Supplementary data

Supplemental material and method

ErbB3 transfection experiments. ErbB-devoid Chinese Hamster Ovary (CHO) cells seeded in DMEM supplemented with 10% FBS were co-transfected with equimolar amounts of pLenti plasmid containing the full human ErbB3 gene fused to an N-terminal FLAG with GFP, HER2, MET, or EGFR (1, 2) using Mirus TransIT-LT1 transfection reagent (Mirus, Madison, WI) according to the manufacturer's protocol. Forty-eight h later, 170 µg/ml of MM-121 was added to the cells and incubated overnight. The next day, cells were treated with the indicated ligands for 10 min and the cells were collected. Detergent-soluble cell lysates were then analyzed for ErbB3 phosphorylation by nitrocellulose Western blot using a phospho-specific ErbB3 antibody (monoclonal rabbit anti-phospho-ErbB3 Y1289, Cell Signaling Technology, Danvers, MA) or immunoprecipitation of ErbB3 using an anti-FLAG antibody (Cell Signaling Technology, Danvers, MA), followed by probing a Western-blotted nitrocellulose membrane with a total phospho-tyrosine (pTyr) antibody(Cell Signaling Technology, Danvers, MA). Membranes were stripped and probed for total ErbB3 (Cell Signaling Technology, Danvers, MA).

***In vitro* signaling studies.** For inhibition studies, cells were seeded at 35,000 cells per well in 96 well tissue culture plates and grown overnight. Cells were serum-starved 20-24 h, and then pre-incubated with 4-fold serial dilutions ranging

from 2 μM to 7.6 pM of MM-121 for 30 min prior to 10 min of stimulation with 25 nM heregulin or BTC. Cells were harvested as described above.

ELISA analyses. For ELISA analysis of cell lysates, capture antibodies against ErbB1 (AF231, R&D Systems,, Minneapolis, MN), ErbB2 (MAB1129, R&D Systems, Minneapolis, MN), ErbB3 (MAB3481,R&D Systems, Minneapolis, MN) and AKT (05-591MG, Millipore, Billerica, MA) were incubated in 384 well black flat-bottom polystyrene high-binding plates overnight at room temperature. ELISAs were blocked with 2% bovine serum albumin (BSA) in phosphate buffered saline (PBS) for one hour and then incubated with lysates diluted in 2% BSA, 0.1% Tween-20 and PBS at RT for 2 h. Between incubation steps, plates were washed three times with 0.05% Tween-20 in PBS. ELISAs measuring phospho-ErbB1, -ErbB2 and -ErbB3 were incubated with phospho-tyrosine horseradish peroxidase (HRP) linked monoclonal antibody (HAM1676, R&D Systems, Minneapolis, MN) for 2 h. ELISAs measuring phospho-AKT were incubated with primary serine 473 specific anti-phospho AKT mouse monoclonal antibody (5102, Cell Signaling Technologies, Danvers, MA) for 2 h, then incubated with streptavidin-HRP for 30 min. All ELISAs were visualized with SuperSignal ELISA Pico Chemiluminescent Substrate (37069, Pierce, Rockford, IL) and luminescent signal was measured using a luminometer.

Fluorescent activated cell sorting (FACS) experiments. Cells were seeded into 6-well plate and cultured in RPMI medium containing 10% FBS. At 70%

confluence, the media was changed to 2 % FBS-RPMI and cells were treated with 200 nM of MM-121 for the indicated time. At each indicated time points (0, 0.5, 2, and 24 hours of MM-121 treatment), cells were washed with ice-cold PBS and incubated with 0.05% trypsin/EDTA on ice. When cells detached, 1ml of PBS/20%FBS RPMI (equal volume) were added to each well and the cells were transferred to eppendorf tube and washed twice with ice-cold binding buffer. Cells were incubated with 100 nM of Alexa 647-conjugated ErbB3 antibody SGP1 (MS-725-PABX, Lab Vision, Fremont, CA) and 1 mg/ml propidium iodide, or buffer only (as unstained control) for 45 min on ice. Our previous data has shown that SGP1 did not overlap or interfere with the ability of MM-121 to bind ErbB3. Cells were washed twice with ice-cold binding buffer, re-suspended with binding buffer and analyzed by flow cytometry. Mean fluorescence intensity of 10,000 cells gated for viability by exclusion of propidium iodide was quantified by FACS. For studies assessing MM-121 binding to mouse ErbB3, binding was tested using the following conditions: no ligand, no secondary antibody (baseline), secondary antibody only (background, MM121 at 0.1 nM, 10 nM , 100 nM and 1000 nM and soluble ErbB3 as negative control at 1000 nM.

Xenograft efficacy studies. Tumor xenografts were established by subcutaneous (s.c.) injection of 5-7 x 10^6/mouse ACHN, 8 x 10^6 DU145, 8 x 10^6 NCI-87, 1-1.2 x 10^7 OvCAR8, 1 x 10^7 SkOV-3, 5 x 10^6 ADRr, 2 x 10^7 BT-474, 5 x 10^6 MALME3M and 1.5 x 10^7 IGROV diluted 1:1 with Matrigel™ Basement Membrane Matrix, Growth Factor Reduced (354230, BD Biosciences, San Jose,

CA) in the right flank of 5-6 wk old female athymic (nu/nu) mice (Charles River Lavatories, Wilmington, MA). In the BT-474 xenograft model, an estrogen pellet containing 0.72 mg of 17β-estradiol (SE-121, Innovative Research of America, Sarasota, FL) was implanted s.c. into the shoulder area of mice 24 h before tumor cell injection. Tumors were allowed to reach 150-300 mm^3 in size. Subsequently, mice were randomized into groups of 10 mice each. Mice were treated intraperitoneally every three days with 100µl MM-121 at indicated dose or vehicle control (PBS). Treatment of animals was continued for the duration of the study. Tumors were measured twice weekly and tumor volume was calculated as pi/6 x length x $width^2$, where the width was the shorter measurement. After final tumor measurements, mice were sacrificed and tumors excised, snap frozen in liquid nitrogen, and stored at -80^0C for biochemical and immunohistochemical analysis. In addition, tumors from untreated mice were collected and flash frozen at the beginning of the study for receptor and ligand profiling studies. The difference in average tumor volume between vehicle control treated mice and mice treated with the highest dose of MM-121 was statistically assessed using Student t-test at each measurement day and computed using Excel (Microsoft, Redmond, WA).

Quantitation of ErbB receptor and ligand expression in tumor lysates.

Three untreated flash-frozen tumors in the range of 200-500 mm^3 per cell line were individually lysed and the receptor and ligand protein expression levels were assessed for each tumor separately. EGFR, ErbB2, ErbB3 and ErbB4

levels were quantified using Luminex™ multi-color bead based multiplex ELISA technology. The capture antibodies from the Duoset ELISA kits (R&D Systems, Minneapolis, MN) for EGFR, ErbB2, ErbB3 and ErbB4 were conjugated to the beads which allowed for the simultaneous quantification of the receptors. The respective ligand expression levels or heregulin and BTC were determined by ELISA. In the heregulin ELISA recombinant ErbB3-His ECD was used as a capture reagent and has been cloned, expressed and purified at Merrimack. Detection of BTC in tumor cell lysates was carried out using a BTC-specific ELISA kit from R&D Systems, Minneapolis, MN (cat# DY261,) following the manufacturer's recommendations.

Binding of MM-121 to mouse ErbB3. CHO-K1 cells were transiently transfected with ErbB3 mouse cDNA using pExpress1-ErbB3 (mouse) from OPEN Biosystems, Huntsville AL (MMM1013-98478677,). Cells were cultured in a 10cm dish until they reached 90% confluency. 24 h before the transfection cells were split to reach 70% confluency. 1.2 ml of Opti-MEM and 80 μl of TransIT-CHO Reagent were mixed and 40 μl (12 μg) plasmid DNA was added. After incubating at RT for 5-20 min, 12μl of CHO Mojo Reagent were added to the complex mixture. Medium was replaced with 13 ml of fresh complete growth medium before the complex mixture was added and cells were incubated for 48 h.

MRI Scanning and tumor volume measurement. Mice were anesthetized by 1% isoflurane inhalation. Biotrig Software was employed to monitor respiratory

and cardiac rates of the mice. MRI scanning was performed in coronal planes with a rapid acquisition with relaxation enhancement (RARE) sequence (TR = 2000 ms, TE effect = 25 ms). Seventeen coronal slices of 1mm thickness were used to cover the entire lung. Matrix size was 128x128 and field of view (FOV) was 2.5 x 2.5 cm^2. Each area of tumor was manually segmented by ImageJ (ver. 1.33, National Institute of Health). Size of the areas on all 17 slices were calculated and used to estimate relative tumor volume regression. Details of MRI methods have been reported previously (3).

Determination of IC_{50} values. Inhibitor IC_{50} values were calculated by least-squares fitting the dose response data to a sigmoidal curve (GraphPad Prism, CA).

Immunohistochemistry. Slide deparaffinization, conditioning and staining with pErbB3 (4791, Cell Signaling Technology, Danvers, MA) and tErbB3 (C-17) (sc-285, Santa Cruz Biotechnology, Santa Cruz, CA) antibodies was performed on the Ventana Discovery XT autostainer. Primary antibodies were diluted in Dako Antibody Diluent with Background Reducing Component (S3022, Dako, Carpinteria, CA) and incubated for 3 h at RT or 37^oC respectively. Detection was performed using Ventana DAB kit with UltraMap anti-Rabbit HRP secondary antibody (Ventana Medical Systems, Tucson, AZ). Hematoxylin and eosin (H&E) and IHC staining were performed on 5 μm paraffin sections in the Department of Pathology at Brigham and Women's Hospital. Terminal deoxynucleotidyltransferase- mediated dUTP-biotin nick end labeling (TUNEL)

assay (ApopTag kit; Chemicon, Billerica, MA) and ki67 (Vector laboratories, Burlingame CA) staining were performed according to manufacturer's protocol. Details of staining procedures were described elsewhere (3).

Statistics. Statistical analyses were performed using unpaired 2 tailed student's *t* test. P values of less than 0.05 were considered significant.

Figure Legends

Figure S1. Ratio of pErbB3 (Y1289) to total ErbB3 in ErbB3 expressing CHO cells co-transfected with GFP (control), EGFR, MET, or ErbB2/HER2 stimulated with different ligands. Densiometric analysis of Western blots depicted in Fig. 1A to relative effects of MM-121 on total and phospho-ErbB3. Blue bars indicate the ratio of the untreated controls (normalized to 1.0) and the red bars the normalized MM-121 treated samples.

Figure S2. MM-121 inhibits hergegulin-induced activation of ErbB-3 even in the absence of internalization.. *A,* MALME 3M cells were starved with 1% FBS for 2 hours. They were then treated with 250 nM MM-121 for 30 min prior to HRG stimulation. To block receptor internalization, the experiment was conducted at 4°C or after 40 minutes pretreatment with 100 μM of monodansyl cadaverine (MDC). ErbB3 and AKT phosphorylation was by immunoblot. *B,* The ErbB3 cell surface levels under the conditions described above (A) were determined by FACS. *C,* Densiometric analyses of western of phosphot-ErbB3 immunoblots in (A).

Figure S3. MM-121 differentially alters cell surface expression of ErbB3 in various cell lines. The indicated cell lines were incubated for 0, 0.5, 2 and 24 hours with 200 nM MM-121, and surface ErbB3 levels were assessed by FACS and presented as a bar graph. Corresponding Western blots are shown for total ErbB3 expression after incubation with MM-121 for 0 and 24 hours.

Figure S4. The Inhibition of BTC-induced activation of ErbB3, AKT and ERK by MM-121 is cell line dependent. Cells were serum-starved for 24 hours, and then pre-incubated with 4-fold serial dilutions ranging from 2 μM to 7.6 pM of MM-121 for 30 minutes prior to 10 minutes of stimulation with 25 nM BTC. ErbB3, AKT and ERK phosphorylation was measured by ELISA.

Figure S5. Effect of ErbB and MET inhibitors on the cell line models used in xenograft experiments. The indicated cell lines were treated with either vehicle, 1μM gefitinib (G), 1μM PHA-665,752 (P) (MET inhibitor), 1 μM lapatinib (L) or 170 μg/ml MM-121 (M) ~16 hours (overnight). The cells lysates were probed with the indicated antibodies.

Figure S6. Summary of xenograft experiments. ACHN, DU145, OvCAR8, SKOV3, NCI-ADRr, NCI- N87, MALME 3M, IGROV1 and BT474 were injected s.c. as a Matrigel suspension into nude mice and allowed to grow to at least 150 mm^3. Tumors were allowed to reach 150-300 mm^3 in size. Subsequently, mice were randomized into groups of 10 mice each. Mice were treated i.p. every three days with MM-121 (black triangle) or vehicle control, PBS, (gray square). Treatment of animals was continued for the duration of the study. Tumors were measured twice weekly. Responders are named in red, partial responders in green and non-responders in blue. The star indicates that the MM-121 treatment

cohort is statistically different to the PBS treated cohort as assessed by Student t-test at each measurement day.

Figure S7. MM-121 blocks ErbB3 phosphorylation in NCI-N87 tumors. *A,* NCI-N87 tumors were treated twice with either vehicle or MM-121, and vehicle (alone) every three days. Twenty four hours after the second treatment, the xenografts were harvested. IHC with an anti-pErbB3 antibody was performed on the vehicle and MM-121 treated xenografts. No primary antibody was used on the vehicle treated xenografts as a control for specific phospho-ErbB3 staining (*left*). *B*, Ki67 and TUNEL staining in NCI-N87 tumors from two vehicle control treated tumors and one MM-121 treated tumor.

Figure S8. MM-121 binds to both mouse and human ErbB3. *A*, Binding of MM-121 to control CHOK-1 cells. MM-121 was used at 0.1 nM, 1 nM, 10 nM and 100 nM. MM-121 binding was measured by FACS. *B*, CHOK-1 cells were transfected with mouse ErbB3 and assessed as in (A). *C,* CHOK-1 cells were transfected with human ErbB3 and assessed as in (A). Specific binding was demonstrated by pre-incubating MM-121 with soluble human ErbB3, which led to the abrogation of the signal (yellow line in *B* and dark blue line in *C*).

Supplemental references

1 Engelman, J.A., Zejnullahu, K., Mitsudomi, T.,et al. MET amplification leads to gefitinib resistance in lung cancer by activating ErbB3 signaling. *Science* **316**, 1039-1043 (2007).

2 Vapnik, V. The nature of statistical learning theory: Springer (1999).

3 Li, D., Ji, H., Zaghlul, S., et al. Therapeutic anti-EGFR antibody 806 generates responses in murine de novo EGFR mutant-dependent lung carcinomas. *J. Clin. Invest.* **117**, 346-352 (2007).

Figure S1

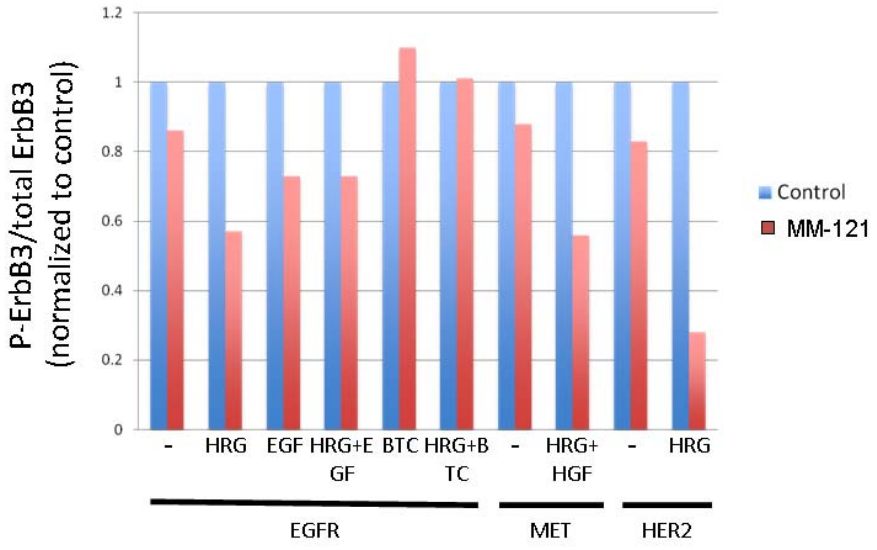

P-ErbB3/total ErbB3
(normalized to control)
1.2
1
0.8
0.6
0.4
0.2
0
-
HRG
EGF
HRG+EGF
BTC
HRG+BTC
-
HRG+HGF
-
HRG
EGFR
MET
HER2
Control
MM-121

Figure S2

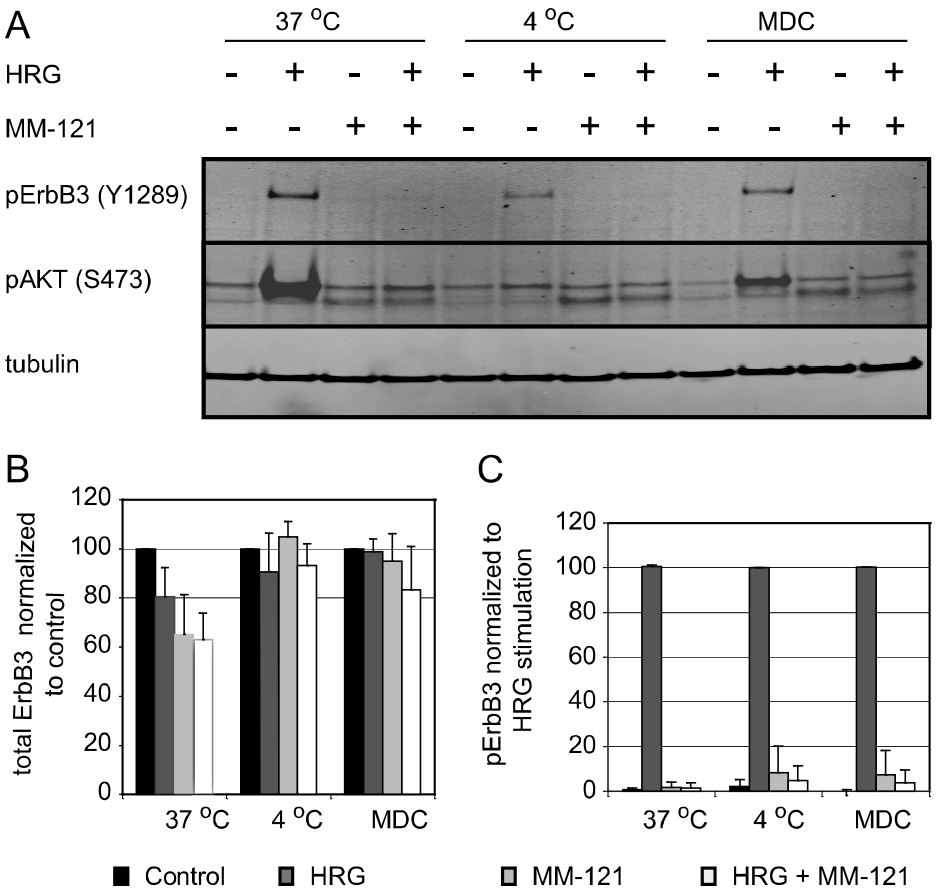
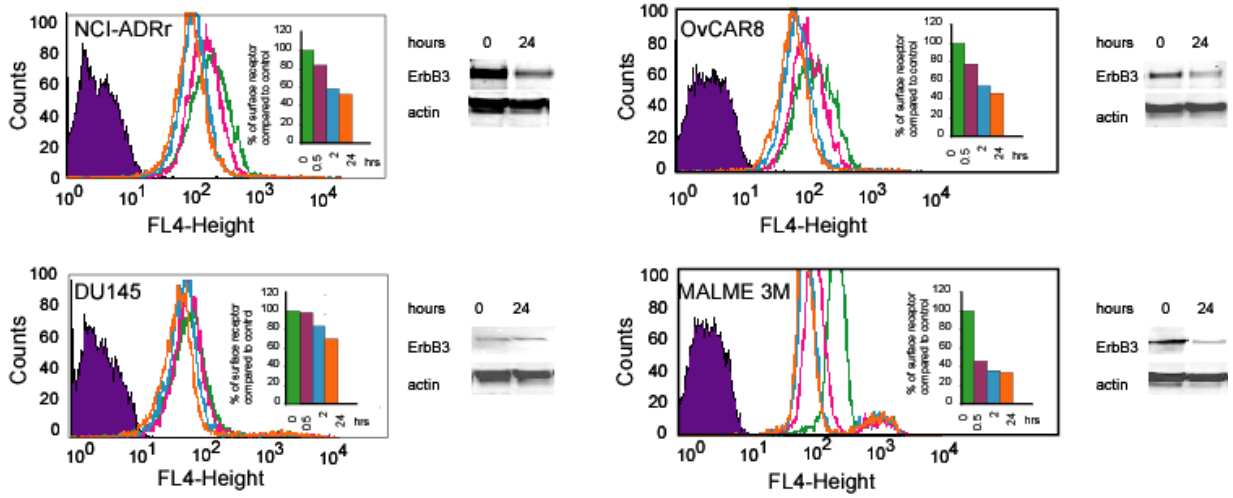


Figure S3



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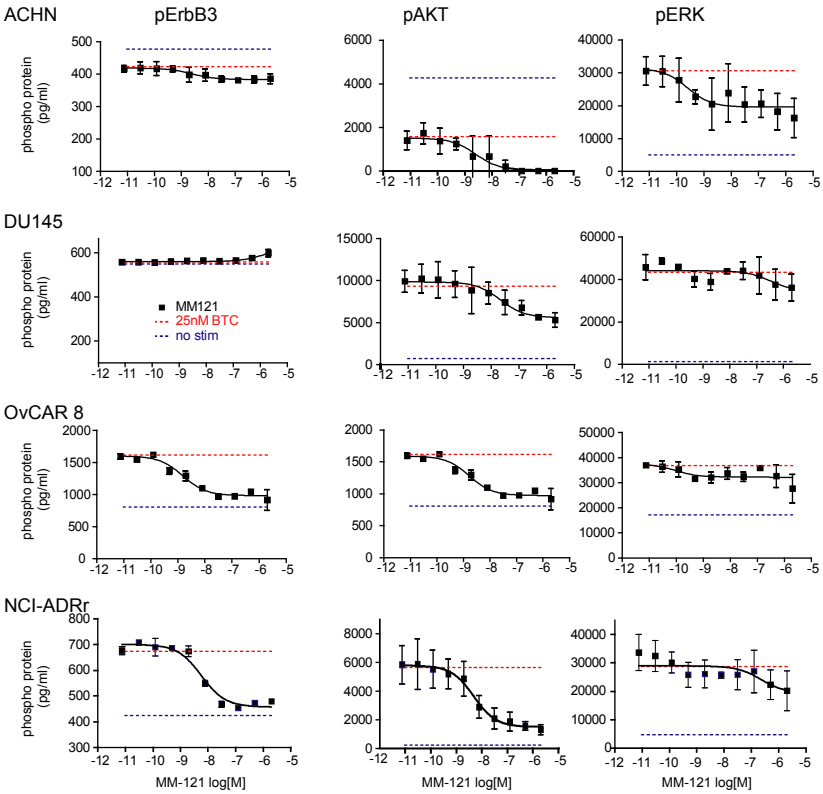
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pERK
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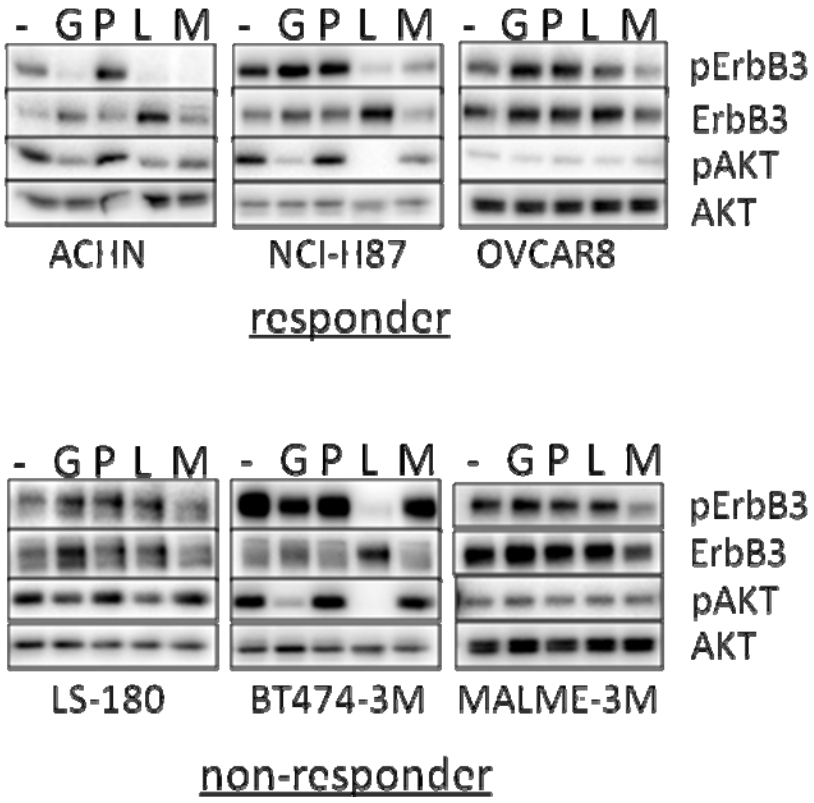
MM121
25nM BTC
no stim
MM-121 log[M]

Figure S5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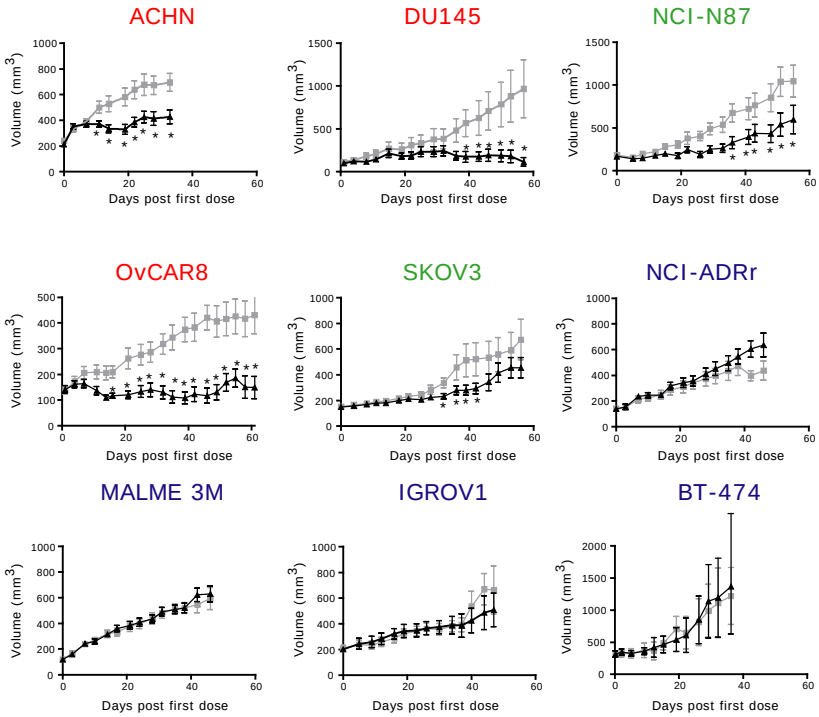
non-responder

Figure S6


ACHN
DU145
NCI-N87
OvCAR8
SKOV3
NCI-ADRr
MALME 3M
IGROV1
BT-474
Volume (mm3)
Days post first dose

FigureS7

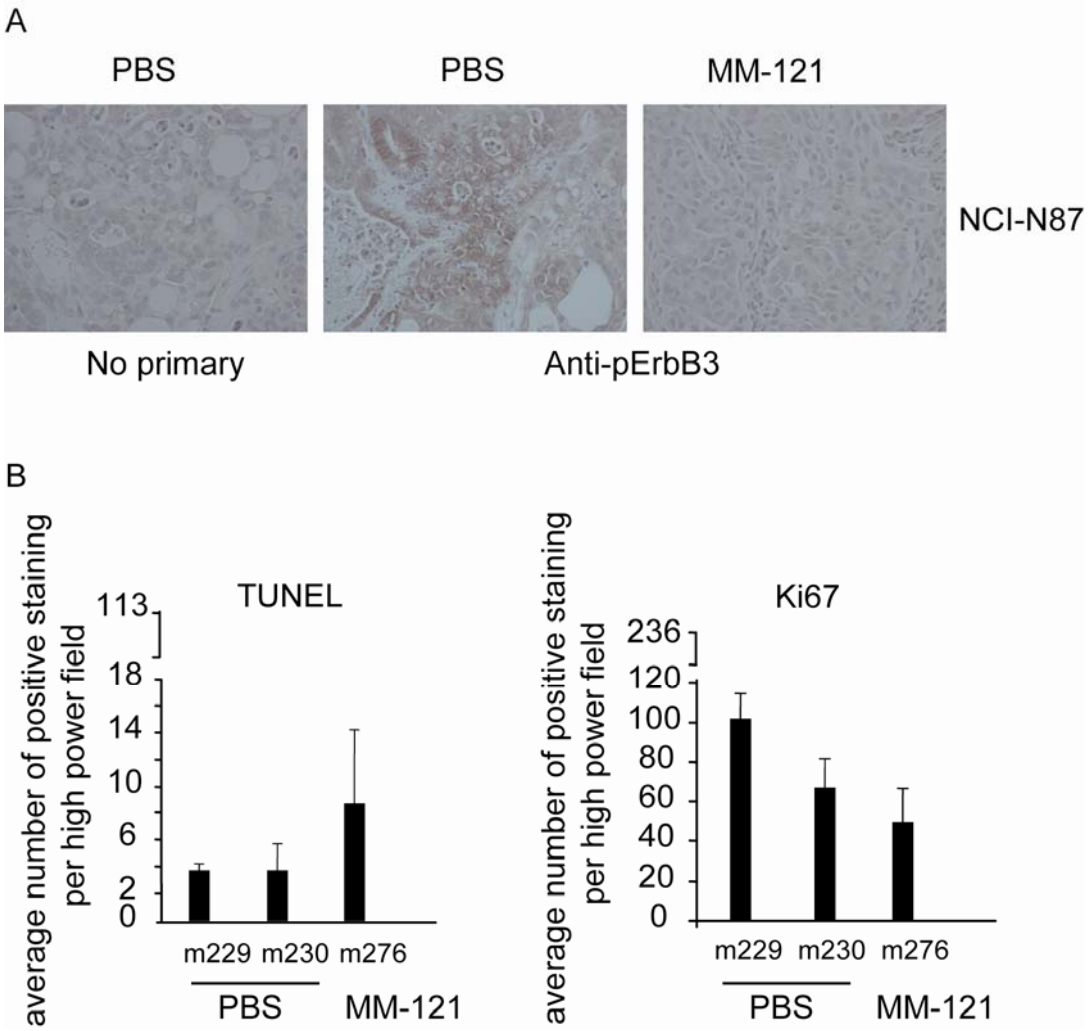


Figure S8

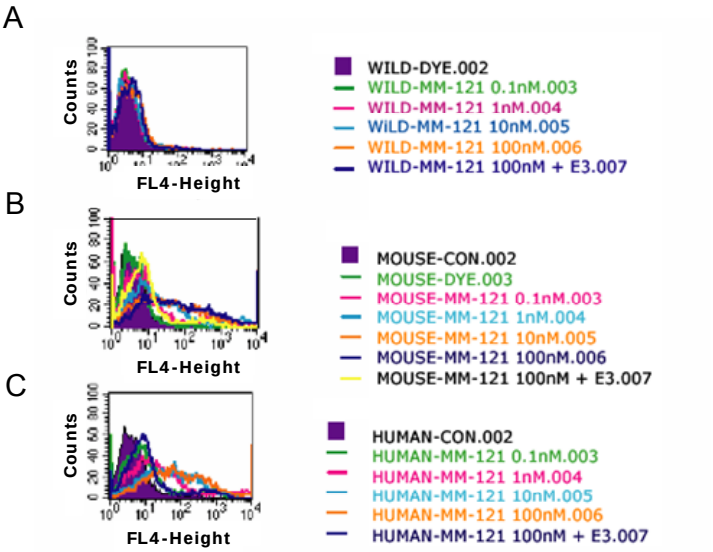


Table S1

Expression levels of ErbB family members and ligands in untreated xenografts evaluated for sensitivity to MM-121

	BTC [pg/μg]	STDEV [pg/μg]	HRG [pg/μg]	STDEV [pg/μg]	EGFR [pg/μg]	STDEV [pg/μg]	HER2 [pg/μg]	STDEV [pg/μg]	ErbB3 [pg/μg]	STDEV [pg/μg]	HER2 copy number
ACHN	2.17E-02	6.41E-03	3.82E-01	1.76E-01	4.85E+01	2.02E+01	5.01E-01	7.85E-01	9.22E-01	1.06E+00	
NCI-ADRr	5.61E-04	2.28E-04	1.38E-02	4.62E-03	7.16E+00	3.62E+00	1.88E-01	1.12E-01	9.69E-01	5.59E-02	
A549	1.75E-02	1.40E-03	2.03E-01	9.22E-02	1.13E+01	3.45E+00	6.99E-03	5.56E-03	2.22E+00	4.64E-01	
BT474-M3	2.73E-03	3.59E-04	7.19E-03	4.03E-03	8.69E+00	2.45E-00	2.00E+01	1.23E+00	1.22E+00	5.24E-01	47
DU145	1.05E-02	9.79E-03	7.17E-02	7.93E-02	1.36E+01	4.47E+00	9.35E-01	4.31E-01	2.05E+00	9.44E-01	3
H1975	3.31E-03	5.36E-04	0.00E+00	0.00E+00	2.98E+01	7.08E+00	2.07E-02	2.90E-03	4.19E-01	1.54E-01	
HCT-116	7.89E-02	5.76E-03	0.00E+00	0.00E+00	1.97E+01	1.17E+01	8.33E-02	2.26E-02	1.23E+00	2.31E-01	
HT29	4.01E-02	2.82E-02	0.00E+00	0.00E+00	3.76E+00	1.39E+00	1.54E-01	7.22E-02	5.59E+00	2.83E+00	
IGROV1	1.78E-02	4.77E-03	0.00E+00	0.00E+00	6.16E+00	1.52E+00	1.46E-01	1.64E-01	6.06E-01	3.31E-01	
MALME 3M	1.91E-03	5.17E-04	1.21E-03	9.81E-04	9.99E+00	2.14E+00	3.32E-01	1.95E-02	3.50E+00	1.76E+00	
NCI-N87	5.58E-03	1.58E-03	9.14E-03	1.48E-03	1.91E+01	5.26E+00	1.28E+02	1.69E+02	4.01E+00	1.99E-01	6.8
OvCAR8	1.35E-03	2.24E-04	5.35E-03	3.50E-03	8.81E+00	2.55E+00	9.46E-02	3.77E-02	1.08E+00	5.65E-01	
SKOV3	2.00E-02	6.87E-03	1.79E-02	1.05E-02	7.12E+00	1.08E+00	3.70E+01	1.76E+01	1.34E-01	3.29E-02	4.2
ZR75	4.10E-03	1.60E-03	1.79E-03	2.19E-03	2.26E+00	4.24E-01	4.10E-01	1.60E-01	7.89E-01	9.70E-02	

The mean of betacellulin (BTC), heregulin (HRG1-β1), EGFR, HER2, and ErbB3 expression levels were determined from three different tumors per cell line. In addition, HER2 copy number was determined for the indicated cell lines.