Supplemental Materials for
"Pre-existence and clonal selection of *MET* amplification in *EGFR* mutant NSCLC"

Inventory of Supplemental Materials

Supplemental Figures

- Figure S1 contains the viability curves from which IC50 values were calculated for Figure 4A.
- Figure S2 contains in vitro characterization of the HCC827-HGF cell line used for the xenograft model in Figure 3E.
- Figure S3 contains supporting data for Figure 5A, B.
- Figure S4 contains additional data corresponding to Figure 6.
- Figure S5 contains a model for the development of MET amplification and FACS data corresponding to Figure 7D.
- Figure S6 contains additional patient data and HGF immunohistochemistry scoring corresponding to Figure 8.
- Table S1 contain quantification of the data presented in Figure 4B.
- Tables S2 and S3 contain quantification the data presented in Figures S4C and S4D-F respectively, and referenced in Figure 6.

Supplemental Experimental Procedures

Supplemental References

Supplemental Figures

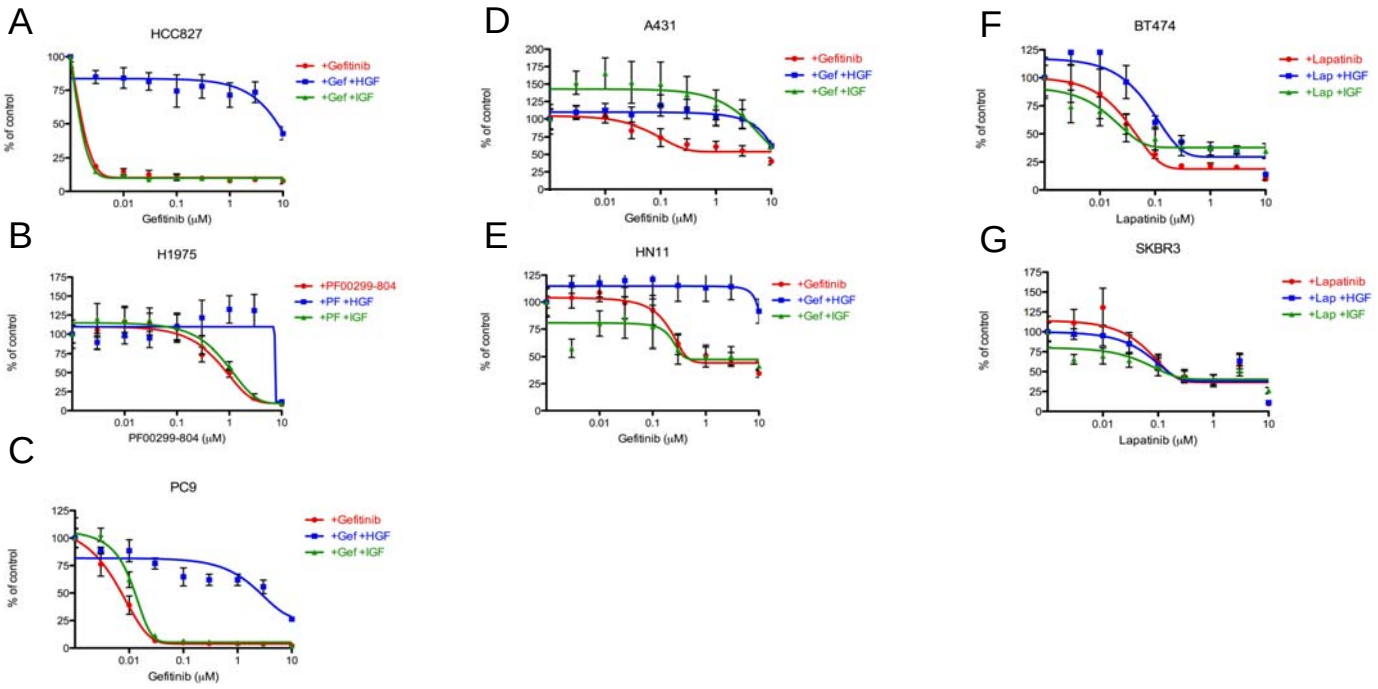


Figure S1. HGF is more effective than IGF in inducing TKI resistance in EGFR-driven cancer cell lines. Cells were grown in media containing full serum unless otherwise noted, and treated with increasing concentrations of the indicated TKI, alone or in combination with HGF (50ng/mL) or IGF (75ng/mL). Cell viability relative to untreated controls was measured after 72 hours of treatment. Each data point represents the mean ±SD of 6 wells. A431 cells were grown in media containing 0.5% FBS as previously described (Guix et al., 2008).

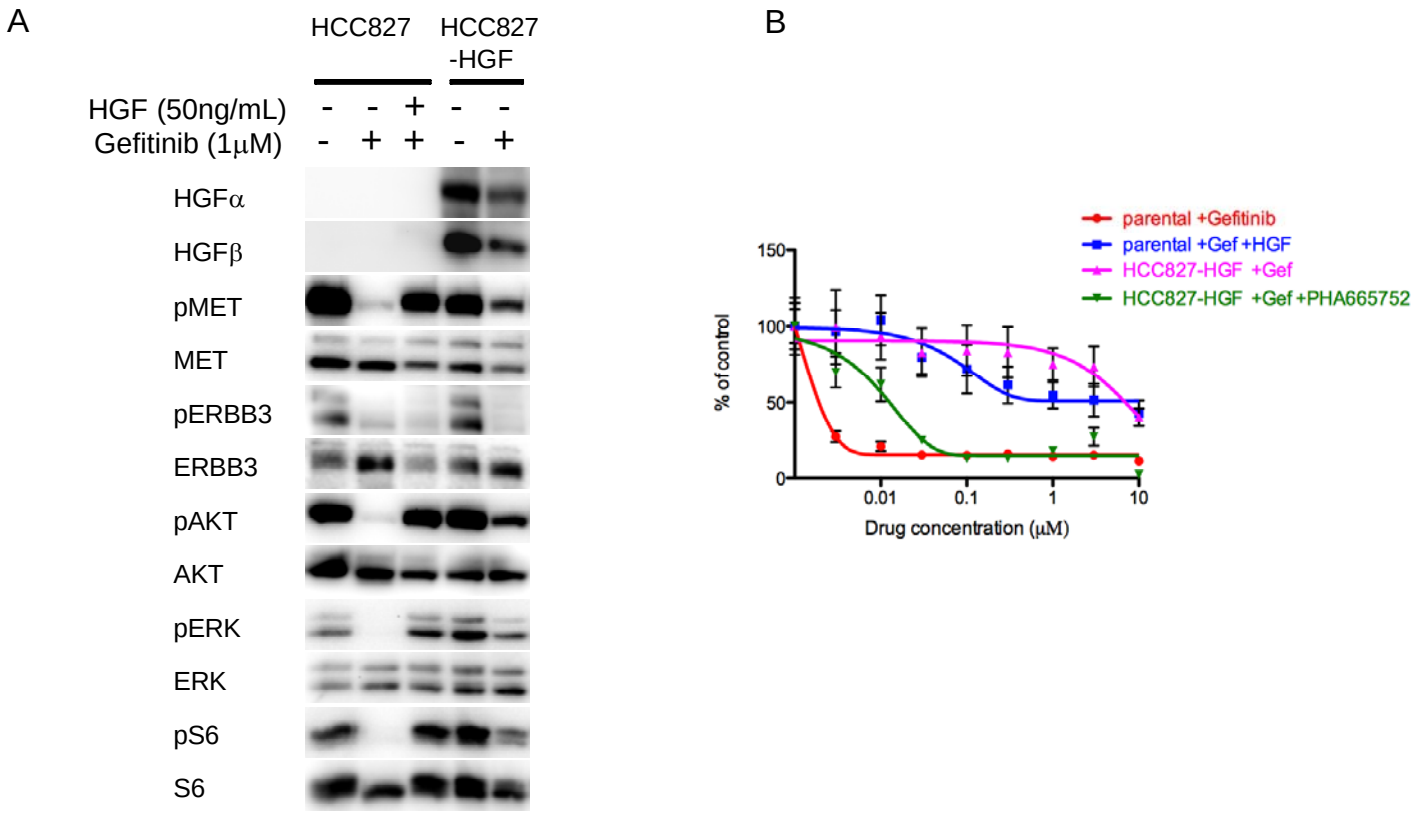


Figure S2. HGF expression in HCC827 cells maintains signaling and induces gefitinib resistance. (A) HCC827 cells were infected with a lentivirus engineered to express HGF (HCC827-HGF). Parenal HCC827 and HCC827-HGF cells were treated for 6 hours with gefitinib (1μM) alone or in combination with HGF (50ng/mL) where indicaed. Cell lysates were immunoblotted to detect indicated proteins. **(B)** Parental HCC827 cells were treated with increasing concentrations of gefitinib, alone or in combination with HGF (50ng/mL) for 72 hours. HCC827-HGF cells were treated increasing concentrations of gefitinib, alone or in combination with the MET inhibitor PHA665752 for 72 hours. Cell viability was measured relative to untreated controls. Each data point represents the mean ±SD of 6 wells.

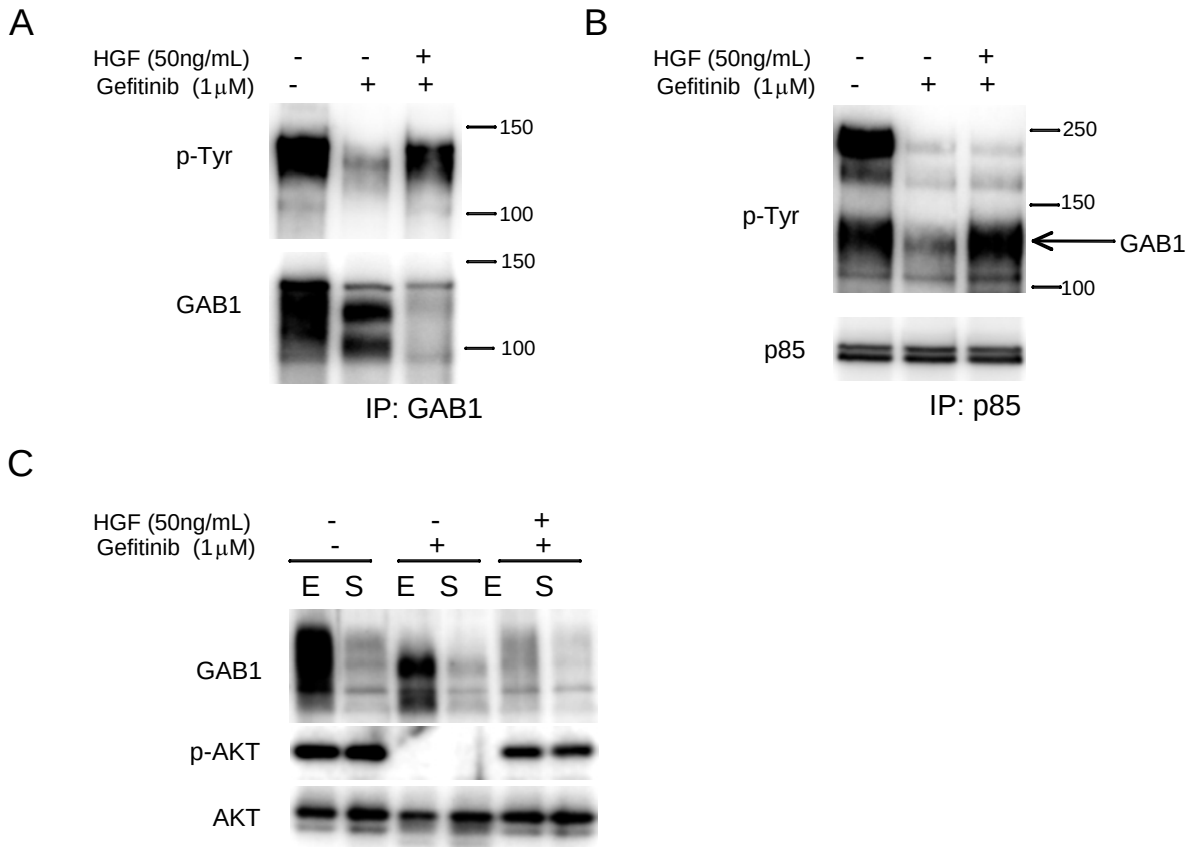
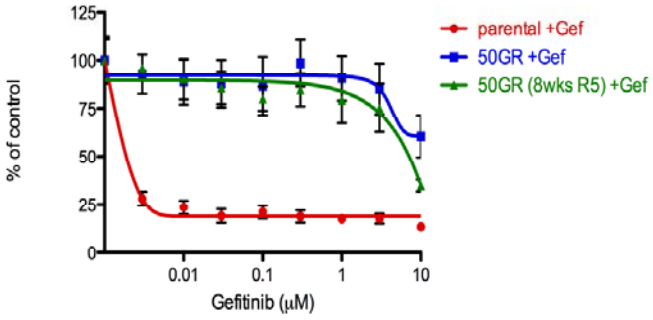
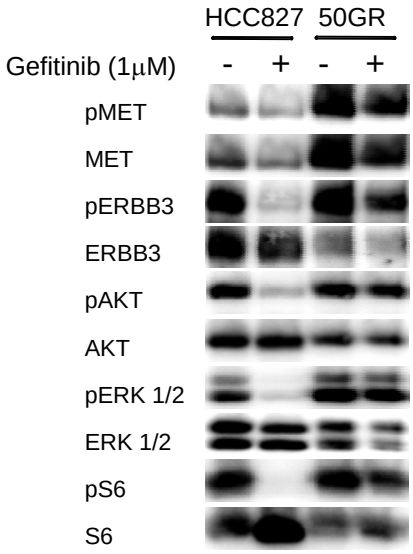


Figure S3. Treatment with gefitinib in combination with HGF dramatically increases the ratio of tyrosine phosphorylated to total GAB1. HCC827 treated for 6 hours with gefitinib (1μM), alone or in combination with HGF (50ng/mL). **(A)** Cell extracts were immunoprecipitated with an anti-GAB1 antibody followed by Western blot with an anti-p-Tyr antibody. Blots were stripped and re-probed with an antibody against total GAB1. **(B)** Cell extracts were immunoprecipitated with an anti-p85 antibody followed by Western blot with an anti-p-Tyr antibody. Blots were stripped and re-probed with an antibody against total p85. **(C)** Cell lysate extracts (E) and supernatants (S) from the immunoprecipitation shown in *(A)* were run in equal amounts and analyzed by Western blot to show clearance of GAB1. Blots were stripped and re-probed with the indicated proteins to confirm that treatment with gefitinib in combination with HGF rescues AKT phosphorylation in these lysates.

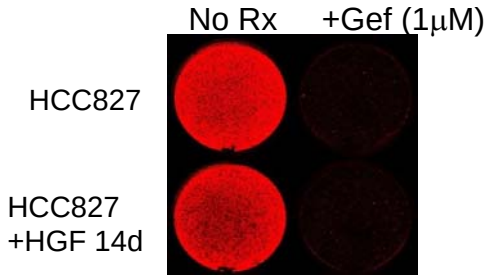
A


% of control
125
100
75
50
25
0
0.01
0.1
1
10
Gefitinib (μM)
parental +Gef
50GR +Gef
50GR (8wks R5) +Gef

B


HCC827
50GR
Gefitinib (1μM)
- + - +
pMET
MET
pERBB3
ERBB3
pAKT
AKT
pERK 1/2
ERK 1/2
pS6
S6

C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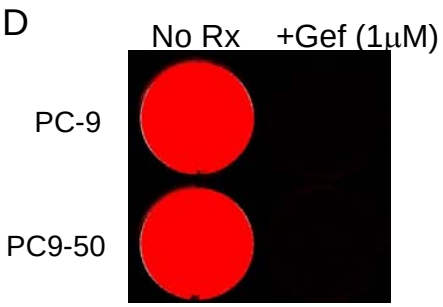
+Gef (1μM)
HCC827
HCC827
+HGF 14d

D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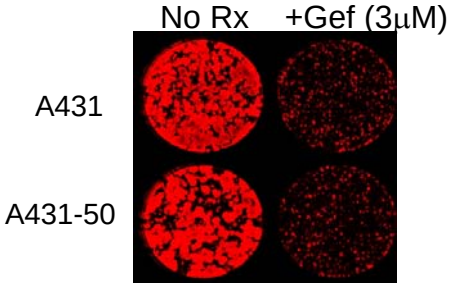
+Gef (1μM)
PC-9
PC9-50

E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+Gef (3μM)
A431
A431-50

F

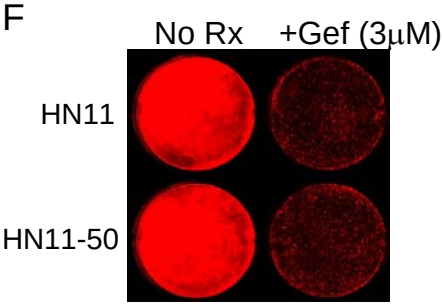
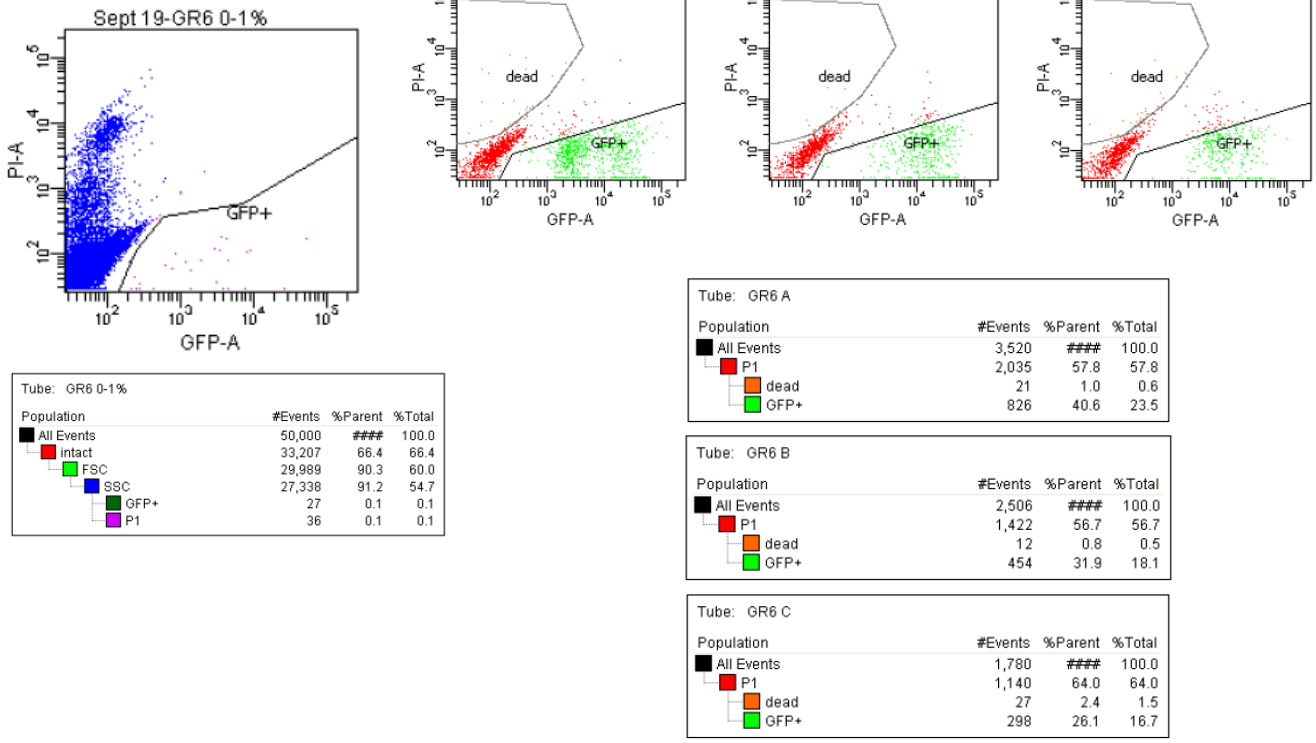

No Rx
+Gef (3μM)
HN11
HN11-50

Figure S4. HCC827 cells are uniquely poised to develop-ligand independent resistance after transient exposure to HGF in the presence of gefitinib. (A) Parental HCC827, HCC827-50GR cells and HCC827-50GR cells grown in media alone (without gefitinib) for 8 weeks, 50GR (8wks R5), were treated with increasing concentrations of gefitinib for 72 hours. Cell viability was measured relative to untreated controls. Each data point represents the mean ±SD of 6 wells. **(B)** HCC827 cells and stably resistant HCC827-50GR cells were treated for 6 hours with gefitinib (1μM). Cell lysates were immunoblotted to detect indicated proteins. **(C)** Selective pressure is necessary for HGF-mediated gefitinib resistance in HCC827 cells. Parental HCC827 cells, and HCC827 pre-treated with HGF (50ng/mL) in the absence of gefitinib for 14 days (827 +HGF 14d), were seeded on a 6-well plate and grown in media alone (No Rx) or treated with 1μM gefitinib for 9 days. Viable cells were visualized using Syto60 staining. **(D)** Transient HGF exposure does not promote stable ligand-independent resistance in other gefitinib sensitive cancer cell lines. Parental PC-9 cells, and PC9-50 cells pre-treated with gefitinib in combination with HGF (50ng/mL) for 14 days, were seeded on a 6-well plate and grown in media alone (No Rx) or media treated with 1μM gefitinib (+Gef) for 7 days. Viable cells were visualized using Syto60 staining. **(E, F)** A431 and HN11 cells were treated with the indicated concentration of gefitinib and assayed as described in (D

A



Tube: GR6 0-1%

Population	#Events	%Parent	%Total
All Events	50,000	####	100.0
intact	33,207	66.4	66.4
FSC	29,989	90.3	60.0
SSC	27,338	91.2	54.7
GFP+	27	0.1	0.1
P1	36	0.1	0.1

Tube: GR6 A

Population	#Events	%Parent	%Total
All Events	3,520	####	100.0
P1	2,035	57.8	57.8
dead	21	1.0	0.6
GFP+	826	40.6	23.5

Tube: GR6 B

Population	#Events	%Parent	%Total
All Events	2,506	####	100.0
P1	1,422	56.7	56.7
dead	12	0.8	0.5
GFP+	454	31.9	18.1

Tube: GR6 C

Population	#Events	%Parent	%Total
All Events	1,780	####	100.0
P1	1,140	64.0	64.0
dead	27	2.4	1.5
GFP+	298	26.1	16.7

B

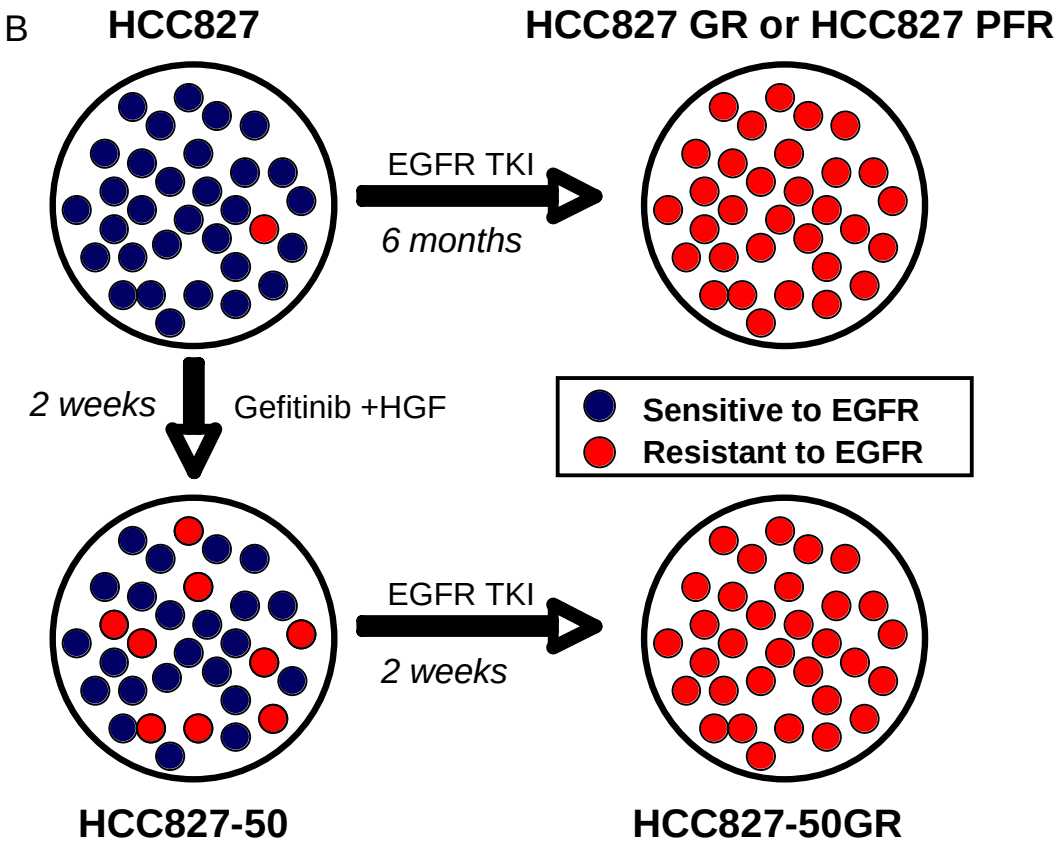
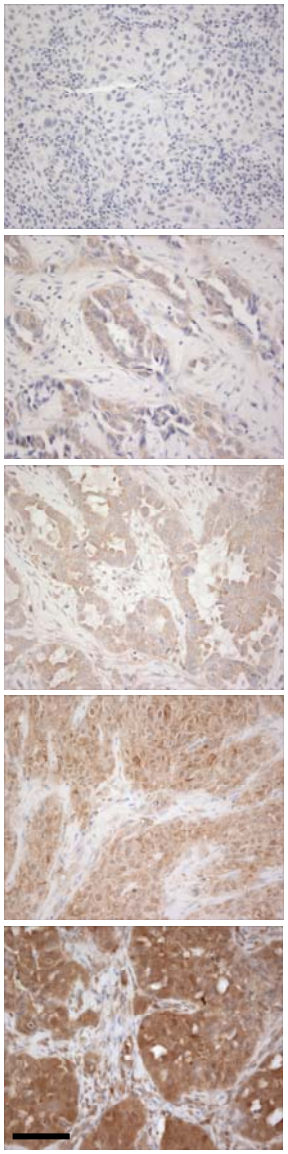
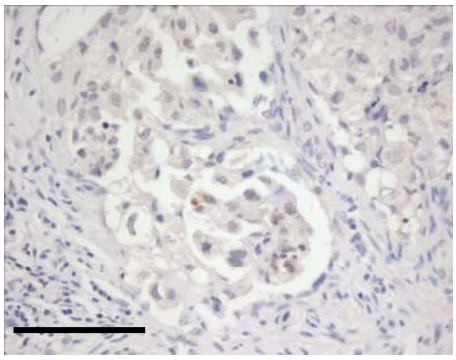


Figure S5. A model for *MET* amplification in HCC827 cells. (A) FACS analysis of HCC827 cells spiked with GFP labeled *MET* amplified HCC827 GR6 cells after 19 days of selection in HGF and gefitinib. Flow cytometric analysis of HCC827 cells spiked with 0.1% GFP labeled HCC827 GR6 cells and treated in triplicate (GR6-A, B, and C) with growth media containing gefitinib (1μM) and HGF (50ng/mL) for 19 days. Cells were analyzed by flow cytometry to detect GFP and propidium iodide (PI) staining (see Supplemental Experimental Procedures). Gates were set and data corrected for results obtained with cells stained with PI alone or unstained HCC827 cells expressing high levels of GFP. Representative scattergrams for each analysis are shown. **(B**) *MET* amplification pre-exists at low frequency in the parental HCC827 cells. These *MET* amplified clones can be selected by exposure to an EGFR TKI (either gefitinib or PF00299804) to generate resistant (HCC827 GR or HCC827 PFR) cell lines. Concomitant exposure of HCC827 cells to gefitinib HGF dramatically increases the appearance of the *MET* amplified clones.

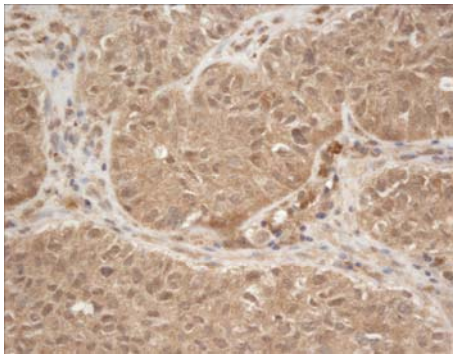
A



B



Pre-treatment



Drug Resistant

C

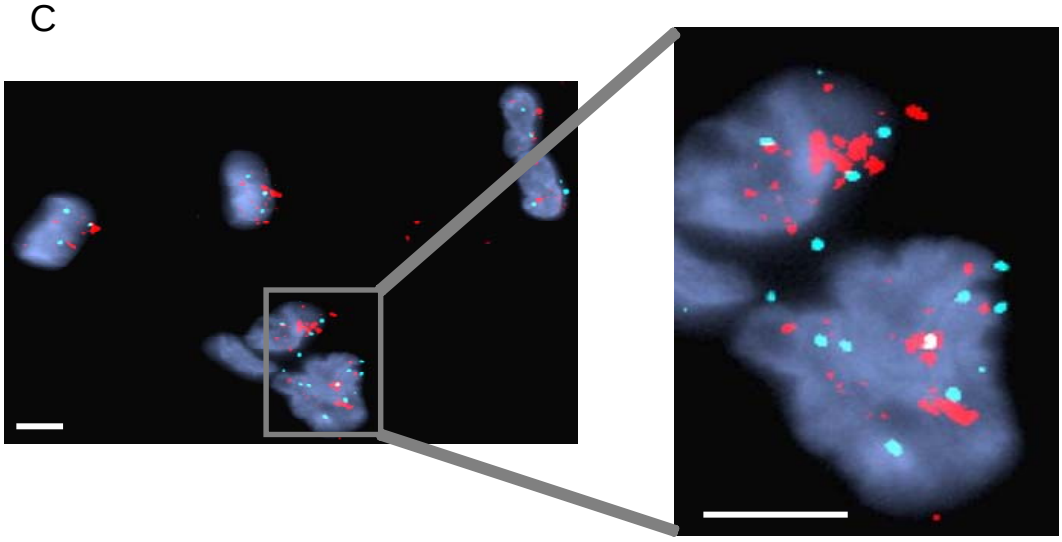


Figure S6. High HGF expression and MET amplifiecation correlate with resistance to EGFR TKIs in NSCLC patient samples. (A) Patient samples were analyzed for HGF expression using immunohistochemistry. Samples were scored from 0 (no detectable HGF) to 4+ (high HGF expression) and a scoring system was developed (see Supplemental Experimental Procedures). Table of results are shown in Figure 8A. Scale bars represent 100μm. **(B)** Pre-treatment *(upper)* and drug resistant *(lower)* tumor samples from patient 1 show low HGF expression before treatment but high HGF expression after relapse and development of resistance to the EGFR TKI. There was no *EGFR* T790M or *MET* amplification detected from the drug resistant specimen. Scale bars represent 100μm. **(C)** FISH analysis of the post-treatment drug resistant sample from patient 10 (Figure 8A) shows evidence of *MET* amplification. *MET* (RP-11-95I120; red); *CEP 7* (aqua). The pre-treatment ample from this patient showed evidence of a small sub-population of pre-existing *MET* amplified cells (Figure 8B). Scale bars represent 10μm.

	IC50 Values (uM)			HGF Rescue			IGF Rescue			Short-term resistance		Stable HGF-induced
Cell Line	TKI	TKI+HGF	TKI+IGF	AKT	ERK 1/2	S6	AKT	ERK 1/2	S6	HGF	IGF	resistance
HCC827	0.008	8.46	0.01	++	+++	++	-	-	-	++	-	+++
H1975	0.979	7.756	1.109	+++	++	+++	-	-	+++	+++	-	+
PC-9	0.008	4.351	0.015	+++	+++	+++	+++	-	++	++	-	-
A431	5.41	>10	>10	+++	+++	+++	+++	-	+++	+++	+++	-
HN11	1.601	>10	3.496	++	+++	+++	+	-	-	+++	-	-
BT-474	0.052	0.264	0.069	-	++	+	-	-	-	-	-	-
SKBR3	0.231	0.199	0.219	-	-	-	-	-	-	-	-	-

Table S1. HGF and IGF rescue in several ERBB-driven cell lines. Quantification of Western blots results (Figure 4B) and viability data (Figure S1) respectively. Short-term rescue was determined as the percentage of viable cells treated with 1μM TKI normalized to untreated cells (See Supplemental Experimental Procedures). Symbols indicate <20% rescue (-), 20-45% rescue (+), 45-75% rescue (++) and >75% rescue (+++).

Cell Line	No Rx	+TKI	+TKI / NoRx (%)
HCC827	2.21E+08	1.42E+07	6.4
HCC827 +HGF14d	1.84E+08	1.41E+07	7.7

Table S2. Selective pressure is necessary for HGF-mediated gefitinib resistance in HCC827 cells. Quantification of viable cells shown in Figure S4C.

Cell Line	No Rx	+TKI	+TKI / NoRx (%)
PC-9	3.47E+08	2893852	0.8
PC9-50	3.45E+08	3907657	1.1
A431	5.99E+07	1.20E+07	20.0
A431-50	6.28E+07	1.04E+07	16.6
HN11	4.39E+07	8.74E+06	19.9
HN11-50	4.35E+07	1.01E+07	23.2

Table S3. Transient HGF exposure does not promote stable ligand-independent resistance in other gefitinib sensitive cancer cell lines. Quantification of viable cells shown in Figure S4D-F.

Supplemental Experimental Procedures

Cell culture and reagents

The *EGFR* mutant NSCLC cell lines HCC827 (del E746_A750), HCC827 GR (del E746_A750/*MET* amplified), PC-9 (del E746_A750) and H1975 (L858R/T790M) have been previously characterized (Amann et al., 2005; Engelman et al., 2007a; Engelman et al., 2007b; Mukohara et al., 2005; Ono et al., 2004). The *EGFR* wild type epidermoid carcinoma cell line A431 and head and neck cancer cell line HN11 have been described previously (Guix et al., 2008). The *HER2* amplified breast cancer cell lines BT-474 and SKBR3 cells were kind gifts from Dr. Carlos L. Arteaga (Vanderbilt University School of Medicine, Nashville, Tennessee). HCC827 and HCC827 GR cell lines were maintained in RPMI 1640 (Cellgro; Mediatech Inc., Herndon, CA) supplemented with 5% FBS; H1975, PC-9, A431 and HN11 cell lines were maintained in RPMI 1640 supplemented with 10% FBS. BT-474 cells were maintained in Dulbecco's Modification of Eagle's Medium (DMEM) (Cellgro; Mediatech Inc., Herndon, CA) and SKBR3 cells were maintained in McCoy's 5A Medium (Invitrogen, Carlsbad, CA), both supplemented with 10% FBS. H3255 cells were maintained in ACL-4 media (Invitrogen, Carlsbad, CA) supplemented with 5% FBS. All growth medium was supplemented with 100 units/mL penicillin, 100 units/mL streptomycin, and 2 mM glutamine.

Recombinant human HGF and the Quantikine ELISA Kit for quantification of HGF in cell culture medium were purchased from R&D Systems (Minneapolis, MN). Recombinant human IGF-1 was purchased from Austral Biologicals (San Ramon, CA).

Cell viability assays

Growth and inhibition of growth was assessed by Syto60 staining (Invitrogen). Cells were fixed with 4% formaldehyde for 20 min at 37^{o}C and incubated with a 1:5000 dilution of Syto60 stain for 60 min. Cell density in each well was determined with an Odyssey Infrared Imager (LiCor Biosciences), corrected for background fluorescence from empty wells and normalized to untreated wells, as described previously (Rothenberg et al., 2008).

For 72 hour viability assays, cells were seeded in 96-well plates and exposed to the indicated TKIs (3.3 nM to 10 μM) alone or in combination with the indicated concentrations of HGF or IGF ligand. The number of cells used per experiment was determined empirically and has been previously established (Mukohara et al., 2005). All experimental points were set up in six to twelve wells and all experiments were repeated at least three times. The data was graphically displayed using GraphPad Prism version 5.0, (GraphPad Software; www.graphpad.com). The curves were fitted using a non-linear regression model with a sigmoidal dose response.

Antibodies and Western Blotting

Cells grown under the previously specified conditions were lysed in the following lysis buffer: 20 mM Tris, pH 7.4/150 mM NaCl/1% Nonidet P-40/ 10% glycerol/1 mM EDTA/1 mM EGTA/5 mM sodium pyrophosphate/50 mM NaF/10 nM β -glycerophosphate/1 mM sodium vanadate/0.5 mM DTT/4 μg/ml leupeptin/4 μg/ml

pepstatin/4 μg/ml apoprotein/1 mM PMSF. Lysates were centrifuged at 16,000 × *g* for 5 min at 4°C. The supernatant was used for subsequent procedures. Western blot analyses were conducted after separation by SDS/PAGE electrophoresis and transfer to nitrocellulose or PVDF membranes. Immunoblotting was performed according to the antibody manufacturer's recommendations. Antibody binding was detected using an enhanced chemiluminescence system (PerkinElmer, Waltham, MA). Anti-phospho-Akt (Ser 473), anti-phospho-ERBB-3 (Tyr-1289), anti-phospho-p42/44 MAP kinase (Thr 202 Tyr 204), anti-p42/44 MAP kinase, anti-phospho-S6 ribosomal protein (Ser 335/236), anti-S6 ribosomal protein, anti-phospho MET (Tyr 1234/1235), anti-MET (25H2), anti-phospho HER2 (Tyr 1211/1222) and anti-phospho-Tyr-100 antibodies were from Cell Signaling Technology (Beverly, MA). Anti-ERBB3, anti-AKT, and anti-EGFR antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The phospho-specific EGFR (Tyr1068) antibody was from AbCam (Cambridge, MA). The anti-HER2 antibody was from Oncogene Research Products now Calbiochem (SanDiego, CA). The anti-p85 antibody used for immunoprecipitations was from Millipore (Billerica, MA).

Western Blot quantification

Western blots images were captured using GeneSnap image acquisition software and analyzed using GeneTools manual band quantification (SynGene: www.syngene.com). To determine the % rescue values used in Table S1, we used the following equation: (experimental value- value with TKI alone)/(untreated value- value with TKI alone)*100, where experimental value is defined as the value with TKI treatment in combination with

the indicated ligand.

Xenograft Studies

Nude mice (nu/nu; 6-8 weeks old; Charles River Laboratories) were anesthetized using a 2% Isoflurane (Baxter) inhalation oxygen mixture. A suspension of $5x10^6$ HCC827 PFR6 or HCC827-HGF cells (in 0.2 ml of PBS) was inoculated subcutaneously into the lower-left quadrant of the flank of each mouse. Mice were randomized to 4 treatment groups (n=5 per group) once the mean tumor volume reached ~500 mm^3. PF2341066 was dissolved in sterile water and administered at 25mg/kg/day. PF00299804 was dissolved in a 0.05N lactate buffer and administered at 10 mg/kg/day. Gefitinib was dissolved in polysorbite vehile and administered at 150mg/kg/day. For combination studies oral administration of the two agents was separated by 1 hour. Tumors were measured twice weekly using calipers, and volume was calculated using the formula (length x $width^2$ x 0.52). Mice were monitored daily for body weight and general condition. The experiment was terminated when the mean size of either the treated or control groups reached 2000 mm^3.

Statistical Analyses

For xenograft studies, tumor volume was analyzed by a mixed linear model, assuming an unstructured covariance matrix for the random effects and an AR(1) structure for the within-mouse correlation. Volume measurements were transformed into the log scale for analysis in order to approximate a linear fit, reflecting the exponential growth of tumors. A quadratic effect was included in the model for Figure 3E to account for the initial

tumor shrinkage followed by subsequent re-growth in the treatment group with gefitinib alone. For comparison of HGF immunohistochemistry results (Figure 8A), the HGF scores in paired NSCLC patient specimens were compared using the Wilcoxon signed rank test. A one sided p-value was used to test the hypothesis that HGF scores were higher in the drug resistant compared to the pre-treatment specimens.

sRNA transfections and Lentiviral Infections

Hs_GAB1_6 HP validated siRNA and AllStars Negative Control siRNA were obtained from Qiagen (Valencia, CA). HCC827 cells were seeded 200K cells/well on a 6-well plate in RPMI media without penicillin or streptomycin. Cells were transfected with 50nM siRNA with using 18µL HiPerFect Transfection Reagent (Qiagen) in Opti-MEM Reduced Serum Media from Gibco Invitrogen (Frederick, MD) and incubated at 37^{o}C for 48 hours. HGF cDNA was obtained from Open Biosystems and cloned into a lentiviral destination vector, pWPI, using the Gateway cloning system (Invitrogen), and infections were performed as previously described (Rothenberg et al., 2008).

EGFR and MET genomic analyses

The *EGFR* tyrosine kinase domain (exons 18-21) from the HCC827 PFR clones were examined for genetic alterations using a modification of previously described sensitive gene scanning methods (Engelman et al., 2006; Janne et al., 2006). The PCR primers and conditions are available upon request. The relative copy number for *MET* was determined using quantitative real time PCR using a PRISM 7500 sequence detection kit (Applied

Biosystems) and a QuantiTect SYBR Green PCR Kit (Qiagen, Inc., Valencia, CA) and as previously described (Engelman et al., 2007b).

FACS analysis for GFP positive cells

Cells were harvested, resuspended in 0.5% FBS in phosphate buffered saline, and stained with 1μg/mL Propidium Iodide (PI) Staining Solution (BD Biosciences, San Jose, CA). Cells were analyzed on a FACSAria flow cytometer (BD Biosciences, San Jose, CA) equipped with a 30mW Argon air-cooled laser and signals were detected through a 530/30 bandpass filter for GFP and 575/26 bandpass for PI.

HGF immunohistochemistry

Immunohistochemistry for HGF protein was performed on positively charged glass slides containing 5-micron sections of formalin-fixed, paraffin-embedded tissue. Slides were deparaffinized in Hemo-De for 10 min and rinsed (6 washes) into absolute ethanol, before endogenous peroxidase was blocked with 1.05% hydrogen peroxide in ethanol for 30 min. Blocked slides were rinsed in water and microwave antigen retrieval was performed in citrate buffer (pH 6.0). The sections were incubated overnight, at room temperature, with primary antibody (mouse monoclonal anti-HGF, generously contributed by Dr. George Vander Woude) diluted 1:300 in TBS. After incubation, excess primary antibody was rinsed off with TBS, and the sample was washed in TBS with 0.02% BRIJ for 10 min. Signal was amplified by secondary incubation with

EnvisionPlus (DAKO, Carpinteria, CA), for 30 min. Bound antibodies were detected by DAB chromogenic (brown) reaction, after 5 min incubation.

Slides were interpreted by a board-certified anatomic pathologist (NIL), who evaluated the percentage of cancer cells with positive cytoplasmic and/or membranous staining (0 – 100%), and the modal intensity of the positively-staining cells on a scale from 0 to 4+ (Figure S6A). The percentage and the intensity were multiplied to give a scoring index ranging from 0 – 400.

Supplemental References

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