SUPPLEMENTARY EXPERIMENTAL PROCEDURES

Reagents

For the *in vitro* experiments, erlotinib and gefitinib were obtained from Enzo Life Sciences, docetaxel and cisplatin from Sigma, and the MEK inhibitors CI-1040, PD-0325901 and AZD-6244 from Axon MedChem.

For the *in vivo* experiments, erlotinib was kindly provided by OSI Pharmaceuticals, AZD-6244 was obtained from Selleck Chemicals and GSK-1120212 from Active Biochem. For administration, erlotinib was suspended in 0.5% (w/v) methylcellulose (Colorcon) and injected intraperitoneally. AZD-6244 was suspended in 0.5% (w/v/) methylcellulose and 0.4% polysorbate (Tween 80; Sigma) and administered via oral gavage. The MEK inhibitor GSK-1120212 was suspended in methylcellulose-polysorbate and administered via oral gavage.

Plasmids

The lentiviral pGIPZ shRNAmir constructs targeting NF1 or non-silencing control (SC) were from Thermo Scientific. For packaging, we used pCMV-VSVg (Addgene #8454) and pCMV-ΔR8.2 (Addgene #12263). The retroviral vectors MEK-DD (Addgene #15268) and Myr-AKT1 (Addgene #15294) are described elsewhere (1). The full-length NF1 expressing construct was kindly provided by Dr. D. Lowy (2). The GAP related domain of NF1 (GRD-NF1) expression construct was generated by subcloning this domain into pcDNA-NeoI, which was used as empty vector (ev) control.

Cell survival assays

Cells were seeded into 96-well plates (2000 cells/well). After 24hr, culture medium was replaced by medium containing drugs at indicated concentrations. Cell viability was measured after 72hr using the CellTiter-Blue assay (Promega). Values were normalized to untreated control wells.

Colony formation assay

Single cell suspensions were seeded into 6-well plates (200 cells/well) and cultured in the absence or presence of erlotinib. At the point that untreated cells reached confluency, cells were fixed with 10% TCA, washed and stained with Sulphorhodamine B as previously described (3). The protein-bound dye was dissolved in 10 mM Tris base solution for OD determination at 530 nm using a microplate reader, and normalized to untreated control wells.

Cell competition assays

Non-infected cells (GFP negative) were mixed with shSC- or shNF1-infected cells (GFP positive) in a 100:1 ratio and cultured in the absence or presence of erlotinib for four weeks for PC9 and H4006 or twelve weeks for H3255, after which cells were harvested and analyzed for the percentages of GFP-positive cells by FACS analysis.

RAS activity assay

Cells were cultured in the absence or presence of 30nM erlotinib for 1hr. RAS-GTP levels were detected using a RAS activation assay, following the manufacturer's instructions (Pierce Biotechnology). RAS pull-down assays were resolved by SDS-PAGE alongside total cell lysates as loading controls.

Data Analysis genome-wide siRNA screen

Each screening plate was normalized using a robust Z-score calculation where the median value of samples on each plate was subtracted from each well. Each well was then divided by the median absolute deviation (MAD) of all sample wells on that plate. In order to account for positional and edge effects a smoothed Z-score was also calculated. This was based on the median and MAD calculated when comparing the distribution of Z-scores at each well position across all plates within the screen. These smoothed Z-scores are included in Table S1. In order to identify differences between drug and control screens, the smoothed Z-score from the drug screen was plotted against the smoothed Z-score from the control screen for each replicate (nine comparisons in total). The residual difference for each data point was then calculated as the perpendicular distance between the data point and the line of best fit (as calculated by linear regression). Negative residual differences represent those genes where the viability score within the drug screen is lower than would be expected based on the

control viability, whereas positive scores indicate genes with viability within the drug screen that is higher than expected based on the control viability.

We selected siRNAs for further validations if the median residual difference was greater than or equal to 2, or smaller than or equal to -2, and excluded the siRNAs with a smoothed Z-score smaller than -2 in the control plates, as these siRNAs substantially kill the cells in the absence of the drug. These siRNAs are included in Table S2. We furthermore excluded the siRNAs that did not validate in the top 5% of the independently performed repeat genome-wide screen. The resulting selection of siRNAs (indicated in bold in Table S2) was screened in a deconvoluted siRNA screen.

Data analysis deconvolution siRNA screen

As the siRNAs within the second round deconvolution screen were pre-selected for likely differences, a Z-score normalization based on background plate medians was not applicable. Instead we used a percent of control normalization where each well value was divided by the median SC control value on each plate. To establish the influence of siRNA-induced knockdown of gene expression on drug sensitivity, the individual effects of drug and siRNAs on viability were taken into account. For this, an index of antagonism or sensitivity (SI) for each siRNA was calculated by subtracting the observed combined effect of drug and siRNA from the expected total viability effect: $SI = (Rc/Cc*Cd/Cc) – Rd/Cc$, as previously published (4). In order for the SI to usefully predict antagonism or sensitivity, an additional criteria that $Rd/Cd > 1.05$ (for antagonistic siRNAs) and $Rd/Cd < 0.95$ (for sensitizing siRNAs) was employed for hit selection.

Desensitizing siRNAs ($SI \leq -0.10$) and sensitising siRNAs ($SI \geq 0.10$) were included in the final dataset (presented in Figure 1B) if the siGENOME SMARTpool and at least two out of four of the individual deconvoluted siRNAs had a reproducible effect on the erlotinib response.

Transfections and viral transductions

For the studies using retroviral infections, PC9 cells expressing the murine ecotropic receptor were generated. Retroviral transductions were performed using Phoenix cells as producers of viral supernatants containing MEK-DD or myr-AKT. For the studies re-expressing NF1-GRD, cells were transfected using Lipofectamine 2000 (Invitrogen) and selected for successful integration of the constructs.

For the studies using lentiviral infections, 293T cells cultured in DMEM, supplemented with 10% FBS, streptomycin and penicillin, were infected with the lentiviral shRNAmir construct in combination with the packaging plasmids VSVg and Δ8.2 using Lipofectamine 2000 (Invitrogen). Viral supernatants were concentrated by ultracentrifugation, and added to the culture medium. Cells with successful lentiviral integration were selected by puromycin. A FACS sort for the top 25% GFP expressing cells was performed to obtain cells with a high expression of the lentiviral shRNAmir construct.

Western blot analysis

Cells were plated in 6-well plates ($1.5x10^5$ cells/well). The following day, cells were treated with the indicated dose of drugs for 1hr. Cells were lysed in 1% Triton-X100 lysis buffer, incubated at 4°C for 20 min and insoluble material was removed by centrifugation. The amount of protein in the supernatant was determined using the Bio-Rad Bradford protein assay (Bio-Rad Biotechnologies). Equal amounts of total protein were resolved by electrophoresis on NuPage 4-12% Bis-Tris gels (Invitrogen) and blotted on Immobilon-P transfer membranes (Millipore). Immunoblots were developed using the ECL Western Blotting Detection Reagent (Amersham).

The following antibodies were used: pEGFR (Y1068, #2236), EGFR (#2232), pErk1/2 (#9101), Erk1/2 (#9102), pAKT (T308 #9257; S473 #9271), AKT (#2920), pS6 Ribosomal Protein (#2211) and S6 Ribosomal Protein (#2217), all from Cell Signaling Technologies; NF1 (#A300-140) from Bethyl and tubulin (T5168) from Sigma.

Quantitative RT-PCR of cell lines and murine samples

Total RNA was isolated from cells using RNeasy Mini Kit (Qiagen). Quantitative RT-PCR assays were performed with QuantiTect SYBR Green RT-PCR Kit (Qiagen), using 7500 Fast Real-Time-PCR system (Applied Biosystems) according to manufacturer's instructions. All primers were QuantiTect Primers obtained from Qiagen, with the exception of NF1-GRD primers that were as described by Holzel et al. (5). Relative mRNA levels were analyzed using the comparative C_T method and normalized to the housekeeping gene GAPDH.

For the mouse samples, total RNA extracted from macrodissected tumors or adjacent lung was used to synthesize cDNA. The cDNA was then amplified using Nf1,

Sftpc (Surfactant Protein C) or Met Taqman Assays (Applied Biosystems) on a viiA7 Real Time PCR Machine. Normalization to Sftpc was used to ensure that the representation of lung epithelial cells in each tumor was accounted for.

Analysis of EGFR or Ras mutations in murine tumors

RNA from flash-frozen tumor nodules was extracted using the AllPrep DNA/RNA kit as per manufacturer's instructions (Qiagen). cDNA was synthesized using the Superscript III First-Strand Synthesis SuperMix for qRT-PCR kit from Invitrogen, and used as a template to amplify the regions of interest (T790M and L858R of the EGFR transgene and codons 12,13 and 61 of Kras, Hras and Nras) for Sanger sequencing. PCR products were sequenced on an Applied Biosystems 3730xl DNA Analyser.

EGFR and MEK inhibitor treatment of CCSP-rtTA / EGFR^{L858R} animals

The *CCSP-rtTA* and *TetO-EGFR*^{L858R} mice were obtained from the Jackson Lab and Mouse Repository respectively, and have been described previously (6-8). Doxycycline was administered by feeding mice with doxycycline-impregnated food pellets (625 ppm) (Harlan-Teklad). Erlotinib, kindly provided by OSI Pharmaceuticals, was suspended in 0.5% (w/v) methylcellulose and injected intraperitoneally at times indicated in the experiments, 5 days a week at 25mg/kg/day. Tumor responses were followed using micro-CT. Once tumors failed to respond to erlotinib, erlotinib was either continued for another 4 weeks alone, or combined with the MEK inhibitor GSK-1120212 (purchased from Active Biochem), resuspended in methylcellulose-polysorbate) and administered by oral gavage 5 days a week at 25mg/kg/day for erlotinib and 2.5mg/kg/day for GSK-1120212.

Micro-CT

Mice were anesthetized with 2% isofluorane oxygen gas, and images of the lung region were acquired on a SkyScan 1176 micro-CT analyzer. Data were sorted using Tsort (SkyScan) and reconstructed using the NRecon program (SkyScan). Reconstructed data were subsequently imaged using DataViewer and CTAn programs. The volume of individual tumor nodules was quantified using CTAn as per manufacturer's instructions (SkyScan).

Xenograft experiments

Animals were maintained and bred in the London Research Institute Biological Resources. All experimental procedures were approved by London Research Institute ethics committees and conform to the UK Home Office regulations.

1 x 10^6 PC9 cells stably infected with shRNA targeting NF1 or control shRNA, or $PC9^{T790M}$ cells were injected subcutaneously in one flank of 6-week-old Balb/c female mice (Charles Rivers). Tumors were measured twice a week with callipers, and tumor volume in mm^3 was calculated according the formula: $width^2$ x length/2. Once tumors were detectable, mice were randomized to receive the following treatments for 30 days; 1: alternating days erlotinib (25mg/kg/day) by intraperitoneal (i.p.) injection and methylcellulose-polysorbate by oral gavage (o.g.), 2: alternating days methylcellulose by i.p. and AZD-6244 (25mg/kg/day) by o.g., 3: alternating days with erlotinib (i.p.) and AZD-6244 (o.g.) or 4: alternating days with the vehicles methylcellulose (i.p.) and methylcellulose-polysorbate (o.g.). Erlotinib was suspended in 0.5% (w/v) methylcellulose (Colorcon) and AZD-6244 in 0.5% (w/v/) methylcellulose and 0.4% polysorbate (Tween 80; Sigma).

For statistical analysis of the growth curves, data were analyzed using R 2.12.2. Data were rounded using the function ceiling. The function glm.nb was used to estimate the dispersion parameters, which were used to fit models with the function glm and the family negative.binomial. Nested models were compared using anova, and the same function was used to test for significant differences between groups. Difference from zero of model coefficients was assessed using the summary function. For clarity, the final model formula used for both data sets was volume ~ day + group:day.

SUPPLEMENTARY TABLE AND FIGURE LEGENDS

Table S1. Tabulated complete data from the first genome-wide screen.

Table S2. Tabulated data from the 242 siRNA pools identified in the first genome-wide screen with the Z-scores obtained in the repeat genome-wide screen. siRNAs that scored in the top 5% of the repeat screen are marked in bold.

Table S3. Summary of the individual tumors with treatment response.
T790M status, presence of high *Met* expression (>4-fold upregulation compared to adjacent normal tissue) and *Nf1* expression relative to adjacent normal lung are indicted.

Table S4. Clinical characteristics of human lung adenocarcinoma samples.
Treatment response is tabulated for each tumor pair used in Figure 7A, as well as the tumor cell content in each analyzed sample.

Figure S1, related to Figure 1. Genome-wide siRNA screen identifies determinants of resistance to erlotinib treatment.
Plot of the Residual Z-scores of all siRNA pools ranked by size of effect. The siRNA pools that validated in the repeat screen are depicted in red.

Figure S2, related to Figure 2. Reduced *Nf1* expression in erlotinib-resistant EGFR-driven lung tumors.
Quantitative RT-PCR analysis for Nf1 expression in mouse erlotinib-resistant mouse tumors using two additional Taqman assays (Nf1a and Nf1b). The samples were analyzed as described in the legend to Figure 2.

Figure S3, related to Figure 3. *NF1* silencing confers resistance of lung adenocarcinoma cells to erlotinib.
A. Knockdown of NF1 increases cell survival upon treatment with the EGFR TKI gefitinib, but not docetaxel. PC9 cells are uninfected or stably infected with shRNAs containing a non-silencing control sequence (shSC) or an NF1-targeting sequence

(NF1#1 or #2). Cells were treated with indicated concentrations of gefitinib or docetaxel for 72 hours before cell viability was measured and survival normalized to untreated controls. Error bars denote SEM.

B. Western blot confirming NF1 protein downregulation in cells infected with an shRNA construct targeting NF1 (shNF1) compared to a non-targeting control (shSC) in NSCLC cell lines H3255, HCC4006 and HCC827.

C. Indicated cell lines were uninfected or stably infected with shRNA containing a non-silencing control sequence (shSC) or an NF1-targeting sequence (shNF1#2). Cells were treated with the indicated concentrations of erlotinib for 72hr before cell viability was measured by Cell Titer Blue and normalized to untreated controls. Error bars denote standard error of the mean (SEM). Drug concentrations inducing 50% inhibition in survival (IC_{50}-values; nM) are indicated for each cell line.

D. GFP negative parental cells were spiked with approximately 1% GFP positive shRNA-infected cells containing a non-silencing control sequence (shSC) or an NF1-targeting sequence (shNF1#2) and cultured for 1 month (HCC4006, HCC827 and H3255) or 3 months (H3255) in the presence or absence of 30nM erlotinib. Cells were collected and the percentage of GFP positive cells was determined using FACS.

E. Knockdown of endogenous NF1 in cells infected with shRNAs targeting NF1 (#1 or #2) is confirmed by immunoblotting with an antibody detecting the C-terminus of NF1, and not the GAP-related domain (GRD) of NF1. qRT-PCR using primers that specifically detect the NF1-GRD confirm the expression of this construct. Error bars denote SD.

Figure S4, related to Figure 4. : *NF1* downregulation activates MAPK pathway signaling.

A. *NF1* silencing prevents complete erlotinib-induced ERK dephosphorylation in multiple EGFR mutant lung adenocarcinoma cell lines. HCC4006 and H3255 cells expressing control shRNA (shSC) or *NF1* targeting shRNA (NF1#2) were left untreated or treated with 30nM erlotinib for 1 hr. Cell extracts were immunoblotted to detect the indicated proteins.

B. *NF1* silencing prevents complete erlotinib-induced ERK dephosphorylation in PC9 cells treated with up to 1μM of erlotinib. Cells were treated with indicated concentrations of erlotinib for 6 hr. Cell extracts were immunoblotted to detect the indicated proteins.

C. Expression of constitutively active MEK (MEK-DD) or AKT (myr-AKT) in PC9 cells. PC9 cells stably expressing the ecotropic receptor (Eco) were infected with MEK-DD or myr-AKT. Cells were incubated for 1 hour with 30nM erlotinib and the phosphorylation status of ERK and AKT were analyzed by immunoblotting. Whereas erlotinib affects the endogenous pERK in the parental Eco-expressing PC9 cells, there is still pERK detectable in the cells transfected with the MEK-DD construct (upper panel). AKT is highly phosphorylated in the myr-AKT transfected cells, which is not affected by erlotinib (lower panel).

D. Cells expressing an active MEK are sensitive to combined EGFR and MEK inhibitor treatment. PC9 cells expressing the ecotropic receptor (Eco) were left uninfected or infected with MEK-DD. Cells were treated with indicated concentrations of erlotinib alone (solid line) or in combination with 1 μM AZD-6244 (dotted line). MEK-DD expressing cells, while being insensitive to erlotinib treatment alone, are sensitive to combined EGFR and MEK inhibition. Error bars denote SEM.

Figure S5, related to Figure 5. Low neurofibromin expressing cells respond to erlotinib when combined with MEK inhibition.

A. shNF1 cells are sensitive to erlotinib when combined with a MEK inhibitor. PC9 cells expressing a non-targeting shRNA construct (shSC) or an shRNA construct targeting NF1 (shNF1#2) were treated with the indicated concentrations of MEK inhibitors CI-1040 or PD0325901 alone (straight line) or in combination with 30nM erlotinib (dotted line) for 72hr before cell viability was measured and survival normalized to untreated controls. Error bars denote SEM.

B. DNA sequencing chromatograms reveal the presence of a minor T790M peak in the population of PC9 cells that acquired resistance by culturing the cells in the presence of 1 μM erlotinib for 3 months ($PC9^{T790M}$). This T790M peak is not detectable in the parental PC9 cell population. Of note, this T-peak is smaller than the wild-type M-peak. This is also the case when we analyze single-cell sorted cells, indicating that one of the EGFR alleles contains the T790M mutations while the other overexpressed EGFR alleles do not.

Figure S6, related to Figure 6. Erlotinib-resistant murine *EGFR*^{L858R} lung adenocarcinomas respond to combined EGFR and MEK inhibition.

Micro-CT images (upper panel) and corresponding 3D reconstructions of air (lower panel) in the lungs and detected tumor nodule from mouse M11; air is visualized in white and tumor nodule in red.
At week 8, diffuse disease was visible and little air remaining, making it impossible to reliably measure tumor volume. A tumor nodule (indicated by a white arrow) was detectable at week 12. However, as the amount of air in the lungs was still low and mouse showed breathing difficulties at week 12, erlotinib treatment was continued for another 4 weeks. The tumor nodule continued to grow in the presence of erlotinib, but did respond to 4 weeks of erlotinib in combination of MEK inhibitor.
Tumor volume measurements are indicated.

Figure S7, related to Figure 7. Reduced *NF1* expression in erlotinib-resistant human NSCLC samples.
A. *NF1* silencing increases cell survival upon treatment with the irreversible EGFR inhibitor afatinib (BIBW-2992). Uninfected PC9 cells or cells stably infected with shRNAs containing a non-silencing control sequence (shSC) or an NF1-targeting sequence (shNF1) were treated with indicated concentrations of afatinib for 72 hours before cell viability was measured and survival normalized to untreated controls. Error bars denote SEM.
B. Selective outgrowth of NF1 silenced T790M-expressing PC9 cells ($PC9^{T790M}$) in the presence of an EGFR TKI. GFP negative $PC9^{T790M}$ cells were spiked with approximately 2.5% GFP positive shRNA-infected cells, non-silencing control (shSC) or targeting NF1 (shNF1#2), and grown for 6 weeks in the presence or absence of a high dose erlotinib (1µM) or afatinib (100 or 250nM). Cells were collected and analyzed for GFP expression by FACS (x-axis). The percentage of GFP positive cells is indicated.

SUPPLEMENTARY REFERENCES

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