Supplementary Data

Acquisition of mutations and development of bypass tracks cause acquired resistance to MET inhibitors.

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Supplementary Figures

Supplementary Figure Legends

Supplementary Materials and Methods

Supplementary Figures

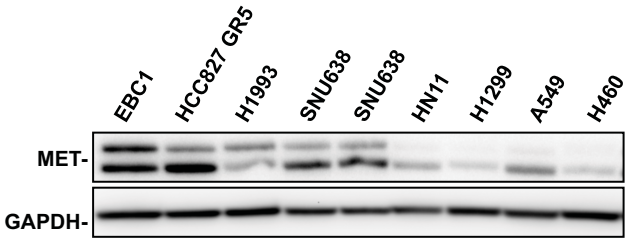


Fig. S1

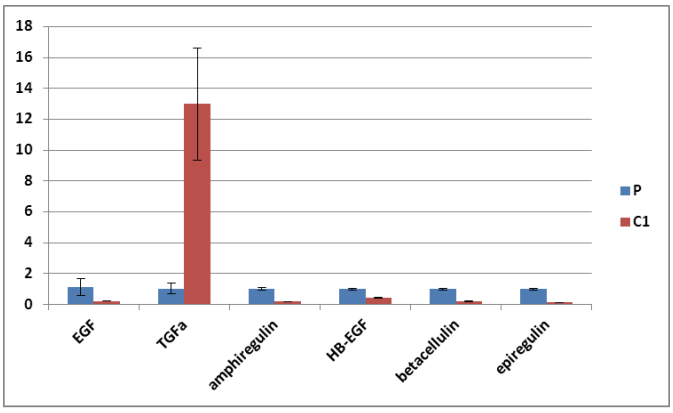
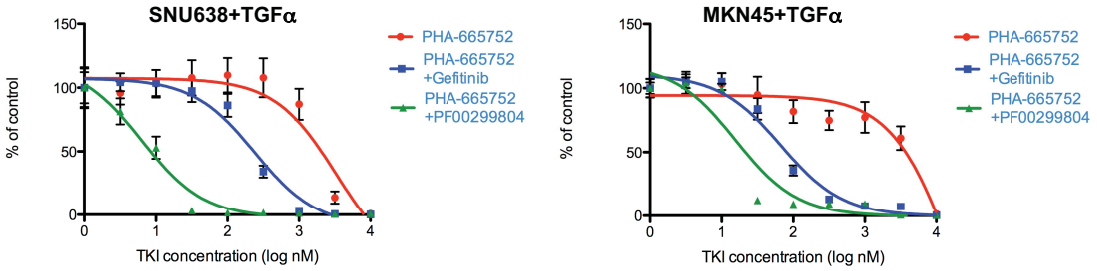


Fig. S2

(A)


SNU638+TGFα
% of control
150
100
50
0
0
1
2
3
4
TKI concentration (log nM)
PHA-665752
PHA-665752
+Gefitinib
PHA-665752
+PF00299804
MKN45+TGFα
% of control
150
100
50
0
0
1
2
3
4
TKI concentration (log nM)
PHA-665752
PHA-665752
+Gefitinib
PHA-665752
+PF00299804

(B)

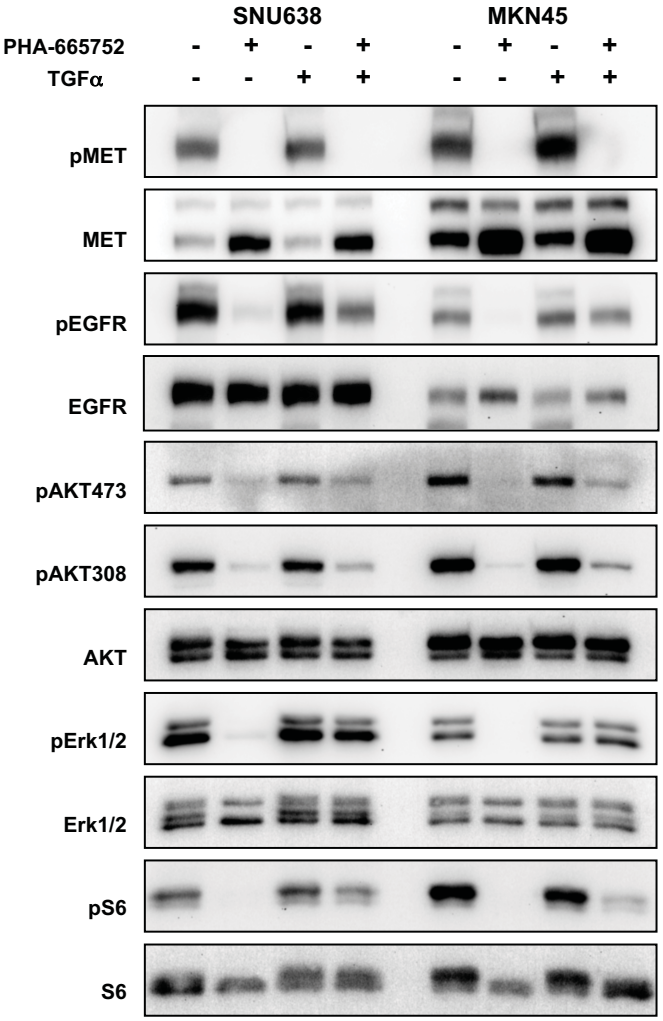

SNU638
MKN45
PHA-665752 - + - + - + - +
TGFα - - + + - - + +
pMET
MET
pEGFR
EGFR
pAKT473
pAKT308
AKT
pErk1/2
Erk1/2
pS6
S6

Fig. S3

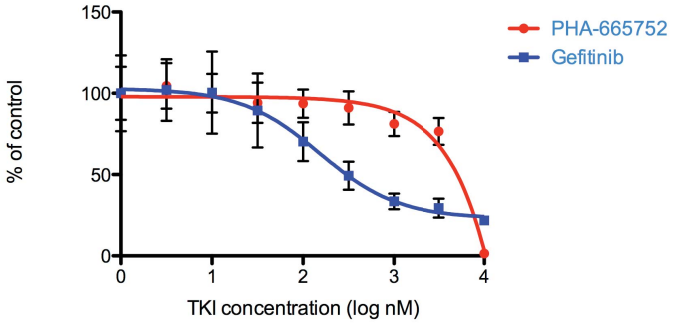

150
100
50
0
% of control
0
1
2
3
4
TKI concentration (log nM)
PHA-665752
Gefitinib

Fig. S4

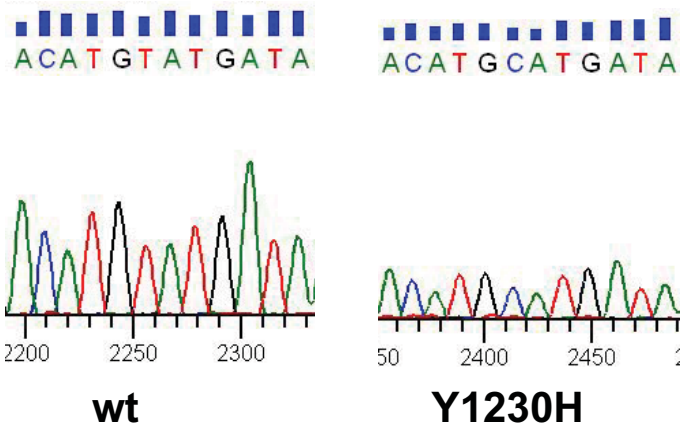

ACATGTATGATA
ACATGCATGATA
2200
2250
2300
50
2400
2450
wt
Y1230H

Fig. S5

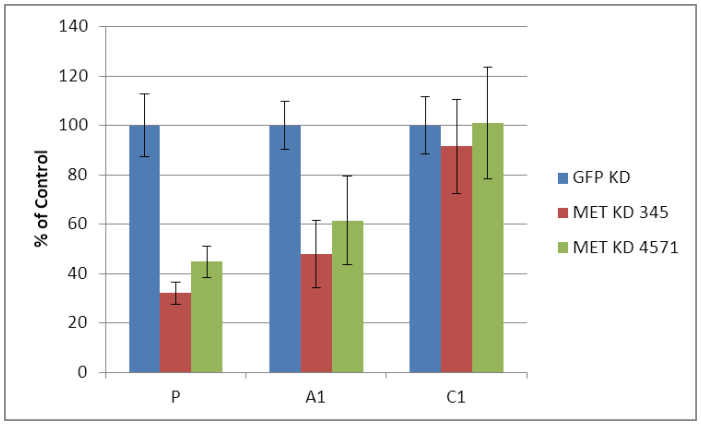

140
120
100
80
60
40
20
0
% of Control
P
A1
C1
GFP KD
MET KD 345
MET KD 4571

Fig. S6

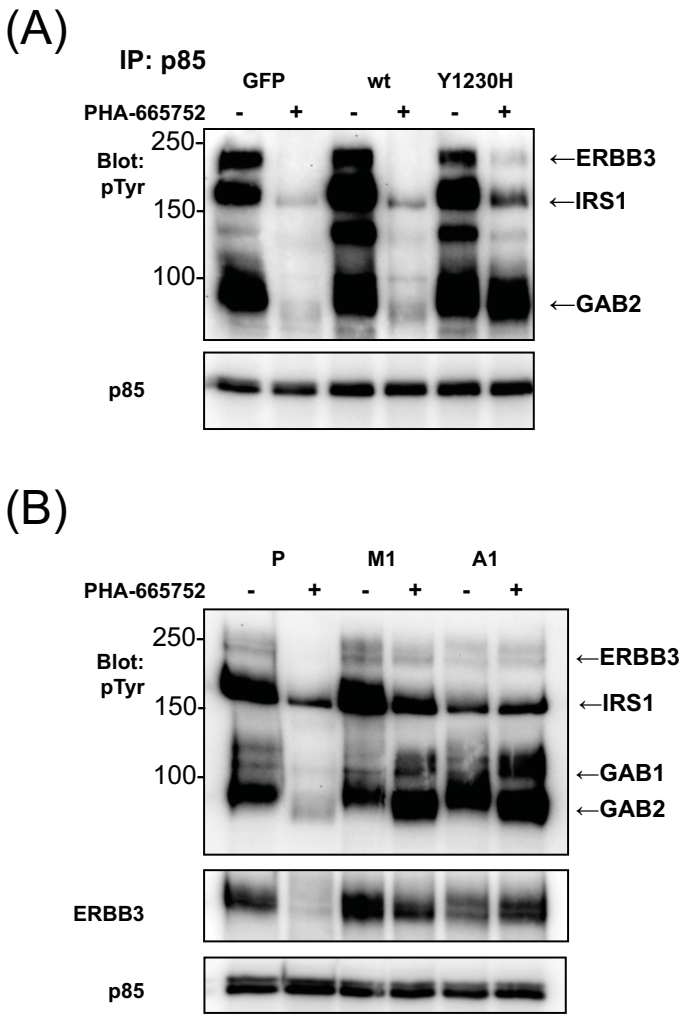
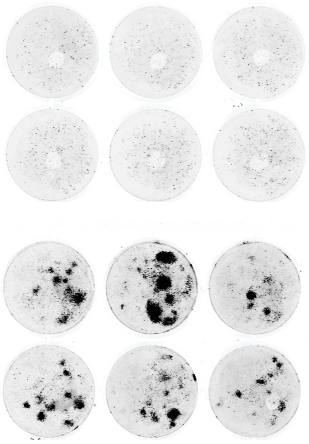
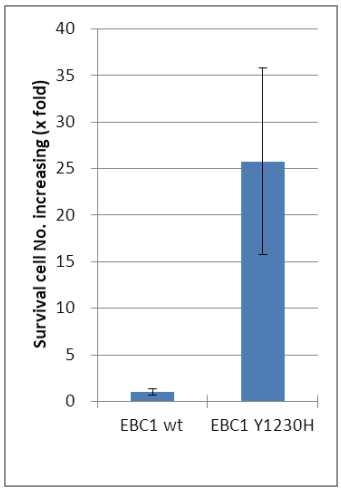

(A)
IP: p85
GFP
wt
Y1230H
PHA-665752
- + - + - +
250
150
100
Blot:
pTyr
←ERBB3
←IRS1
←GAB2
p85
(B)
P
M1
A1
PHA-665752
- + - + - +
250
150
100
Blot:
pTyr
←ERBB3
←IRS1
←GAB1
←GAB2
ERBB3
p85

Fig. S7

(A)


EBC1
MET wt
MET
Y1230H


Survival cell No. increasing (x fold)
40
35
30
25
20
15
10
5
0
EBC1 wt
EBC1 Y1230H

(B)

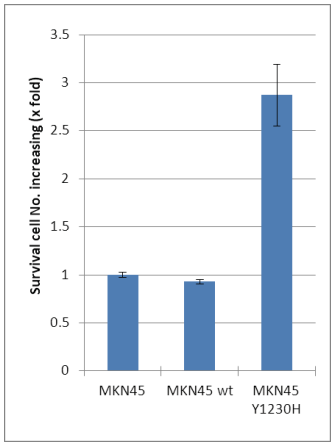
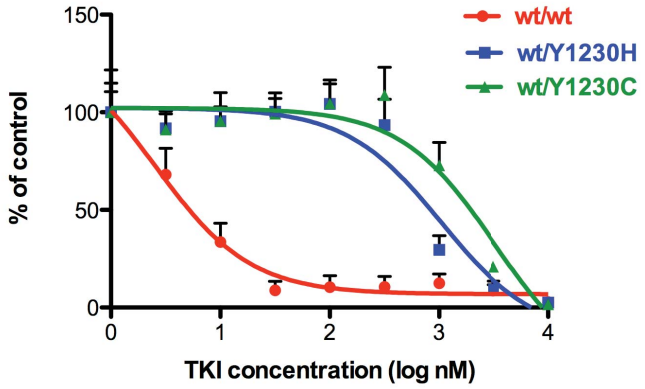
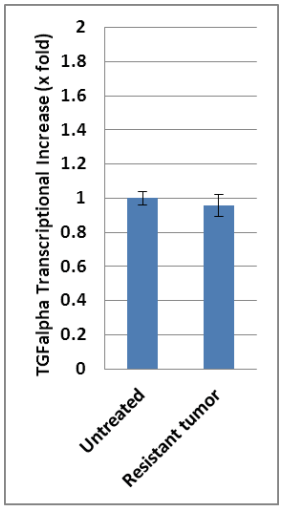

Survival cell No. increasing (x fold)
3.5
3
2.5
2
1.5
1
0.5
0
MKN45
MKN45 wt
MKN45 Y1230H

Fig. S8

(A)


wt/wt
wt/Y1230H
wt/Y1230C
% of control
150
100
50
0
0
1
2
3
4
TKI concentration (log nM)

(B)


TGFalpha Transcriptional Increase (x fold)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Untreated
Resistant tumor

(C)

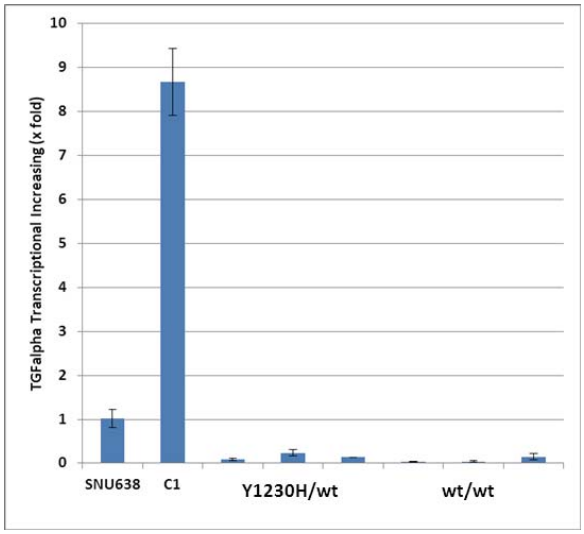
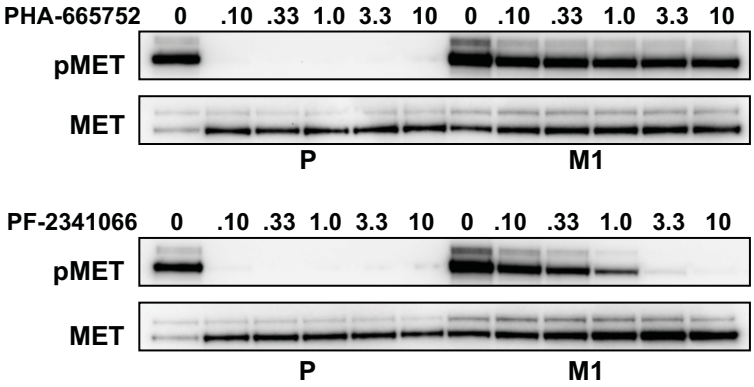

TGFalpha Transcriptional Increasing (x fold)
10
9
8
7
6
5
4
3
2
1
0
SNU638
C1
Y1230H/wt
wt/wt

Fig. S9

(A)


PHA-665752 0 .10 .33 1.0 3.3 10 0 .10 .33 1.0 3.3 10
pMET
MET
P
M1
PF-2341066 0 .10 .33 1.0 3.3 10 0 .10 .33 1.0 3.3 10
pMET
MET
P
M1

(B)

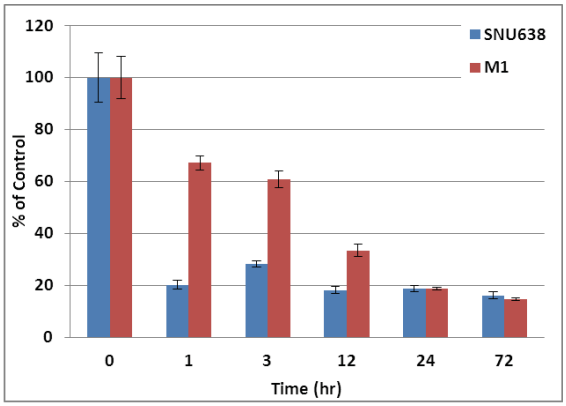

120
100
80
60
40
20
0
% of Control
SNU638
M1
0
1
3
12
24
72
Time (hr)

Fig. S10

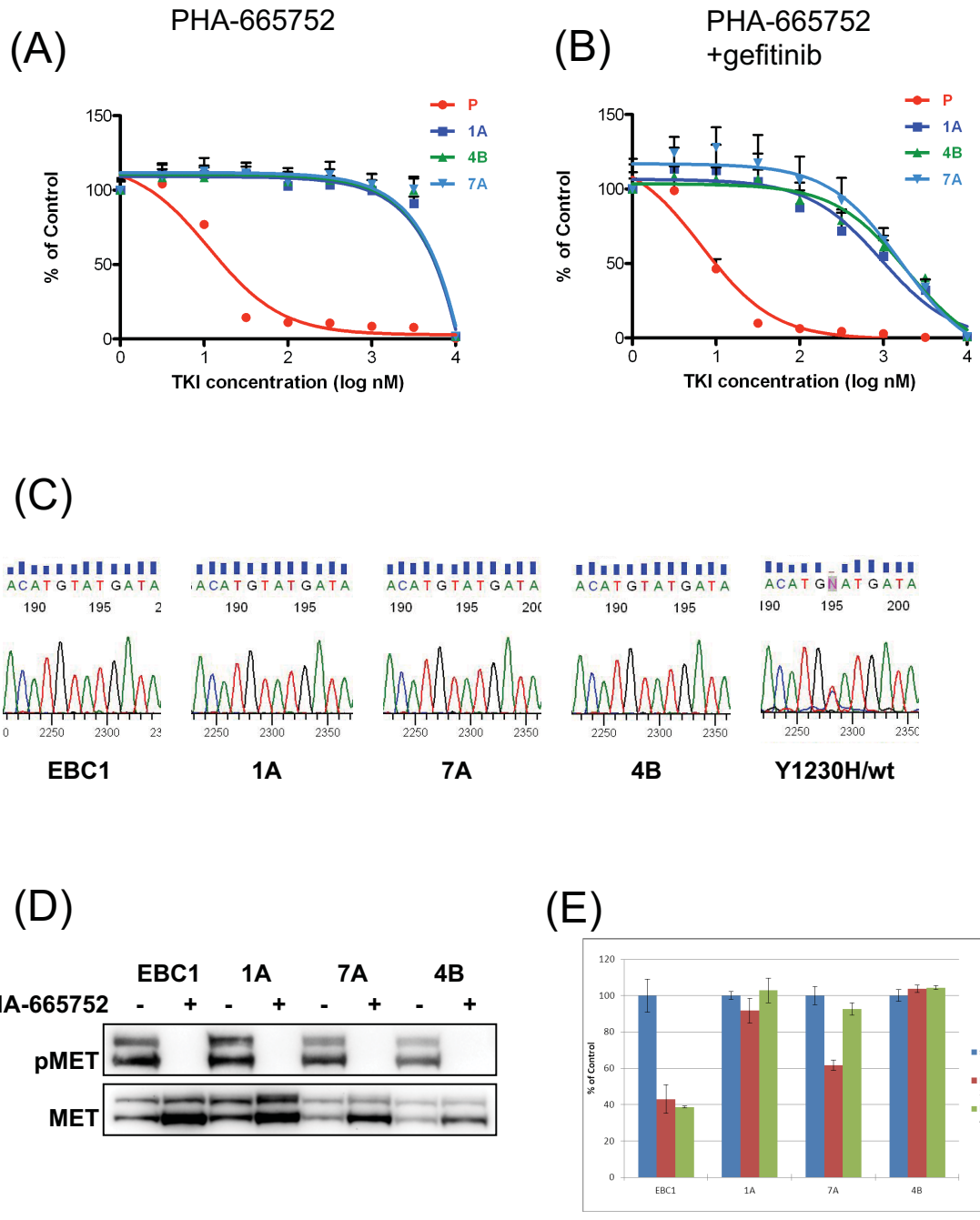

(A)
PHA-665752
P
1A
4B
7A
% of Control
TKI concentration (log nM)
(B)
PHA-665752
+gefitinib
(C)
EBC1
1A
7A
4B
Y1230H/wt
(D)
EBC1
1A
7A
4B
PHA-665752
pMET
MET
(E)
% of Control
scRNA
MET KD 345
MET KD 4571

Fig. S11

Supplementary Figure Legends

Figure 1. SNU638 cells express high levels of MET.

Protein extracts from cells harboring MET amplification (EBC1, HCC827GR6 and H1993) and lines without MET amplification (HN11, H1299, A549 and H460) were assessed for expression of MET. GAPDH is shown to demonstrate comparable protein loading

Figure 2. TGFα is increased in the C1 resistant cells.

Abundance of mRNA was assessed by quantitative real-time PCR using primers targeting EGF, TGFα, amphiregulin, HB-EGF, betacellulin and epiregulin.

Figure 3. Addition of TGFα induces resistance to PHA-665752 in SNU638 and MKN45 cells.

(A) SNU638 and MKN45 cells were treated with 10 ng/mL TGFα in increasing concentrations of PHA-665752 alone or in combination with gefitinib or PF-00299804 for 72 hrs then analyzed by Syto60 survival assays as in Figure 2C.

(B) TGFα activates EGFR and maintains downstream signaling in the presence of PHA-665752. SNU638 and MKN45 cells were treated with +/- PHA-665,752 +/- 10 ng/mL TGFα for 6 hrs, and lysates were assessed by western blot analyses with the indicated antibodies.

Figure 4. PHA-665752 resistant clone C1 cells are not sensitive to single-agent EGFR inhibitors.

C1 cells were treated with increasing concentrations of PHA-665752 or gefitinib for 72 hrs, and survival was measured by Syto60 assay as in Figure 1.

Figure 5. Bacterial clone sequencing confirms the Y1230H mutation.

The RT-PCR product of the kinase domain from A1 cells was sub-cloned into cloning vectors and then transformed into E.coli. for amplification. Individual bacterial clones then picked up for DNA sequencing. One clone with a Y1230H mutation is shown.

Figure 6. A1 cells, but not C1 cells, are sensitive to MET knockdown (KD).

Parental, A1 and C1 cells were infected by lentivirus carrying shRNA against MET (345) and (4571) or GFP control. 2 days after infection, puromycin was added, and numbers of viable cells assessed were normalized to cells infected with control shRNA. Titers were used that gave the same results when experiments were performed in the presence or absence of puromycin selection.

Figure 7. PI3K is activated through ERBB3 and GAB2 in resistant cells despite MET inhibition.

(A) SNU638 cells expressing GFP, MET wt and MET Y1230H (Fig. 4A) were treated with 1μM PHA-665752 for 6 hrs. The PI3K regulatory subunit, p85, was immunoprecipitated from lyates and probed with the indicated antibodies. The pTyr bands that are ERBB3 and GAB2 are demonstrated. (B) SNU638 parental, M1 and A1 cells were treated as in (A), and subjected to immunoprecipation with anti-p85 antibodies. The IP was probed with the indicated antibodies. The pTyr bands that are ERBB3, GAB1, and GAB2 are demonstrated.

Figure 8. EBC1 and MKN45 cells become resistant to PHA-665,752 when they are engineered to express MET Y1230H.

(A) EBC1 cells expressing exogenous MET wt or Y1230H were treated with PHA-665752 for 3 weeks. Viable cells were stained by Syto60 (left panel) and counted (right panel).

(B) MKN45 parental, or cell expressing MET wt or Y1230H were also tested as EBC1. Cell viability was determined by CellTiter-Glo.

Figure 9. Resistance of individual M1 subclones to PHA-665752 *in vitro*.

A) Single clones derived from the M1 cells comprising each genotype (Fig. 5E) were assessed for sensitivity to PHA-665752 by Syto60 assay as in Figure 1.

B) SNU638 xenografted tumors from mice treated with vehicle or after the acquisition of resistance was excised and lysed for RNA exaction. The abundance of TGFα RNA was assessed by quantitative RT-PCR.

C) Abundance of TGFα RNA was determined by quantitative real-time PCR in SNU638 parental cells, the TGFα overexpressing C1 cells (Fig. 2), and clones derived from the resistant tumor developed *in vivo* (Fig. 5).

Figure 10. High- PF-2341066 is required to inhibit MET Y1230H/C.

(A) SNU638 parental and M1 cells were exposed to increasing concentration of PHA-665752 and PF-2341066 for 6hrs. Phospho- and total MET were probed in lysates.

(B) SNU638 parental and M1 cells were exposed to 10 μM PF-2341066 for various durations followed by 3 washes in PBS and repletion with growth media lacking inhibitor. Viable cells were assessed in triplicates after a total 72hrs by Syto60 assay.

Figure 11. EBC1 clones with acquired resistance to MET inhibitors do not acquire a Y1230 mutation and are not sensitive to a combination of MET and EGFR inhibitors.

(A,B) EBC1 parental and resistant cells (1A, 7A and 4B) were assessed for sensitivity to PHA-665752 alone or to PHA-665752 and gefitinib combination treatment as described in Figure 1.

(C) The kinase domain of MET from EBC1 parental and resistant (1A, 7A and 4B) cells was sequenced. Please note that no mutations were observed at Y1230. The sequencing result from MET Y1230H/wt cells is shown as positive control.

(D) EBC1 parental and resistant cells were treated with 1μM PHA-665752 for 6hrs, and lysates were probed with the indicated antibodies.

(E) EBC1 parental and resistant cells were infected by lentivirus expressing control shRNA (scRNA) or MET shRNA (MET KD 345 or 4571). Cell viability was determined 4 days after infection using Syto60 assay as described in Fig 3D.

Supplementary Materials and Methods

Survival assays

Assessment of cell viability was performed as follows. Cells were counted in an automated video-based cytometer (Nexcelom), and seeded in 96 well plates so that the control cells would reach ~80% confluency at the end of the assay. On the following the day, the cells were treated with increasing concentrations of the indicated drugs for 72 hrs. Each concentration was performed in sixplicate (n=6). Cell viability was determined using the Syto60 assay as previously described (1).

The effect of shRNA on cell viability was measured as previously described (2) with minor modifications. Cells were seeded then infected by the indicated shRNA the next day. Media was changed the following day. Cells assessed by Syto60 3 days later. Titers of virus were used that gave the same results in the absence and presence of puromycin.

Site-directed mutagenesis and MET Y1230H construct

The mutation Y1230H of MET was introduced by site-directed mutagenesis following the protocol of Stratagene's QuikChange Site-directed Mutagenesis Kit. The following oligonucleotides were used: sense 5'-ttttggtcttgccagagacatgcatgataaagaatactatagtgta-3' and antisense 5'-tac act ata gta ttc ttt atc atg cat gtc tct ggc aag acc aaaa-3'. The mutation was confirmed by sequencing. The mutant MET gene was recombined into a pLENTI vector (3) and then confirmed by sequencing again.

MET and PHA-665,752 co-crystal structure

The crystal structure of MET kinase domain complexed with PHA-665,752 and experimental methods are available from the Protein Data Bank (wwPDB) with accession codes 2WKM. Human MET protein comprising the kinase domain (residues 1051–1348) was prepared as previously described (4). Crystals were obtained at 13 °C in 1-5 days from hanging drops containing 1.2 μL of purified, nonphosphorylated MET KD (7-13 mg/mL): PHA-665752 solution (~1:5 molar ratio) and 1.2 μL of precipitating solution (0.05 M citrate-phosphate, pH 4.6, 0–275 mM NaCl, 21 % (w/v) PEG-3350). Crystals obtained under these conditions grow as thin plates and belong to space group $P2_12_12$ with cell dimensions: a = 77.4 Å, b = 95.8 Å, c = 46.6 Å, $\alpha=\beta=\gamma=90°$. Prior to data collection crystals were transferred for > 1 minute into a cryoprotectant solution (crystallization buffer + 20% glycerol) and flash frozen in liquid N2. Diffraction data were collected on beamline 5.0.1 at the Advanced Light Source (Berkely, CA). Molecular replacement was performed using a model derived from the previously published PF-2341066 complex structure (PDB ID = 2WGJ). Structural refinement was done using Refmac5 and CNX

version 5.0 (5, 6). The PHA-665752 complex structure has a refined R-factor of 0.215 in the resolution range 500 – 2.2Å.

Sequencing

RNA was isolated using the Qiagen RNeasy kit. The cDNA was transcribed from 1 µg of total RNA with Transcriptor Reverse Transcriptase (Roche Applied Science, Mannheim, Germany). The cDNA was used as template for subsequent PCR amplifications of the full sequence of MET. The primers used for amplification were:

5'-gacagctgacttgctgagagg-3' and 5'-cgaatgcaatggatgatctg-3' for amplicon 1; 5'-atagaagagcccagccagtg-3' and 5'-gctgcaaagctgtggtaaac-3' for amplicon 2; 5'-gatctgccatgtgtgcattc-3' and 5'-gtgcagctctcatttccaag-3' for amplicon 3; 5'-tctgcctgcaatctacaagg-3' and 5'-attcagctgttgcagggaag-3' for amplicon 4; 5'-accgtgaagatcccattgtc-3' and 5'-cgggcacttacaagcctatc-3' for amplicon 5; 5'-ggattgattgctggtgttgtc-3' and 5'-ttcatgcctttggctacttg-3' for amplicon 6; 5'-cgctggtggtcctaccatac-3' and 5'-agtgcaattttggcaagagc-3' for amplicon 7.

Quantitative real-time PCR

Quantitative real-time PCR was performed as described previously (29) with minor modifications. The reaction was carried out on Lightcycler 480 (Roche Diagnostics) using FastStart Universal SYBR Green Master reagent (Roche Diagnostic). The primers for quantification were as follows, 5'-GGCCCTGGCTGTCCTTATC-3' and 5'-AGCAAGCGGTTCTTCCCTTC-3' for TGF , 5'-AAGGTACTCTCGCAGGAAATGG-3'

and 5’-ACATACTCTCTCTTGCCTTGACC-3’ for EGF, 5’-GTGGTGCTGTCGCTCTTGATA-3’ and 5’-ACTCACAGGGGAAATCTCACT-3’ for amphiregulin, 5’-CCCTCCCACTGTATCCACG-3’ and 5’-AGTGACTCTCAAAAGGTCCAGA-3 for HB-EGF, 5’-CCTGGGTCTAGTGATCCTTCA-3’ and 5’-CCTTTCCGCTTTGATTGTGTG-3’ for betacellulin, 5’-CTGCCTGGGTTTCCATCTTCT-3’ and 5’-GCCATTCATGTCAGAGCTACACT-3’ for epiregulin, 5’-TGGCTACTCCTTCGTGACCA-3’ and 5’-GCCGACTCCATACCGATGAA-3’ for actin.

Pulse administration

Cells were seeded at equal number in 6-well plates and exposed to 10 M PF-2341066 the next day for various durations of time from 1hr to 24hrs. The cells were washed 3 times by PBS and cultured in fresh medium without drug. Variable cells were assessed at 72hrs from the initial exposure to pulse administration by Syto60 assay.

Reference

1. Faber AC, Li D, Song Y, et al. Differential induction of apoptosis in HER2 and EGFR addicted cancers following PI3K inhibition. Proc Natl Acad Sci U S A 2009;106:19503-8.

2. Engelman JA, Mukohara T, Zejnullahu K, et al. Allelic dilution obscures detection of a biologically significant resistance mutation in EGFR-amplified lung cancer. J Clin Invest 2006;116:2695-706.

3. Rothenberg SM, Engelman JA, Le S, Riese DJ, 2nd, Haber DA, Settleman J. Modeling oncogene addiction using RNA interference. Proc Natl Acad Sci U S A 2008;105:12480-4.

4. Timofeevski SL, McTigue MA, Ryan K, et al. Enzymatic characterization of c-Met receptor tyrosine kinase oncogenic mutants and kinetic studies with aminopyridine and triazolopyrazine inhibitors. Biochemistry 2009;48:5339-49.

5. Brunger AT, Adams PD, Clore GM, et al. Crystallography & NMR system: A new software suite for macromolecular structure determination. Acta Crystallogr D Biol Crystallogr 1998;54:905-21.

6. Murshudov GN, Vagin AA, Dodson EJ. Refinement of macromolecular structures by the maximum-likelihood method. Acta Crystallogr D Biol Crystallogr 1997;53:240-55.