SKBR-3, and MCF-7), gastric cancer (NCI-N87), head and neck cancer (CAL-27), and lung cancer lines (A549, NCI-H1935, NCI-H322M) were from ATCC. The NIH/3T3-R1, -R2, -R2R3, -R3, -R1R4, and BXPC3-Luc cells were provided by C. Larbouret (Institut de Recherche en Cancérologie de Montpellier, INSERM-U896). Ovarian and lung cancer cells were cultured in RPMI 1640 medium. Other cells were cultured in DMEM. Media were supplemented as recommended by ATCC, usually with 10% (vol/vol) FCS (Life Technologies).

Abs and Reagents. Abs XC252 and XC90 were generated in our laboratory (1). Trastuzumab was from Genentech Inc. For Western blotting, anti-HER3, anti-AKT and anti-ERK were from Santa Cruz Biotechnology; anti-pAKT and -pHER3 Abs were from Cell Signaling Technology. Abs against pERK were provided by Rony Seger (Weizmann Institute). The secondary Abs used for isotyping were purchased from SouthernBiotech. NRG labeled with the d2 dye was provided by CisBio. Unless indicated, all other materials were from Sigma. The mouse polyclonal IgG used as control was from MP Biomedicals.

Production of IgB3. To produce IgB3 (a recombinant HER3's extracellular domain fused to a human IgG Fc domain), the cDNA sequence was derived from the already-made plasmid pCDM7-IgB3 (1). We cloned the IgB3 cDNA sequence into the pENTR/D-TOPO vector, before processing to recombination into the pLenti6/V5-DEST vector (Invitrogen), following the vendor's recommendation. The stop codon was maintained to avoid the V5-Tag. HEK-293FT cells were cotransfected by using Jet-PEI (Polyplus) with the pLenti6-IgB3 vector and the ViraPowerTM Packaging Mix (Invitrogen). After 3 d of production, the supernatant containing the lentiviral particles was used to infect HEK-293 cells. A stable cell line was established out of the infected cells by further selection with Blasticidine (10 μg/mL). The HEK-293/IgB3 cells were maintained in DMEM-1% FCS for 6 d at 32 °C. The supernatant was then loaded on an Agarose-Protein G column (2 mL). After intensive washes with PBS, the IgB3 protein was eluted from the column with 0.1 M glycine buffer (pH 2.7). The more concentrated fractions were pooled and dialyzed for 24 h against PBS.

Flow Cytometry. NIH/3T3-R2R3 cells were treated with trypsin and washed twice in PBS containing BSA (weight/vol). The cells were then incubated for 1 h at 4 °C with the mAbs directed to EGFR, HER2, HER3, or HER4 (10 μg/mL). After two washes, the cells were incubated for 1 h at 1 °C with an anti-mouse Ab coupled to Alexa Fluor 488. The capacity of the anti-HER mAbs to bind with the indicated HER protein at the cell surface was correlated with the fluorescence intensity measured by using the LSRII flow cytometer.

Competition Assays. NG33 and XC252 were labeled with Lumi4 Tb cryptate (K2) and NRG with the d2 dye. Ab competition assays were performed in 96-well black plates coated with IgB3 (1.5 μg/mL). After blocking with PBS/BSA, plates were incubated for 1 h with various concentrations of competitors under gentle shaking at room temperature. The labeled mAb, NG33-K2 or XC252-K2, was then added at 1 nM final concentration. After 1 h of incubation, the plate was washed four times by using KREBS buffer (146 mM NaCl, 4 mM KCl, 0.5 mM MgCl2, 1 mM CaCl2, 10 mM Hepes, 1 g/L glucose, and 1 mg/mL BSA, pH 7.4), and the fluorescent intensity (excitation, 340 nm; emission, 610 nm) was measured by using a Pherastar FS reader. NRG competition assays were performed by using NIH/3T3-R2R3 cells plated in 96-well black plates (50,000 cells per well) and incubated for 24 h in full medium. After overnight serum starvation, the cells were washed with KREBS buffer (100 μL) and incubated with increasing concentrations of competitors for 45 min at 4 °C. After the addition of NRG-d2 (10 nM), the cells were incubated for an additional 45 min at 4 °C. Finally, after two washes with KREBS buffer, the fluorescence intensity was measured at 670 nm (excitation: 620 nm).

Cell Proliferation Assays. Cells were plated on 96-well plates (5,000 cells per well) in triplicates. After 1 d, the cells were treated as indicated in starvation medium supplemented with NRG (1 or 10 ng/mL). After 3 d, the MTT reagent was added to the cells, and 1 h later, the formazan crystals were dissolved in SDS/dimethylformamide solution for 30 min. Absorbance was measured at 570 nm.

Migration Assays. OVCAR-5 cells (1 × 10E6) were seeded in the upper compartment of migration chambers (Transwell; Corning). The lower compartment was filled with medium supplemented with NRG (10 ng/mL) in the absence or presence of mAb NG33 (10 μg/mL). After 24 h, cells that reached the lower side of the filter were fixed, permeabilized with Triton X-100 (0.1%), and stained with a Giemsa solution. Images were quantified by using ImageJ.

Data and Statistical Analysis. FACS data were analyzed by using the FlowJo software. The other data were represented by using the Prism GraphPad software, and statistical analysis was performed by using this same software.

1. Chen X, et al. (1996) An immunological approach reveals biological differences between the two NDF/heregulin receptors, ErbB-3 and ErbB-4. *J Biol Chem* 271(13):7620–7629.

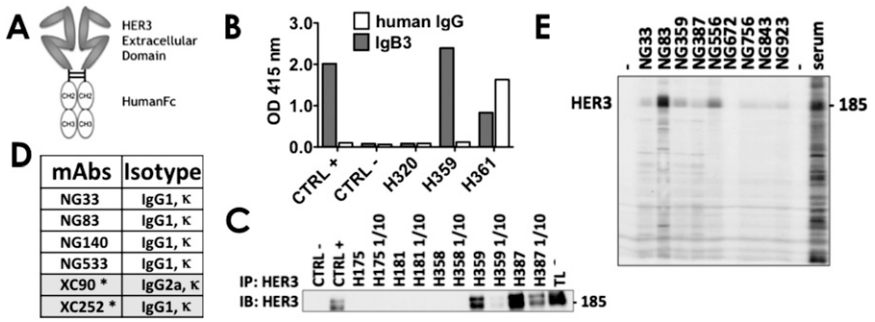


Fig. S1. Hybridoma screening and mAb isotyping. (*A*) Mice were immunized with recombinant IgB3. (*B*) The hybridoma supernatant screening using ELISA was performed on a 96-well plate coated with IgB3 (1 μg/mL) or with a human IgG. The plates were blocked with PBS-1% BSA and incubated for 1 h with hybridoma supernatants, followed by a second incubation for 1 h with HRP-labeled anti-mouse IgG and subsequently detected by 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) addition. The OD at 415 nm was then measured by using an ELISA microplate reader. (*C*) The second step of the screening was performed by immunoprecipitation (IP). Anti-mouse IgG beads were incubated first with the hybridoma supernatant and subsequently with total cell lysate from HER3-expressing T47D cells. (*D*) The mAbs directed to HER3 were isotyped by using ELISA. The 96-well plates were coated with IgB3 (1 μg/mL) and, after blocking, incubated with the indicated mAbs for 1 h. After washing, the plates were incubated for 1 h with various secondary HRP-coupled Abs able to bind specifically, IgG1, IgG2a, IgG2b, IgG3, IgM, IgA, Kappa chain, or Lambda chain. The detection was performed as shown in *B*. (*E*) Finally, the ability of the mAbs to detect HER3, used as primary Ab in a Western blot experiment, was determined on cell lysate from T47D cells.

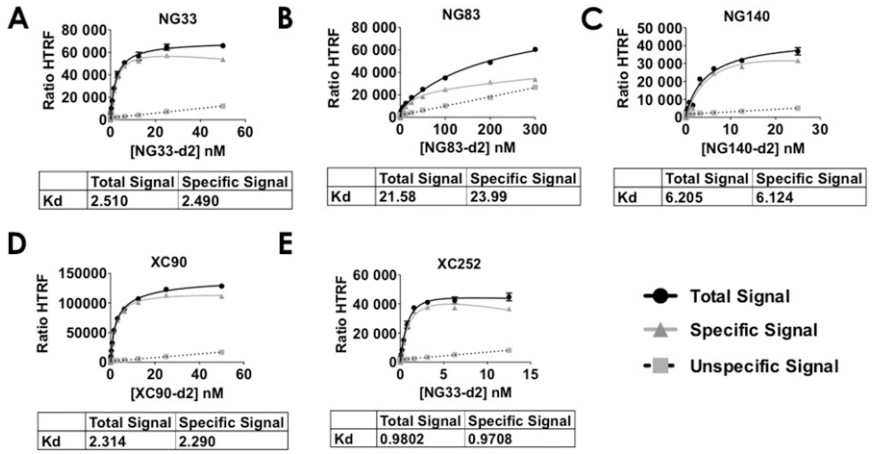
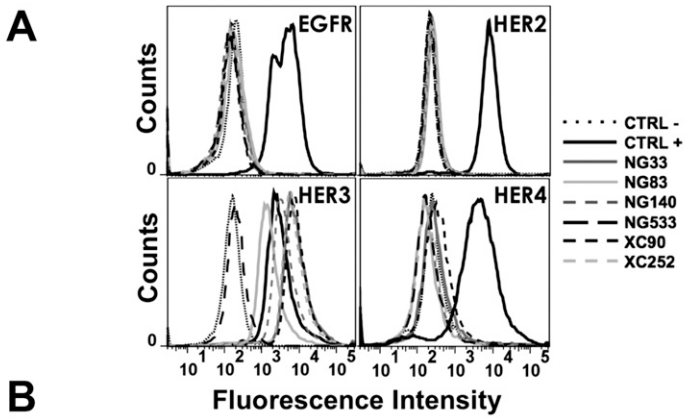


Fig. S2. K_d determination of mAb to HER3 using the Tag-Lite technology (*A*–*C*) of the newly generated mAbs and (*D* and *E*) of the previously generated mAbs. Cells are transfected with HER3–SNAP-Tag and labeled withBG-Lumi4(Tb), a SNAP-tag subtract. After incubation with increasing concentrations of indicated d2-labeled mAb directed to HER3, the K_d was determined from the binding curve fitting. The binding curve was obtained by measuring the TR-FRET between the donor Lumi4(Tb) and the acceptor d2-dye ($n = 3$). The unspecific binding was evaluated by adding an excess of unlabeled Ab.



mAbs	EGFR			HER2			HER3			EGFR/HER4		
CTRL+	3986	+/-	83	6869	+/-	51	2832	+/-	93	3349	+/-	129
CTRL -	170	+/-	59	205	+/-	38	147	+/-	55	245	+/-	89
NG33	142	+/-	77	238	+/-	41	7365	+/-	85	249	+/-	88
NG83	164	+/-	75	242	+/-	37	1524	+/-	79	326	+/-	96
NG140	120	+/-	70	223	+/-	37	3980	+/-	87	184	+/-	92
NG533	119	+/-	74	212	+/-	39	200	+/-	61	177	+/-	84
XC90	122	+/-	61	193	+/-	37	8031	+/-	76	157	+/-	86
XC252	128	+/-	77	220	+/-	40	6593	+/-	80	177	+/-	98

Fig. S3. Specificity of the mAbs directed to HER3. (*A*) NIH/3T3-EGFR, -HER2, -HER3, or -EGFR/HER4 cells were incubated with a concentration of 25 μg/mL of each mAb for 1 h and 30 min at 4 °C. After two washes, the cells were incubated for 1 h at 4 °C (in the dark) with a secondary anti-mouse IgG Ab coupled to Alexa Fluor 488. The fluorescence intensity (F.I.) was measured on the LSRII flow cytometer. The negative control was made by using an irrelevant mouse IgG as primary Ab. The positive controls are the following: mAb 565 (anti-EGFR), mAb L26 (anti-HER2), mAb 9F7 (anti-HER3), and mAb 77 (anti-HER4). *B* presents the geometric mean and the coefficient of variation of the fluorescence intensity.

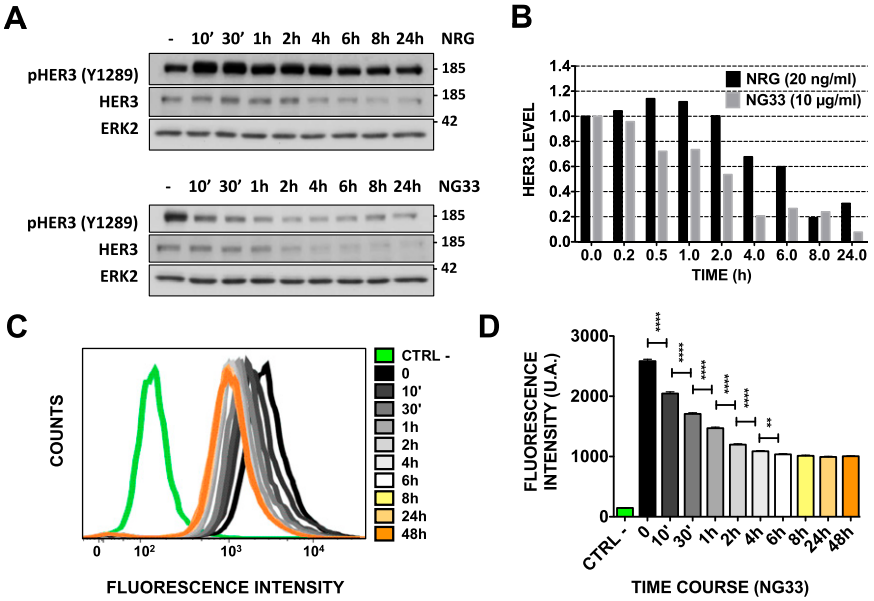


Fig. S4. NG33 mAb treatment induces HER3 internalization and degradation. (*A* and *B*) N87 cells were treated for the indicated time periods at 37 °C with 10 μg/mL mAb or 20 ng/mL NRG. After cell lysis and protein extraction, the samples were subjected to immunoblotting by using the indicated Ab. HER3 levels were quantified and presented in a histogram. (*C* and *D*) N87 cells were incubated for different time intervals with NG33 mAb (10 μg/mL). After treatment with trypsin and washing in saline containing albumin (1 mg/mL), cells were incubated for 30 min at 4 °C in the dark with a noncompetitive anti-HER3 mAb labeled with PE. Fluorescence intensity signals of 10,000 cells per sample were determined by using the LSRII flow cytometer. ****$P < 0.0001$; **$P < 0.01$ (ANOVA and post hoc tests).

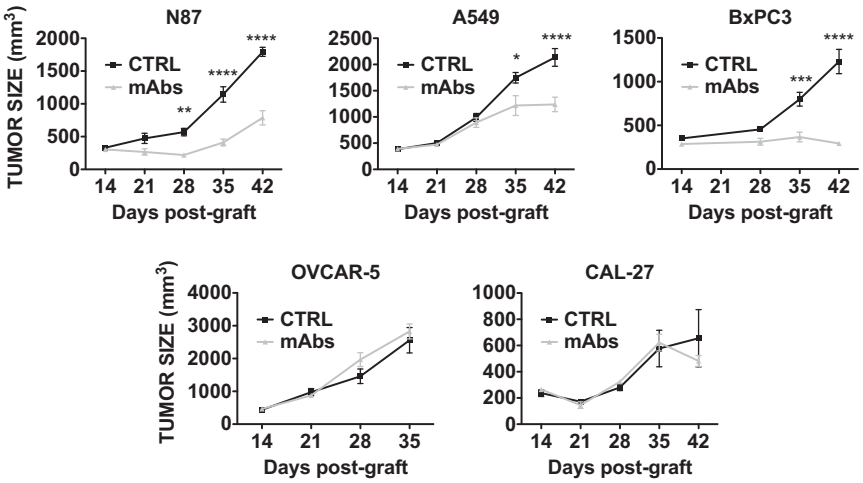


Fig. S5. In vivo tests determining the effect of anti-HER3 mAb combinations on several types of carcinomas. CD1-Nude mice were grafted s.c. with 5×10^6 cells. Once tumors became palpable, the mice were randomly divided into groups of three mice and were injected twice a week, intraperitoneally (IP) with the indicated treatments for 5 wk. The control group (CTRL) was injected with 200 μL of PBS, and the mAbs group were treated with the NG33+XC252 combination at a final concentration of 0.2 mg/0.2 mL PBS per mouse. The mice were weighed and the tumors were measured once a week. The average tumor size of three mice (±SEM) is reported.