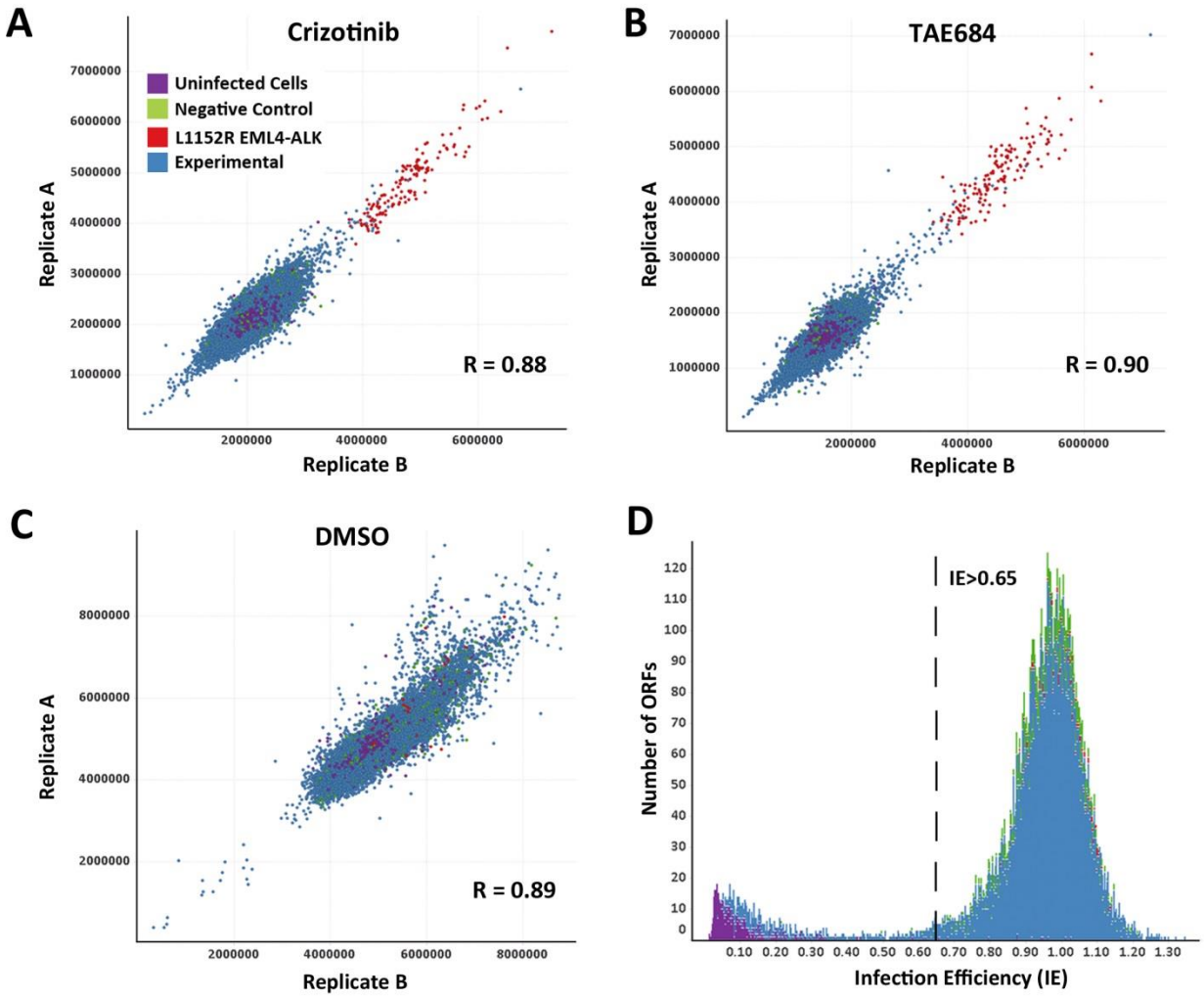


Figure S1, related to Figure 1. Statistical analysis of primary screening dataset and determination of lentiviral infection efficiency. **A.** Raw Cell Titer-Glo luminescence values for both replicates of ORF-expressing H3122 cells treated with crizotinib. Data is shown for all experimental ORFs, negative control ORFs (Lac Z, luciferase, enhanced green fluorescent protein, HcRed, and blue fluorescent protein), the L1152R EML4-ALK positive control, and uninfected control H3122 cells assayed in the screen. Correlation between replicate values for each ORF was determined using Pearson's R. **B.** As in A, except with luminescence values of cells treated with TAE684. **C.** As in A, except with luminescence values of cells treated with DMSO. **D.** Infection efficiency (IE) for ORFs assayed in the screen. IE was expressed as the ratio of raw luminescence of cells treated with blasticidin (selectable marker harbored by the lentiviral expression vector) to luminescence of cells in DMSO. The mean IE of ORF-expressing cells was 93.4% (including experimental ORFs, negative controls, and L1152R EML4-ALK). Uninfected H3122 cells lack the blasticidin selectable marker and are therefore associated with low calculated IE. Experimental ORFs with IE <0.65 were considered failed infections and were not further analyzed (see Supplemental Experimental Procedures).

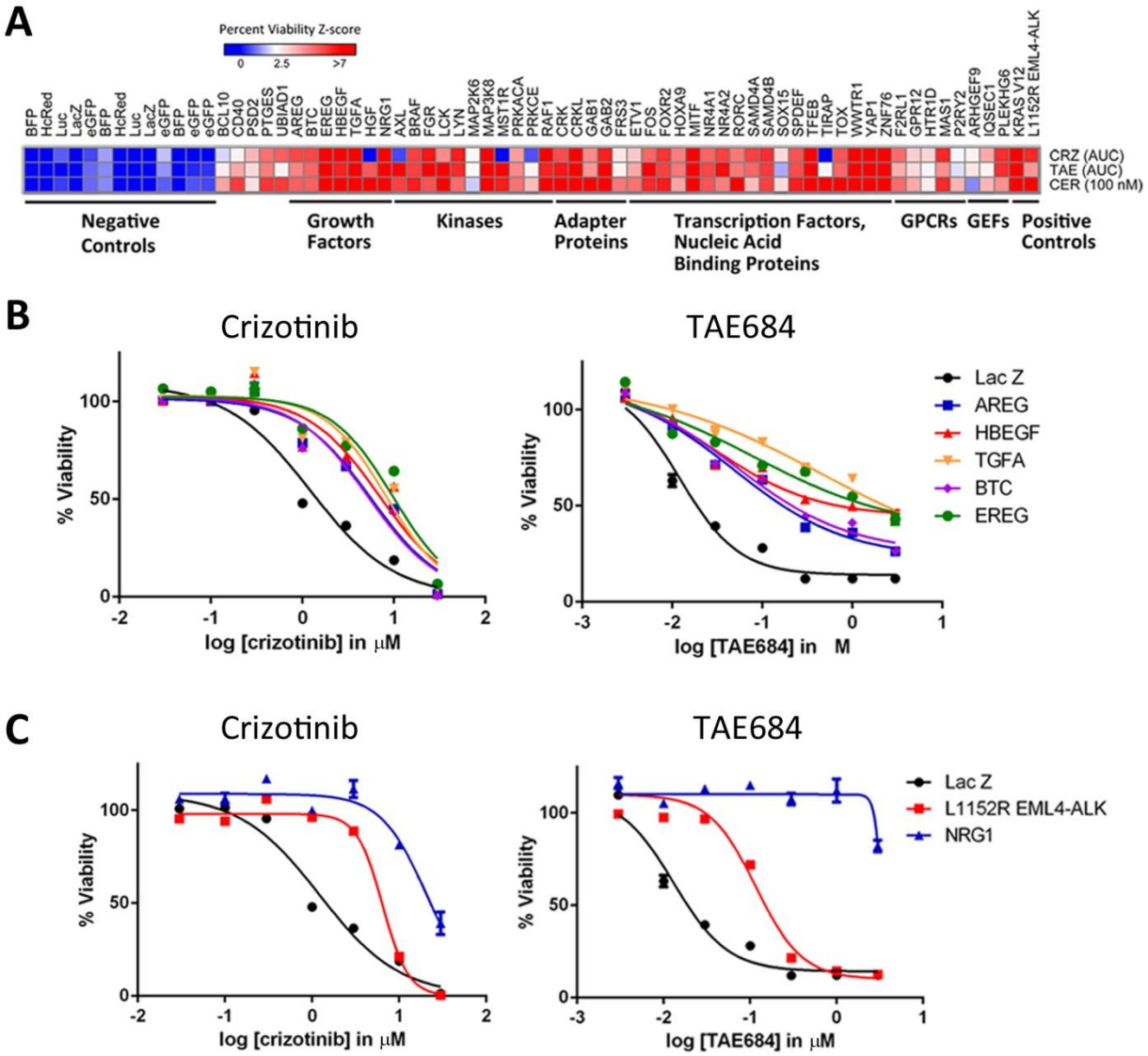


Figure S2, related to Figure 2. A. Resistance ORFs validated against crizotinib and TAE684 in H3122 also drive resistance to ceritinib. As in Figure 2C, a heat map is displayed with normalized AUC values derived from growth curves for validated drivers of resistance to crizotinib (CRZ) and TAE684 (TAE) in H3122. In addition, the normalized % viability of each ORF in the presence of 100 nM ceritinib (CER) in H3122 is shown. GPCRs, G-protein coupled receptors; GEFs, guanine nucleotide exchange factors. **B.** Growth factors drive resistance to ALK inhibition in H3122. ORFs encoding Lac Z or each of the 5 EGF ligands identified as candidate resistance drivers from the screen were introduced into H3122 via lentiviral infection. ORF-expressing cells were exposed to crizotinib or TAE684 at the indicated concentrations for 5 days. Cell viability was determined with Cell Titer-Glo. Mean and standard error of 4 replicates are shown. **C.** As in B, except for neuregulin-1 (NRG1). The growth curve for cells overexpressing the L1152R EML4-ALK positive control is shown for comparison.

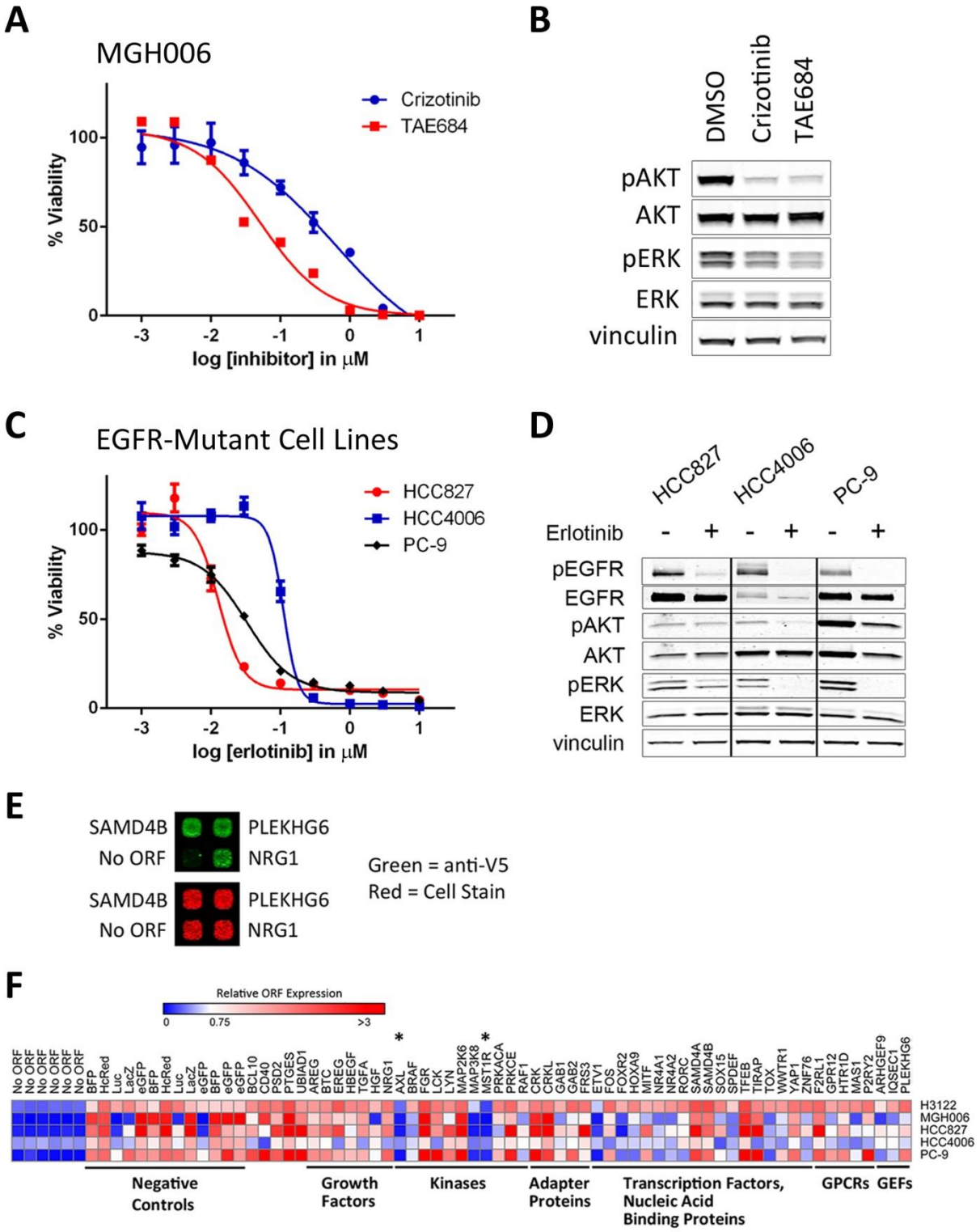


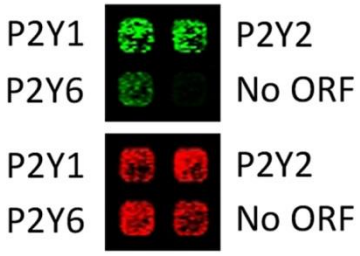
Figure S3, related to Figure 4. A. Cell lines used for validation of screening findings are sensitive to ALK or EGFR inhibition. ALK-rearranged MGH006 cells were exposed to varying concentrations of crizotinib or TAE684 for 5 days. Cell viability was determined with Cell Titer-Glo. Mean and standard

error of 3 replicates are shown. **B.** MGH006 cells were exposed to DMSO, 2 μM crizotinib, or 200 nM TAE684 for 6 hours. Immunoblotting of cell lysates was performed using the indicated antibodies. **C.** EGFR-mutant NSCLC cell lines (HCC827, HCC4006, and PC-9) were exposed to varying concentrations of erlotinib for 3-5 days. Cell viability was assessed as in A. Mean and standard error of 3 replicates are shown. **D.** EGFR-mutant NSCLC cell lines were exposed to 200 nM erlotinib (for HCC827) or 2 μM erlotinib (for HCC4006 and PC-9) for 6 hours. Immunoblotting was performed as in B. **E.** In-cell Western immunostaining confirms ectopic ORF expression in assayed cell lines. V5 epitope-tagged ORFs were introduced via lentiviral infection into H3122 in 384-well format. Staining was performed using an antibody against the V5 epitope tag (green) or with cell staining markers (red) as described in the Supplemental Experimental Procedures. Representative results for expression of 3 ORFs in H3122 are shown. **F.** A ratio of fluorescence intensity (V5/Cell marker) was determined for each ORF in each cell line and displayed as a heat map. For comparison, ratios of V5/Cell marker were determined for 6 wells containing cells without ectopic ORF expression ("No ORF", left). Asterisks indicate 2 ORFs (AXL and MST1R) that lack a V5 epitope tag; expression of these 2 ORFs cannot be demonstrated with this assay. GPCRs, G-protein coupled receptors; GEFs, guanine nucleotide exchange factors.

A

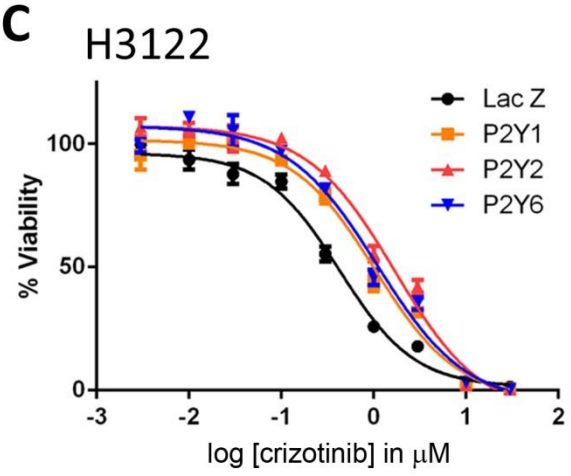
ORF	ORF Infection Efficiency (%)	Viability z-score (crizotinib)	Viability z-score (TAE684)
P2RX1	102.2	-0.14	0.40
P2RX1	99.9	0.63	0.13
P2RX2	104.1	-0.10	-0.36
P2RX4	91.1	-0.29	-0.03
P2RX5	90.9	-0.27	-0.30
P2RX6	87.8	0.40	-0.10
P2RY1	94.9	2.60	1.83
P2RY2	99.1	5.29	4.62
P2RY4	100.2	1.35	0.04
P2RY6	94.7	2.90	2.84
P2RY8	91.3	0.22	0.27
P2RY10	98.1	0.49	-0.40
P2RY11	109.2	0.82	0.28
P2RY12	101.3	0.92	0.56
P2RY13	94.6	0.49	1.41
P2RY14	106.9	-0.20	-0.28

B



Green = anti-V5
Red = Cell Stain

C



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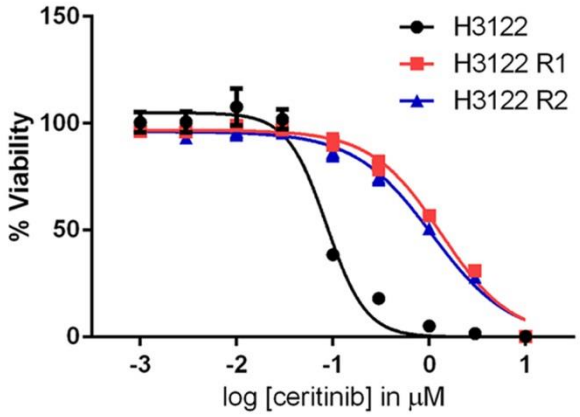

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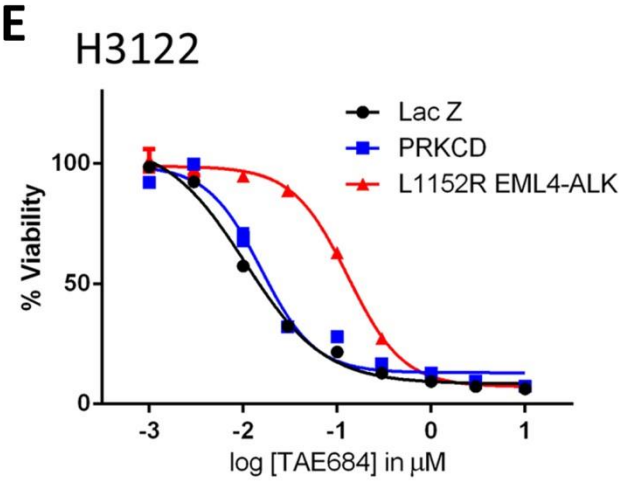

F

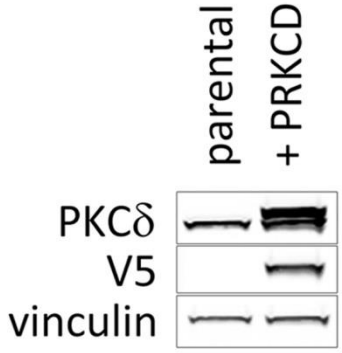

Figure S4, related to Figure 6. A. P2Y1, P2Y2, and P2Y6 mediate resistance to ALK inhibition in H3122 cells. Members of the P2X and P2Y receptor subfamilies represented in the CCSB-Broad Lentiviral Expression library are shown. Percent viability z-scores determined from the screen in the presence of crizotinib or TAE684 are shown for each ORF (along with infection efficiency). Results for *P2RY1*, *P2RY2*, and *P2RY6* (encoding P2Y1, P2Y2, and P2Y6 respectively) are highlighted. **B.** Ectopic expression of P2Y receptors in H3122 was confirmed by in-cell Western. V5 epitope-tagged P2Y receptors were introduced via lentiviral infection into H3122. Staining was performed using an antibody against the V5 epitope tag (green) or with general cell staining markers (red) as described in Supplemental Experimental Procedures. **C.** H3122 cells expressing Lac Z, P2Y1, P2Y2, or P2Y6 were treated with the indicated concentrations of crizotinib for 5 days. Mean and standard error of 3 replicates are shown. **D.** H3122 R1 and R2 cells are insensitive to ALK inhibition with ceritinib. H3122 cells were cultured to resistance with ceritinib to generate H3122 R1 and H3122 R2 cells (see Supplemental Experimental Procedures). H3122, H3122 R1, and H3122 R2 cells were exposed to the indicated concentrations of ceritinib. Cell viability was determined after 5 days of drug exposure. Mean and standard error of 3 replicates are shown. **E.** Overexpression of PKCδ (encoded by *PRKCD*) is not sufficient to drive resistance to ALK inhibition in H3122. H3122 cells were spin-infected with the indicated ORFs and exposed to varying concentrations of TAE684. Cell viability was determined with Cell Titer-Glo after 5 days of drug exposure. Mean and standard error of 4 replicates are shown. **F.** To confirm ectopic expression of PRKCD in H3122, lysates were prepared from parental H3122 cells or from H3122 overexpressing PRKCD. Immunoblotting was performed with the indicated antibodies. The PRKCD lentiviral clone harbors a C-terminal V5 epitope tag.

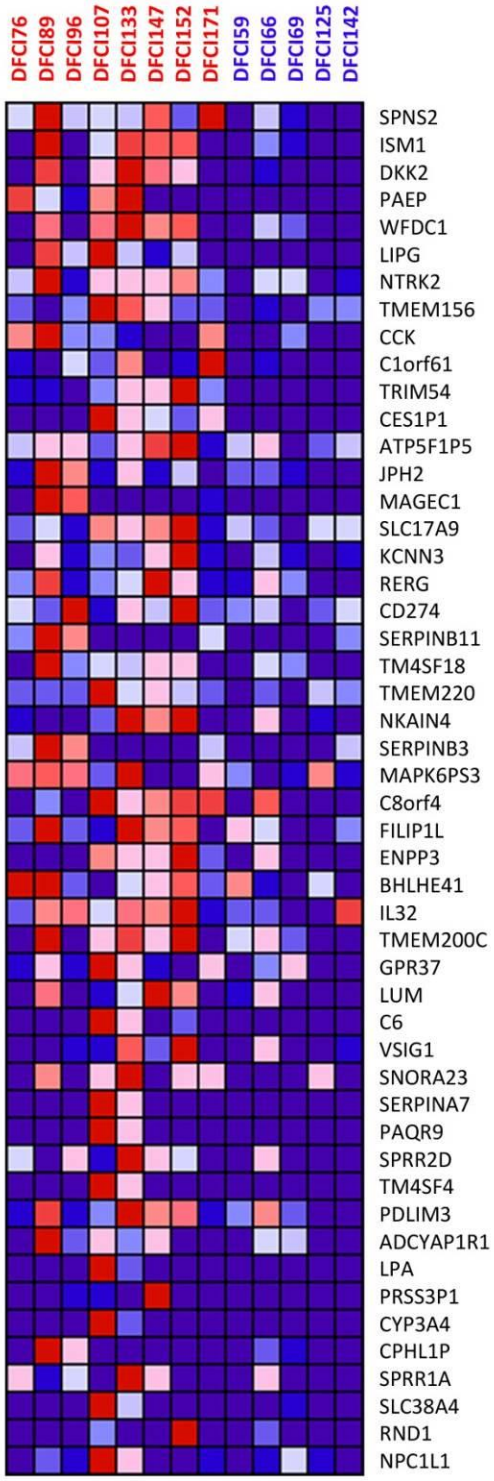

Figure S5, related to Figure 7. A subset of up-regulated genes in the P2Y gene signature have increased expression in ALK inhibitor-resistant tumors compared to controls. Heat map demonstrating increased expression of a subset of up-regulated genes in the P2Y gene signature in ALK inhibitor-resistant tumors (labeled at the top in red) compared to treatment-naïve tumors (labeled in blue).

Table S1, related to Figure 7. Top 200 genes up-regulated in P2Y gene signature (provided as an Excel file).

Table S2, related to Figure 7. 76 genes up-regulated in P2Y gene signature and crizotinib-resistant tumors (provided as an Excel file).

Table S3, related to Figure 7. Probe IDs corresponding to top and bottom 100 genes differentially expressed in DFCI133 compared to DFCI107 (used in CMap query). Provided as an Excel file.

Table S4, related to Figure 7.

Table S4: CMap Compound Connections to DFCI133 Relative to DFCI107

Rank	Compound	Score (Best 4)
1	**phorbol-12-myristate-13-acetate**	**0.999**
2	**prostratin**	**0.996**
3	**ingenol**	**0.993**
4	nocodazole	0.993
5	podophyllotoxin	0.993
6	MST-312	0.991
7	MAZ-51	0.98
8	PAC-1	0.978
9	TW-37	0.977
10	NPI-2358	0.977

Score (Best 4) values range from 1 (most correlated) to -1 (most anti-correlated for 3273 compounds across 4 cell lines.
Compounds ranked 1, 2, and 3 (indicated in red) are known activators of PKC.
See http://www.lincsproject.org for more information about the Connectivity Map (CMap).

Supplemental Experimental Procedures

Center for Cancer Systems Biology (CCSB)-Broad Lentiviral Expression Library. The CCSB-Broad Lentiviral Expression library has been previously described (Yang et al., 2011). All open reading frames (ORFs) in the library are expressed from the lentiviral expression vector pLX304 (http://www.addgene.org/25890) with a cytomegalovirus (CMV) promoter. pLX304 contains a blasticidin selectable marker and encodes a C-terminal V5 epitope tag. HEK293T cells were used to produce lentivirus as described on the RNAi Consortium Portal (http://www.broadinstitute.org/rnai/public/resources/protocols).

Cloning of EML4-ALK and Site-Directed Mutagenesis. Total RNA was isolated from H3122 using the RNeasy kit (Qiagen). Reverse transcription was performed using Superscript III First-Strand Synthesis SuperMix for qRT-PCR (Life Technologies) according to the manufacturer's instructions. The full-length cDNA encoding EML4-ALK was amplified using gene-specific primers and cloned into the pLX304 lentiviral expression vector. The L1152R mutant form of EML4-ALK was generated by site-directed mutagenesis using the QuikChange system (Agilent) according to the manufacturer's instructions. Sanger sequencing of both the wild-type and mutant clones of EML4-ALK was performed to confirm sequence identity.

Statistical analysis and identification of ORFs as candidate mediators of resistance. For each ORF in the CCSB-Broad Lentiviral Expression library, infection efficiency was expressed as the ratio of raw luminescence for cells selected with blasticidin divided by the raw luminescence for unselected cells (DMSO-treated). ORFs with an infection efficiency of less than 0.65 were considered failed infections and were excluded from the final data analysis (<5% of ORFs assayed). Correlation between replicates of ORF-expressing cells treated with drug vehicle, crizotinib, and TAE684 was assessed. Obvious outlier values among replicates (with >3-fold difference between raw luminescence replicate values) were excluded (<0.1% of ORFs assayed).

Raw luminescence values for cells expressing each individual ORF in the absence of drug were compared to the mean and standard deviation for all ORF-expressing cells on the same 384-well plate by calculating a standard score (or z-score) for each ORF. ORFs that appeared to have either a proliferative or toxic effect on cells in the absence of drug were defined as ORFs associated with a z-score ≥ 2.5 or ≤ 2.5, respectively. These ORFs were excluded from the final data analysis (<1% of ORFs assayed). Differential proliferation in the presence of drug was expressed for each ORF as the ratio of duplicate-averaged raw luminescence for cells treated with 1 μM crizotinib or 30 nM TAE684 divided by duplicate-averaged luminescence for cells in drug vehicle. A z-score for differential proliferation (% viability z-score) was then determined for each ORF as described above. ORFs with a % viability z-score ≥3 were selected as candidate mediators of resistance to crizotinib or TAE684. This criterion (differential proliferation at least 3 standard deviations above the mean) was established to select only ORFs associated with the most significant increase in cell viability in the presence of drug for follow-up. The identity of each ORF nominated as a candidate driver of resistance was confirmed by Sanger sequencing.

Validation of ORFs as candidate mediators of resistance in H3122. H3122 cells were plated and spin-infected with ORFs encoding candidate mediators of resistance as described in the Experimental Procedures. 48 hours after infection, cells were treated with crizotinib or TAE684 at 7 drug concentrations in half-log intervals or with drug vehicle (4 replicates each) for 5 days. Cell viability was determined using Cell Titer-Glo. A growth inhibitory curve was generated for each ORF with GraphPad Prism. The area under the curve (AUC) was determined for each ORF and compared to the AUC for a reference group of 13 negative controls. Negative controls included ORFs encoding Lac Z, HcRed, luciferase (Luc), enhanced green fluorescent protein (eGFP), or blue fluorescent protein (BFP). ORFs associated with an AUC ≥2.5 standard deviations above the mean (as determined from the reference group) for either crizotinib or TAE684 were selected as validated mediators of resistance to ALK inhibition in H3122. As for the primary screen, L1152R EML4-ALK was used as a positive control for validation studies. A constitutively-active KRAS (KRAS V12) was utilized as a second positive control.

Validation of ORFs as candidate mediators of drug resistance in additional cell lines. MGH006, HCC827, HCC4006, and PC-9 cells were seeded in 384-well microtiter plates followed by lentiviral infection the following day. Optimal cell seeding density was empirically determined for each cell line. Spin-infection was performed as for H3122 except that virus was removed after 6 hours (instead of 24 hours) for HCC827 and PC-9 cells. 24-48 hours after infection, cells were treated with a single dose of crizotinib, TAE684, or drug vehicle for MGH006 or with erlotinib or drug vehicle for HCC827, HCC4006, and PC-9 cells (4 replicates each). Drug concentrations used for the assay were determined empirically for each cell line depending on sensitivity to the corresponding drug and are shown in Figure 4. Cell viability was determined after 3-5 days of drug exposure as above. Percent (%) cell viability was determined for each ORF in the presence of drug (relative to DMSO) and compared to the % cell viability associated with a reference group of 13 negative controls as described above. ORFs associated with a % cell viability ≥2.5 standard deviations above the mean (as determined from the reference group) for either crizotinib or TAE684 (for MGH006) or erlotinib (for the EGFR-mutant cell lines) were selected as validated drivers of resistance to ALK inhibition in MGH006 or to EGFR inhibition in EGFR-mutant lines, respectively.

In-cell Western assays. Cells were seeded into black clear-bottom 384-well plates. ORFs were introduced via lentiviral infection as described above. 4 to 5 days after infection, ORF-expressing cells were fixed and permeabilized with 4% formaldehyde / 0.1% Triton X-100. Blocking was performed with Odyssey Blocking Buffer. Expression of V5 epitope-tagged ORFs was demonstrated using an anti-V5 antibody (Life Technologies #R960-25). In the same well, cells were stained with cell staining markers Draq5 and Sapphire700 (LI-COR). Fluorescence detection and quantitation was performed as described in the Experimental Procedures. For each ORF, fluorescence intensity from V5 staining was normalized to fluorescence intensity obtained with cell staining markers Draq5 and Sapphire700.

Generation of ceritinib-resistant H3122. H3122 cells were seeded at 50% confluence in two 15 cm dishes. Cells were cultured in the presence of 150 nM ceritinib for over 3 months with media change and fresh drug addition every 3 days. When cells in the two 15 cm dishes reached confluence, the resistant cell populations were passaged and maintained in 150 nM ceritinib. These ceritinib-resistant (R) cell populations generated from two separate dishes were designated H3122 R1 and R2. Retention of EML4-ALK in the resistant cell populations was confirmed by Western blot (Figure 6F).

Single-sample Gene Set Enrichment Analysis of Patient-Derived Tumor Specimens. We used single-sample Gene Set Enrichment Analysis (ssGSEA) to evaluate the extent to which genes comprising a specific gene signature are coordinately up- or down-regulated within a single tumor (Barbie et al., 2009). This method estimates a separate enrichment score for each sample and gene set that represents the degree to which the gene set's component genes are coordinately up- or down-regulated within a single sample. Gene signatures queried included those deposited in the C6 Oncogenic Signatures collection in the Molecular Signatures Database (MSigDB; version 4.0; http://www.broadinstitute.org/gsea/msigdb/index.jsp) in addition to other published and unpublished gene sets. All signatures indicated in Figure 7B (except the P2Y signature) have been previously reported: "EGFR (1)" and "RAF1" signatures are described in (Creighton et al., 2006) and are included in the MSigDB C6 Oncogenic Signatures collection (version 4.0) as "EGFR_UP.V1_UP" and "RAF_UP.V1_UP", respectively. "EGFR (2)" and "HER2" signatures are both described in (Gatza et al., 2010) and (Gatza et al., 2014).

The P2Y signature was defined as the top 200 genes up-regulated (as determined by the signal-to-noise test statistic as described in the Experimental Procedures) in H3122 cells overexpressing P2Y1 and P2Y2 compared to uninfected H3122 cells and cells expressing Lac Z. Enrichment of this P2Y signature in crizotinib-resistant tumors compared to treatment-naïve controls (Figure 7C) was demonstrated using Gene Set Enrichment Analysis (GSEA; http://www.broadinstitute.org/gsea/index.jsp), which revealed that 76 of the top 200 genes up-regulated in the P2Y gene signature are also up-regulated in the cohort of crizotinib-resistant tumors compared to controls. The 200 genes comprising the P2Y signature (and the 76 genes also up-regulated in crizotinib-resistant tumors compared to controls) are listed in Tables S1 and S2.

Information index. In the heat maps shown in Figure 7B, we assess the degree of association between the gene expression signatures' enrichment scores and the ALK inhibitor-resistant versus treatment-naïve distinction using an information index. This index is a signed rescaled [-1, 1] version of the continuous mutual information computed using a kernel-based method with 1 representing maximum association and -1 maximum anti-association (Linfoot, 1957). The sign is determined by the sign of the correlation coefficient. The p-values shown in the heat maps were obtained by using a permutation test where 1000 permutations of the class distinction are generated and compared against each signature profile to generate a null distribution.

Connectivity Map (CMap) queries. Additional gene expression queries were made using the Connectivity Map, part of the NIH Library of Integrated Cellular Signatures (LINCS) program (http://www.lincsproject.org). Differential gene expression between patient-derived tumors DFCI133 and DFCI107 was calculated as the log2 fold change (after adding a pseudocount of 1). The ranked top and bottom 100 genes enriched in DFCI133 compared to DFCI107 (and included within L1000 well-inferred gene space) were used to query the CMap database with the following parameters: Build ID: A2; Column Space: gold; Gene set size: 100; Metric: wtcs; Row space: bing. Affymetrix probe IDs corresponding to these top and bottom 100 genes are listed in Table S3.

Supplemental References

Linfoot, E. H. (1957). An informational measure of correlation. Information and Control *1*, 85-89.
Yang, X., Boehm, J. S., Yang, X., Salehi-Ashtiani, K., Hao, T., Shen, Y., Lubonja, R., Thomas, S. R., Alkan, O., Bhimdi, T.*, et al.* (2011). A public genome-scale lentiviral expression library of human ORFs. Nat Methods *8*, 659-661.