

CRISPR Screens Reveal YAP/TEAD Axis as a Mediator of Drug-Tolerant Cells in EGFR- Mutant NSCLC

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Abstract

Despite initial responses, most patients with metastatic lung cancer—including those with EGFR mutations—ultimately develop resistance to targeted therapies. To systematically uncover mechanisms underlying this resistance, genome-wide CRISPR knockout and activation screens were conducted in EGFR-mutant lung cancer cell lines treated with EGFR inhibitors such as osimertinib and gefitinib. These screens highlighted a recurrent involvement of genes associated with the Hippo signaling pathway. Notably, a subset of tumor cells, termed 'persister' cells, survive initial osimertinib exposure by engaging non-genetic, transcriptional adaptation mechanisms that promote drug tolerance. Our studies, integrating both genetic and pharmacological approaches, identified Hippo pathway activation as a key driver of this drug-tolerant state. Importantly, co-inhibition of EGFR and the Hippo signaling axis led to a pronounced reduction in cell viability in both established cell lines and patient-derived organoids. These findings propose that dual targeting of EGFR and Hippo signaling may offer a promising therapeutic approach to overcome resistance in EGFR-mutant lung cancer.

1 | INTRODUCTION

Drug resistance remains a major obstacle to effective cancer therapy and is frequently responsible for treatment failure (Shen, 2020). This challenge is attributed to a subpopulation of residual cancer cells—termed drug-tolerant persisters—that survive initial therapy and can eventually evolve into fully resistant clones (Shen, 2020). Although these persister cells have been reported across various cancer types and are thought to rely on non-genetic mechanisms such as transcriptional adaptation, their underlying biology remains incompletely defined (Shen, 2020).

In lung adenocarcinoma, activating mutations in the EGFR gene are observed in approximately 10–20% of cases (Mok, et al., 2009). EGFR-targeted therapies, including tyrosine kinase inhibitors (TKIs) such as gefitinib and osimertinib, initially improve patient outcomes. However, in the metastatic setting, resistance invariably develops (Mok, et al., 2009). Previous research has highlighted several genetic mechanisms of acquired resistance, such as secondary EGFR mutations (e.g., T790M, C797S), gene amplifications (e.g., MET, FGF), activating mutations in other oncogenes (e.g., KRAS, PIK3CA), gene fusions (e.g., BRAF, RET), and histologic transformation (e.g., to small cell or squamous cell carcinoma) (Schoenfeld et al., 2020; Mehlman et al., 2019). In contrast, the early adaptive responses that allow cancer cells to persist immediately after treatment are less well understood. Emerging studies point to diverse non-genetic processes, including epigenetic remodeling, modulation of apoptotic thresholds, and activation of the YAP/TEAD transcriptional axis (Shen, 2020; Kurppa, et al., 2020). Targeting these early persister states may provide a strategy to prevent the evolution of permanent resistance.

To uncover the molecular drivers of drug tolerance and resistance, we employed large-scale CRISPR-based forward genetic screens—using both CRISPR knockout (CRISPRn) and activation (CRISPRa) libraries—in EGFR-mutant lung cancer models treated with EGFR inhibitors. Our comprehensive analysis, spanning first- and second-line therapeutic contexts, identified a restricted set of recurrent pathways

involved in resistance. Notably, a significant proportion of candidate resistance genes converged on the Hippo signaling pathway. This pathway regulates TEAD-mediated transcription by controlling the phosphorylation status of the co-activators YAP1 and WWTR1 (also known as TAZ). When Hippo signaling is suppressed, YAP1/WWTR1 become active and drive TEAD-dependent gene expression, influencing cellular processes such as proliferation, polarity, adhesion, and survival (Varelas, 2014).

Our findings reveal that treatment with EGFR inhibitors rapidly induces YAP1/WWTR1/TEAD-dependent transcription in EGFR-mutant lung cancer cells. Importantly, blocking this transcriptional response significantly impairs cancer cell persistence. Moreover, both genetic and pharmacologic inhibition of the YAP1/WWTR1/TEAD axis not only resensitized resistant cells to osimertinib but also prevented the formation of drug-tolerant persister populations. These results suggest that targeting the Hippo pathway could be an effective strategy to combat osimertinib resistance and highlight the YAP1/WWTR1/TEAD complex as a promising target for combination therapies in EGFR-mutant lung cancer.

2 | MATERIALS AND METHODS

Cell Culture and Drug Resistance Models

PC-9 (RRID:CVCL_B260) and HCC827 (RRID:CVCL_2063) human non-small cell lung cancer (NSCLC) cell lines were obtained from the European Collection of Authenticated Cell Cultures (ECACC) and the American Type Culture Collection (ATCC), respectively. Both lines harbor activating EGFR mutations and are widely used as models for evaluating sensitivity and resistance to EGFR-targeted therapies.

Drug-resistant sublines, designated PC-9^{T790M} and HCC827^{T790M}, were developed in-house through chronic exposure to gefitinib or mutagenesis protocols to enrich for clones harboring the T790M mutation. These derivative cell lines are not registered with RRID databases but were authenticated by short tandem repeat (STR) profiling and confirmed to be free of mycoplasma contamination. Their derivation protocols and molecular characteristics are detailed within the manuscript. The absence of RRIDs for these in-house generated lines does not affect the scientific validity of the study. Cell lines were maintained in RPMI or DMEM/F12 media, following the respective supplier's guidelines, and supplemented with 5% fetal bovine serum (FBS) and penicillin-streptomycin. Cultures were incubated at 37°C in a humidified atmosphere with 5% CO₂. Mycoplasma contamination was assessed using the MycoSEQ Mycoplasma Detection Kit (ThermoFisher). PC-9 and HCC827 cells were obtained from ECACC and ATCC, respectively. All cell lines were authenticated using short tandem repeat (STR) profiling (IDEXX Laboratories).

To model acquired resistance, PC-9 cells were subjected to repeated weekly exposure to IC90 concentrations of gefitinib for six weeks. This approach has been previously shown to enrich for clones harboring the EGFR T790M mutation (25). Clonal populations were isolated using cloning cylinders, expanded, and subjected to whole-exome sequencing. One clone, designated PC9-GR5, was used in further experiments and renamed PC-9^{T790M} for clarity.

In contrast, HCC827 cells are known to commonly acquire MET amplification under similar conditions (Engelman et al., 2007). To bias toward point mutations such as EGFR T790M, the cells were first mutagenized with ENU, then exposed to gefitinib weekly for six weeks. Clones were isolated, expanded, sequenced, and one harboring T790M was selected and termed HCC827^{T790M}. Whole-exome sequencing confirmed no additional driver mutations in either clone compared to the parental lines. Both mutant lines were resistant to gefitinib but sensitive to osimertinib (Supplementary Figs. 1A–D).

Immunoblotting

Cells were lysed at specified time points using RIPA buffer (ThermoFisher Scientific, #89901) supplemented with protease and phosphatase inhibitor cocktails (ThermoFisher Scientific, #78441). Ten micrograms of total protein were resolved via SDS-PAGE and transferred onto nitrocellulose membranes. Immunoblotting was performed using the following antibodies: anti- β -actin (Sigma, A5316), anti-CSK (Cell Signaling Technology, #4980), anti-KCTD5 (Proteintech, 15553-1-AP), anti-NF1 (Abcam, ab17963), anti-NF2 (CST, #6995), anti-PTEN (CST, #9559), anti-YAP1 (CST, #14074), anti-WWTR1 (CST, #70148), anti-phospho-EGFR Y1068 (CST, #2234), anti-EGFR (CST, #4267), anti-phospho-AKT S473 (CST, #4060), anti-AKT (CST, #9272), anti-phospho-MEK1/2 S217/221 (CST, #9154), anti-MEK1/2 (CST, #4694), anti-phospho-ERK1/2 T202/Y204 (CST, #9106), anti-ERK1/2 (CST, #9102), anti-MET (CST, #8198), anti-GAPDH (CST, #2933), anti-mouse-IgG-HRP (CST, #7076), and anti-rabbit-IgG-HRP (CST, #7074). Immunoblot data are available in Supplementary Fig. 12.

Genome-Wide CRISPR/Cas9 Knockout Screens

For loss-of-function screening, cells were transduced with a lentiviral sgRNA library targeting 18,010 human genes (Tzelepis et al., 2016). For gain-of-function (CRISPRa) experiments, a separate library targeting 23,430 isoforms was used (Konermann et al., 2015). Following antibiotic selection, cells were treated with gefitinib or osimertinib (IC80–90, 100 nM) twice weekly for 14 days to select for resistant populations.

Lentiviral particles were generated using the vector pKLV2-U6gRNA5(BbsI)-PGKpuro2ABFP-W (Addgene #50946), and packaging plasmids psPAX2 and pMD2.G (Addgene #12260, #12259) at a 5.4:5.4:1.2 μ g ratio per 10 cm plate. Transductions were conducted in 6-well plates using 8 μ g/mL Polybrene and 1×10^6 cells/well.

To generate Cas9-expressing lines, cells were transduced with pKLV2-EF1a-Cas9Bsd-W (Addgene #68343) and selected with blasticidin (50 μ g/mL). Cas9 activity was validated using a reporter system (Addgene #67980) in which GFP silencing confirmed functional genome editing. Transduction efficiency was assessed via flow cytometry (BD LSRFortessa) and analyzed with FlowJo.

For pooled screening, 6×10^7 cells were transduced at a multiplicity of infection (MOI) of 0.3, ensuring most cells carried only one sgRNA and achieving > 200-fold library coverage. Puromycin selection (2–3

µg/mL) was performed for 5–6 days. After a 14-day drug treatment period, cells were collected for DNA extraction and sequencing.

Illumina Sequencing and MAGeCK Analysis

Genomic DNA (72 µg) from each sample was used to perform 36 PCR reactions (2 µg/reaction) with the following primers:

Forward: ACACTCTTCCCTACACGACGCTCTTCCGATCTCTTGTGGAAAGGACGAAACA

Reverse: TCGGCATTCCTGCTGAACCGCTCTTCCGATCTCTAAAGCGCATGCTCCAGA

PCR products were purified and indexed for Illumina sequencing (29). Guide RNA enrichment or depletion was analyzed using the MAGeCK RRA statistical package (version 0.5.8), which compares read counts from treated versus DMSO control cells, normalized to the plasmid library (30). MAGeCK employs a negative binomial model and α -RRA algorithm to rank significant gene hits.

CRISPR Activation (CRISPRa) Screens

To perform activation screens, cancer cells were first transduced with lentiviruses expressing dCas9-VP64 and MS2-P65-HSF1 using vectors lenti dCas9-VP64_Blast and lenti MS2-P65-HSF1_Hygro (Addgene #61425, #61426), respectively. Packaging was performed in 293T cells using psPAX2 and pMD2.G. Transduced cells were selected with blasticidin and hygromycin.

The pooled gRNA activation library (lenti sgRNA(MS2)_puro; Addgene #1000000074) targeting 18,965 genes with 70,297 guides was used for transduction at MOI < 0.3. After puromycin selection for 10 days, cells were exposed to DMSO, gefitinib, or osimertinib (100 nM) for 21 days. Genomic DNA was isolated and analyzed as above (Koike-Yusa, 2014; Li et al., 2014).

Arrayed CRISPR Screens

Synthetic cr:tracrRNA duplexes (Horizon Discovery) were reconstituted at 10 µM in Tris-HCl (pH 7.4) and diluted to 2.5 µM. A custom 384-well library targeting 155 genes (up to 4 guides per gene) was prepared and dispensed using an Echo acoustic dispenser.

Reverse transfections were carried out by adding 400 nL of duplexed cr:tracrRNA and 10 µL transfection mix (serum-free RPMI + 0.75% RNAiMAX) per well. Cas9-expressing PC-9, HCC4006, and HCC827 cells were seeded at 700 cells/well and incubated for 72 h at 37°C with 5% CO₂. Controls included non-targeting sgRNAs, positive controls (NF2, PTEN), and YAP1/WWTR1 biomarkers.

EGFR Inhibitor Treatment and Immunofluorescence Imaging

After 72 hours of reverse transfection, cells were treated with 50 nM osimertinib or 0.15% DMSO (vehicle control; Merck KGaA, Darmstadt, Germany) using the Tecan D300e Dispenser (Tecan AG, Switzerland) in

384-well plates. The plates were incubated for an additional 24 hours at 37°C, 5% CO₂.

Following a total of 96 hours (72-hour transfection plus 24-hour inhibitor exposure), cells were fixed with 4% paraformaldehyde (PFA) and incubated at room temperature for 2 hours in a blocking/permeabilization buffer containing 0.2% Triton X-100 and 2% bovine serum albumin (BSA) in PBS. Parallel plates were stained using antibodies targeting MAPK/PI3K pathway components (phospho-p44/42 MAPK [E10, #9106], phospho-AKT [D9E, #4075, AF647-conjugated], phospho-S6 [D57.2, #9865, AF594-conjugated]) or Hippo pathway components (YAP1/WWTR1 [D24E4, #8418]), all from Cell Signaling Technology (Danvers, MA). Primary antibody incubation occurred overnight at 4°C.

After washing three times in blocking/permeabilization buffer, cells were incubated for 2 hours at room temperature with a secondary antibody solution containing Hoechst 33342 (2 µg/mL) and either Alexa Fluor 488 anti-mouse or Alexa Fluor 647 anti-rabbit (4 µg/mL; Thermo Fisher, Waltham, MA). Cells were washed in PBS and sealed with black plate seals prior to imaging.

Images were captured using a Cell Voyager 8000 (CV8000, Yokogawa Inc., Tokyo, Japan) with a 20× water objective and 2×2 image binning. Four fields per well were acquired at a single z-plane. Columbus software (PerkinElmer, Waltham, MA) quantified cell number and fluorescence intensity in nuclear and cytoplasmic compartments. Nuclear:cytoplasmic (Nuc:Cyt) ratios for YAP1/WWTR1 were calculated per cell. Genedata Screener v16 (Genedata AG, Basel, Switzerland) was used for data normalization and QC. For MAPK/PI3K data, a two-point normalization method was used with untreated NTC gRNA as maximum signal (0) and osimertinib-treated NTC gRNA as minimum signal (– 100). YAP1/WWTR1 Nuc:Cyt values were expressed as fold change compared to untreated NTC.

Generation of Isogenic Cell Lines

Stable Cas9-expressing cells were transduced with sgRNAs targeting specific gene exons or non-targeting control guides (NTC) using vectors pKLV2-U6gRNA5(BbsI)-PGKpuro2ABFP-W (#67974) or pKLV2-U6gRNA5(BbsI)-PGKpuro2AZsG-W (#67975, Addgene). Transduced cells were selected with puromycin and analyzed by MACSQuant Flow Cytometry (Miltenyi Biotec, Germany). Western blotting confirmed gene knockout 14 days post-transduction (Supplementary Fig. 3).

Endogenous MET activation was achieved in MS2-p65-HSF1/dCas9-VP64 expressing cells using LV06 vectors (Merck KGaA, Germany) targeting specific promoters. Puromycin selection followed, and expression was verified by western blot.

YAP1 and WWTR1 cDNA sequences (NM_001130145.3 and NM_015472.6, respectively) were cloned into modified pKLV2-EF1a-Cas9Bsd-W (#68343, Addgene) replacing the Cas9 cassette with the cDNA linked to a bleomycin resistance marker (GeneScript Biotech, Netherlands). Transduced cells were selected with zeocin (500 µg/mL; Invitrogen, US).

For inducible expression, wild-type and mutant (5SA/4SA) YAP1/WWTR1 cDNAs were inserted into the pTRIPZ vector (Horizon Discovery) and transduced into PC-9 and HCC827 cells. Puromycin selection (1

µg/mL) was applied, and expression was induced with doxycycline.

Clonogenic and Live Cell Proliferation Assays

For long-term proliferation assays, 5,000 cells/well were seeded in 6-well plates. After 24 hours, treatments were applied. Following treatment, cells were fixed using BD Cytofix (#554655) for 20 minutes, stained with 0.01% crystal violet, washed, dried, and imaged using a GelCount colony counter (Oxford Optronix, UK). Colony areas were quantified using ImageJ. Two biological replicates with at least two technical duplicates were performed.

Live cell proliferation was assessed using Incucyte S3 (Sartorius, Germany). Cells were seeded in 96-well plates, grown to exponential phase (3–4 days), then treated. Media was refreshed every 3 days. If inducible constructs were used, doxycycline was added 3 days before drug treatment. Eight images/well were taken every 4 hours, and confluency was analyzed using Incucyte software. Results were plotted using GraphPad Prism.

Viability Assays

Short-term viability was assessed by seeding 2,500 cells/well (PC-9, HCC827, HCC4006) in 96-well plates. Twenty-four hours post-seeding, cells were treated and viability was measured using CellTiter-Glo (Promega) as per manufacturer's protocol. Luminescence was measured with a CLARIOstar plate reader (BMG Labtech) and analyzed with GraphPad Prism.

Organoid Culture and Analysis

Patient-derived xenograft (PDX) tumors were minced into 2–3 mm fragments, washed with PBS, and digested in Liberase DH (2.5 mg; Sigma #7891) at 37°C for 30–60 minutes. Samples were lysed with ACK buffer (Gibco, A10492-01), filtered (70 µm), and frozen in Recovery Cell Culture Freezing Medium (ThermoFisher, 12648010). Organoids were cultured in Advanced DMEM/F-12 (31).

For qPCR, organoids were treated with DMSO or osimertinib (160 or 500 nM) for 48 hours. For IHC, treatments were extended to 5 days. Organoids on chamber slides were fixed with 4% PFA, washed, and embedded in Histogel (Thermo Scientific, HG-4000-012) before paraffin embedding.

TEAD Reporter Assay

Cas9-expressing PC-9 and HCC827 cells were transduced with pLVdCIN (Addgene #34615), a luciferase reporter driven by TEAD binding sites. Cells were selected with G418 (ThermoFisher #10131035). NTC or NF2 knockouts were generated as previously described. To quantify TEAD activity, 2,500 cells/well were seeded, stained with CellTiter-Fluor (Promega), and analyzed for green fluorescence (cell count) and luciferase activity using ONE-Glo EX (Promega). Data were normalized to cell count and averaged across three experiments.

Competitive Proliferation by Flow Cytometry

YAP1/WWTR1 single and double knockouts were generated using pKLV2.2-h7SKgRNA5-hU6gRNA5-PGKpuroBFP-W (Addgene #72666). PC-9 NF2 KO cells expressing ZsGreen were co-transduced with BFP- and GFP-marked guides for NTC, YAP1, WWTR1, or both. Co-cultures were treated with osimertinib or DMSO. Ratios of KO to control cells were measured by flow cytometry and normalized to baseline (day 0). Data were analyzed using FlowJo. Experiments included biological triplicates.

RNA-Sequencing and Analysis

RNA was extracted with the RNeasy 96 QIAcube HT Kit (Qiagen), quantified via Qubit (Invitrogen), and assessed using NanoDrop and Bioanalyzer (Agilent; RIN > 7). Libraries were prepared with Illumina mRNA Stranded kit and sequenced on NovaSeq 6000. Reads were aligned to hg38 using STAR (v2.6.1d). Data analysis was performed using ROSALIND®, with quantification by HTSeq, normalization by DESeq2, and QC via RSeQC. Fold changes and p-values were calculated using DESeq2. Pathway enrichment used MSigDB Hallmark gene sets and Enrichr (FDR < 0.2). GSEA (16199517) was used to evaluate Hippo target signatures (Zhang et al., 2009). Transcription factor activity was inferred using Enrichr with TRANSFAC/JASPAR motifs (p < 0.01; Supplementary Data 18).

Single-Cell RNA-Seq Analysis

Public scRNA-seq datasets (GSE15094914, GSE14938315) were analyzed using Seurat. Data were normalized, log-transformed, scaled, and reduced using PCA and UMAP. Module scores for Hippo signatures were calculated using AddModuleScore. Comparisons across time points were made using the Wilcoxon rank-sum test.

Real-Time PCR

RNA was extracted with RNeasy Plus Mini Kit (Qiagen), quantified via NanoDrop, and reverse-transcribed using the High-Capacity cDNA RT Kit (Thermo Fisher). qPCR was performed with TaqMan master mix and the following assays: GAPDH (Hs99999905_m1), CTGF (Hs00170014_m1), CYR61 (Hs00155479_m1), AMOTL2 (Hs01048101_m1). QuantStudio 7 Flex (Applied Biosystems) was used. Expression was normalized to GAPDH.

Immunofluorescent Staining for YAP1/WWTR1

Cells were seeded in 96-well CellCarrier Ultra plates, treated, and fixed with 4% PFA. Permeabilization/blocking was done in PBS + 0.1% Triton-X-100 + 1.1% BSA. Cells were stained with anti-YAP1/WWTR1 (CST, #8418) at 1:100 overnight at 4°C. Secondary staining was performed with Alexa Fluor Plus647 (A32733) and Hoechst33342 (H3570). Imaging was done using Operetta CLS (PerkinElmer). Nuclear/cytoplasmic ratios were quantified using Columbus software and normalized to controls.

PDX Models and IHC

EGFR-mutant PDX models were sourced from Crown Bioscience, Champions Oncology, and Dana-Farber Cancer Institute. Mice were treated with osimertinib (25 mg/kg daily). Tumors were fixed after 28 days and stained for YAP1/WWTR1.

IHC was performed on Leica Bond RX (Leica Biosystems). Antibodies: YAP1 (CST #14074, 0.07 µg/mL) and WWTR1 (CST #83669, 0.4 µg/mL). Detection was done with Bond Polymer Refine Detection. Sections were scanned on Aperio AT2. Staining specificity was validated using high/low expressor cell lines and KO models.

IHC Analysis

Quantification was performed using HALO software (Indica Labs). Nuclear and cytoplasmic staining was scored (0–3+), and H-scores were calculated: $H = (\%1+ \times 1) + (\%2+ \times 2) + (\%3+ \times 3)$.

Statistical Analysis

Details of statistical methods are provided in figure legends. All replicates are biological. p-values < 0.01 and < 0.001 are indicated by ** and ***, respectively. Error bars represent variability across replicates.

3 | RESULTS

Genome-Wide CRISPRn and CRISPRa Screens to Identify Drug Resistance Mechanisms in EGFR-Mutant Lung Cancer

To systematically map resistance mechanisms, genome-wide CRISPR knockout (CRISPRn) and activation (CRISPRa) screens were conducted in EGFR-mutant lung cancer cell lines PC-9 and HCC827, along with isogenic derivatives PC-9^{T790M} and HCC827^{T790M}. These latter models harbor the secondary EGFR T790M mutation, commonly associated with acquired resistance to first-line gefitinib therapy. All four cell lines were treated with IC90 doses of gefitinib and osimertinib, leading to substantial inhibition of signaling and cell viability (Fig. 1a, b; Supplementary Figs. 1a–h).

The MAGeCK algorithm was used to identify significant resistance genes by comparing treated versus control groups across all six screens, using thresholds of $p < 0.005$ and absolute fold change ≥ 2 (Fig. 1b; Supplementary Data 1, 2–7). Across the six datasets, 1191 resistance-associated genes were identified in at least one experiment, with 250–280 hits typically observed per cell line per drug. A core set of 38 genes emerged as recurrent hits in at least four of six experiments (Fig. 1c; Supplementary Fig. 2), including clinically established resistance genes such as MET, KRAS, PTEN, and NTRK1 (17).

A protein interaction and pathway enrichment analysis of the 38 core resistance genes revealed significant involvement in PI3K, MAPK, mTOR, ubiquitination, and Hippo signaling pathways (Fig. 1d). Four of the top 10 most frequent hits—FOSL1, NF2, WWTR1, and PARD3—are regulators of YAP1/WWTR1-driven transcription and Hippo signaling (Supplementary Data 1).

Experimental Validation of Resistance Genes

A subset of genes identified through the CRISPR screens were selected for functional validation in PC-9, HCC827, and HCC4006 cells (Supplementary Figs. 1i, j). Genes selected for overexpression or knockdown included KCTD5, PTEN, NF1, MED24, CSK, and MET (Supplementary Fig. 4a). Clonogenic assays under osimertinib treatment confirmed the screen results and validated the degree of resistance conferred (Supplementary Figs. 4b–d).

High-Content CRISPR Imaging Links Resistance Genes to Pathways

To delineate downstream signaling alterations, a high-content imaging CRISPR platform was established. Synthetic guides targeting 72 recurrent resistance genes were introduced into PC-9, HCC827, and HCC4006 cells, followed by osimertinib treatment for 24 hours. Cells were stained for pAKT (PI3K), pERK1/2 (MAPK), pS6 (mTOR), and nuclear YAP1/WWTR1 (Hippo) (Fig. 2a). As expected, osimertinib reduced MAPK, PI3K, and mTOR signaling in cells transduced with non-targeting controls (Fig. 2b–d).

Silencing of most resistance genes did not restore PI3K/MAPK/mTOR signaling. However, a notable outcome was increased nuclear localization of YAP1/WWTR1—observed in 20% of PC-9-targeted genes (13/65), 26% in HCC827 (9/34), and 40% in HCC4006 (29/72) (Fig. 2b–d). Eleven genes induced increased YAP1/WWTR1 nuclear presence in at least two lines, including known Hippo regulators (NF2, LATS2, WWC1) and novel genes (C16orf72, CAB39, MED12, DDA1, PPM1F, RNF7, SOX4). Most of these did not significantly affect pAKT or pERK levels (Fisher's exact test), suggesting alternate resistance pathways.

Interestingly, osimertinib itself caused nuclear accumulation of YAP1/WWTR1 in PC-9 cells without changing total expression (Fig. 2e; Supplementary Fig. 4e), implying Hippo pathway engagement is an acute cellular response to EGFR inhibition.

Functional Role of Hippo Pathway in Osimertinib Resistance

To further investigate the Hippo pathway's role in resistance, key regulators were knocked out (e.g., NF2) or overexpressed (YAP1, WWTR1) in EGFR-mutant cell lines (Supplementary Figs. 5–6). NF2, a well-established suppressor of Hippo transcriptional output, emerged as a top resistance gene across all six CRISPRn screens (Supplementary Data 1). Knockout of NF2 in PC-9, HCC827, and HCC4006 enhanced resistance and upregulated canonical Hippo targets (Fig. 3a). This resistance was not associated with MAPK or PI3K reactivation post-treatment, though increased basal EGFR phosphorylation was observed in NF2-depleted cells, potentially explaining enhanced proliferation (Fig. 3b) (8).

NF2 loss promoted nuclear accumulation of YAP1/WWTR1 and enhanced TEAD-dependent transcription, confirmed by TEAD luciferase reporter assays (Fig. 3c–d). Silencing of YAP1 and/or WWTR1 reversed NF2 KO-driven resistance (Fig. 3e; Supplementary Fig. 5b). Additionally, Hippo pathway disruption alone

(even without drug treatment) reduced viability, suggesting NF2 KO creates dependency on YAP1/WWTR1 activity (Fig. 3e, left panel).

High-content imaging reveals Hippo pathway activation as a key feature of osimertinib resistance.

a Schematic of the arrayed CRISPR knockout screening assay coupled with high-content microscopy. Cas9-expressing PC-9, HCC827, and HCC4006 cells were transfected with pools of synthetic sgRNAs targeting candidate resistance genes identified in genome-wide screens. After 72 h, cells were treated with either DMSO or 50 nM osimertinib for 24 h. A multiplexed immunofluorescence approach was used to assess expression of pathway markers: phospho-AKT (S473), phospho-ERK1/2 (T202/Y204), phospho-S6 (S235/236), and nuclear localization of YAP1/WWTR1, a readout of Hippo pathway activity. Data were normalized using a max/min scaling approach for pAKT, pERK1/2, and pS6, and by nuclear-to-cytoplasmic ratio for YAP1/WWTR1.

b–d Heatmaps showing normalized effects of gene knockouts on signaling pathway reactivation in each cell line. For each, the first two columns reflect the baseline impact of osimertinib alone on pAKT, pERK, and pS6, serving as reference points (0 = DMSO, –100 = osimertinib). Remaining columns display the extent to which specific gene knockouts reverse or maintain inhibition. Red asterisks mark genes whose knockout also led to increased nuclear localization of YAP1/WWTR1. Data represent at least two biological replicates per condition.

e Osimertinib treatment for 24 h induces elevated nuclear YAP1/WWTR1 in PC-9 cells compared to cytoplasmic levels, as quantified by the nuclear:cytoplasmic ratio. Quantification method is described in the Methods section.

YAP1 and WWTR1 are key effectors of the Hippo signaling pathway. Stable overexpression of their full-length coding sequences in PC-9 and HCC827 cells led to increased resistance to osimertinib, although the resistance conferred by YAP1 was less pronounced in PC-9 cells (Supplementary Fig. 6a, b). Notably, YAP1 was not identified as a significant resistance gene in the CRISPRa screen in PC-9 cells. Using a doxycycline-inducible system, wild-type or phosphorylation-resistant mutants (YAP1 5SA and WWTR1 4SA) were expressed in PC-9 cells (Supplementary Fig. 6c) (Zhang, H. et al., 2009; Zhao, 2010). Wild-type WWTR1 overexpression enhanced resistance to osimertinib in a doxycycline-dependent and reversible manner (Fig. 3f, g), while the effect for wild-type YAP1 was modest. Constitutively active mutants of both YAP1 and WWTR1 induced stronger resistance (Fig. 3f, g).

Hippo Pathway Components as Sensitizing Genes

While the primary aim of the CRISPR screens was to identify resistance drivers, analysis of genes whose knockout enhanced sensitivity to EGFR inhibitors revealed that Hippo pathway regulators were among the most recurrent sensitizers across four of the six screened cell lines (Supplementary Data 11).

Transcriptional Programs and TF Activity Driven by Hippo Signaling

TEAD transcription factors (TEAD1–4) are central mediators of Hippo pathway transcription. We performed RNA-seq in isogenic PC-9, HCC827, and HCC4006 models (NTC, NF2 KO, WWTR1 OE, YAP1

OE) to identify differentially expressed genes and infer transcription factor (TF) activities (Supplementary Data 12). Gene set enrichment analysis (GSEA) confirmed upregulation of YAP1/WWTR1/TEAD transcriptional signatures (Supplementary Fig. 7a, b) (Zhang, H. et al., 2015). TF activity inference identified TEAD2 and TEAD4 as consistently activated across models, while TEAD1 showed no activity and TEAD3 was absent from the TF panel (Fig. 4a; Supplementary Data 13). Additional TFs such as JUN and FOS (AP-1 complex) were also detected but less frequently.

Distinct transcriptional responses were observed in different isogenic models, indicating redundancy in the mechanisms used to activate Hippo signaling (Fig. 4a, Venn diagrams). TEAD2 and SP1 were strongly activated in PC-9 and HCC4006 cells, while HCC827 cells exhibited broader TF activation profiles (Fig. 4b–d). SP1, a Krüppel-like family member, was among the most consistently activated TFs. However, most of these TFs were not hits in CRISPR screens, in contrast to YAP1 and WWTR1, which were significant sensitizers to both gefitinib and osimertinib. In parental cells treated with osimertinib for 48 hours, TEAD4, SP1, and SP3 were among the most recurrently activated TFs (Fig. 4e).

Hallmark pathway enrichment analyses revealed EMT as the most consistently upregulated pathway across all isogenic models and in response to osimertinib in parental lines (FDR < 0.2) (Fig. 4f–h; Supplementary Fig. 6c; Supplementary Data 14a, b). Although YAP1 and WWTR1 are often co-referenced, they showed divergent transcriptional profiles. EMT-related genes upregulated by each factor overlapped partially but also included uniquely regulated transcripts (Fig. 4f–h).

Hippo Signaling Supports Persister Cell Survival

Although regulators of the Hippo pathway are rarely found as acquired resistance genes in patients, we hypothesized their role might be non-genetic and critical for early-stage drug tolerance. Whole-exome and targeted sequencing confirmed the absence of pre-existing resistance mutations in our models. Increased nuclear localization of YAP1/WWTR1 was previously observed as an acute response to osimertinib (Fig. 2e).

We evaluated nuclear YAP1/WWTR1 levels and canonical Hippo targets (CTGF, CYR61, AMOTL2) post-treatment with osimertinib (160–1000 nM) in both 2D and 3D models. A significant, time-dependent increase in nuclear localization of YAP1/WWTR1 was observed ($P < 0.001$, Wilcoxon rank sum test) (Fig. 5a; Fig. 5b; Supplementary Data 16). WWTR1 expression increased at the protein but not transcript level in PC-9 cells, consistent with regulation via degradation (Supplementary Fig. 7c).

Enrichment of the EMT signature was detected following osimertinib treatment in all three cell lines, with strongest effects in PC-9 and HCC4006 (Supplementary Fig. 7d; Supplementary Data 14). Public single-cell RNA-seq datasets from time-course studies of PC-9 cells treated with osimertinib or erlotinib also showed significant Hippo signature enrichment in persister cells ($p < 0.001$) (Supplementary Fig. 8) (Oren et al., 2021; Aissa et al., 2021).

Targeting Hippo and EGFR Pathways in Combination

The TEAD inhibitor K-975 disrupts the YAP1/WWTR1–TEAD interaction, suppressing Hippo transcriptional output (Kaneda et al., 2020). In TEAD reporter assays, K-975 reduced luciferase activity in NF2 KO PC-9 and HCC827 cells, indicating effective inhibition (Supplementary Fig. 7e). K-975 had minimal impact as monotherapy (Supplementary Fig. 7f), but when combined with osimertinib, significantly reduced the population of persister cells in short-term assays (Fig. 5c).

In 21-day assays, K-975 combined with osimertinib (160 nM) nearly eliminated viable cells, even when osimertinib was used at higher doses (500–1000 nM) to enrich for persisters (Fig. 5d; Supplementary Fig. 7g). Similar effects were observed using MYF-01-037, another TEAD inhibitor. In NF2 KO cells, combining EGFR and Hippo pathway inhibition reversed osimertinib resistance in PC-9, HCC827, and HCC4006 lines (Supplementary Figs. 9a–g) (5).

4 | DISCUSSION

Elevated Nuclear YAP1 Expression in Select Patient-Derived Xenograft Models Following Osimertinib Therapy

To evaluate the role of Hippo signaling in response to osimertinib, a panel of 10 EGFR-mutant lung cancer patient-derived xenograft (PDX) models was examined. These models were categorized as either sensitive or resistant based on their in vitro sensitivity to osimertinib (Fig. 6a; Supplementary Data 15). Notably, all five resistant models were obtained from patients who had progressed during osimertinib treatment and exhibited genomic alterations associated with resistance (Supplementary Data 15).

Immunohistochemical analysis was conducted to assess nuclear localization of YAP1 and WWTR1 proteins across the PDX cohort. Although nuclear YAP1 expression appeared elevated in resistant models, this trend did not reach statistical significance (Fig. 6b). No consistent differences in nuclear WWTR1 expression were observed between sensitive and resistant groups.

To further explore in vivo effects of osimertinib, two osimertinib-sensitive PDX models (CTG-2548 and LU5221) were engrafted into NSG mice and subjected to 28 days of daily treatment with osimertinib or vehicle. Both models demonstrated significant tumor regression during treatment; however, tumors regrew upon drug withdrawal, indicating the persistence of viable drug-tolerant cancer cells (Fig. 6c).

Tumor tissues collected before and after the 28-day treatment period were evaluated for nuclear YAP1 and WWTR1 expression using IHC (Fig. 6c; Supplementary Fig. 10). In the CTG-2548 model, osimertinib treatment led to a statistically significant increase in nuclear YAP1 expression ($p = 0.005$), accompanied by a corresponding reduction in WWTR1 expression (Finch-Edmondson et al., 2015). In contrast, no induction of nuclear YAP1 or WWTR1 was observed in the LU5221 model following treatment.

Therapeutic resistance remains one of the greatest obstacles in the management of metastatic cancers, ultimately leading to treatment failure in the vast majority of cases. Historically, efforts to understand resistance mechanisms have focused on detecting acquired genomic alterations in tumors from

relapsed patients. However, these studies have consistently revealed significant heterogeneity, with different resistance-conferring mutations present in distinct tumor sites within the same individual (Vasan, 2019; Shi, H. et al., 2014). This complexity poses a substantial challenge in designing effective drug combinations for patients with established resistance, prompting investigators to shift focus toward earlier stages of resistance development—specifically, the transient state of drug-tolerant persister (DTP) cells.

DTP cells represent a population that survives initial therapy by activating non-genetic adaptive mechanisms, often of a transcriptional nature, enabling escape from cell death and ultimately allowing for the acquisition of stable resistance mutations. Several mechanisms have been implicated in the persistence phenotype, including alterations in cell cycle progression, shifts in metabolic states, changes in differentiation status, and interactions with the tumor microenvironment [1]. While multiple preclinical strategies have been proposed to eradicate these cells, none have yet reached clinical application.

To identify key regulators of resistance, we employed genome-wide CRISPR/Cas9 loss-of-function screening in EGFR-mutant non-small cell lung cancer (NSCLC) models treated with osimertinib. Our findings revealed that components of the Hippo pathway were among the most recurrent and potent contributors to osimertinib resistance. Interestingly, genes such as NF2, LATS1/2, YAP1, and WWTR1 have not been reported as common resistance mutations in patient-derived tumor samples following relapse, suggesting that their role may be more closely tied to early persistence mechanisms rather than late-stage genetic resistance.

Supporting this notion, we demonstrated that YAP1/WWTR1/TEAD-dependent transcription is acutely activated following osimertinib treatment in both 2D and 3D EGFR-mutant lung cancer models. Pharmacologic or genetic inhibition of this transcriptional complex significantly diminished the survival of persister cells. Importantly, in two osimertinib-sensitive PDX models tested, only one exhibited activation of this Hippo axis, indicating that other persistence mechanisms also exist and contribute to resistance heterogeneity. Across all models, epithelial-to-mesenchymal transition (EMT) emerged as the most consistently upregulated transcriptional program following osimertinib exposure. EMT has previously been linked to resistance to EGFR inhibitors and to enhanced cell survival through inhibition of apoptosis (Raoof et al., 2019; Lamouille, 2014).

Although direct pharmacologic reversal of EMT remains challenging, our findings suggest that targeting upstream regulators of EMT—namely YAP1 and WWTR1—may represent an indirect but effective strategy. TEAD inhibitors that disrupt the interaction between TEAD family transcription factors and their YAP1/WWTR1 co-activators are in development and have demonstrated efficacy in preclinical models of mesothelioma and EGFR-mutant lung cancer (Kurppa et al., 2020; Kaneda et al., 2020; Tang & Post, 2022).

Despite these promising findings, several limitations should be noted. While we confirmed the effectiveness of combining TEAD inhibition with osimertinib in vitro, in vivo validation was constrained by the lack of a TEAD inhibitor with confirmed systemic activity during the study period. However, a

recent report presented at AACR supports the efficacy of this combination in EGFR-mutant xenografts and PDX models (Tang & Post, 2022). Additional studies are needed to determine whether similar effects are observed in vivo, particularly in models incorporating tumor microenvironmental factors or in clinical specimens collected after osimertinib therapy.

Moreover, while our in vitro and in vivo data showed that YAP1/WWTR1/TEAD activation occurs in response to osimertinib, it remains to be demonstrated whether this also holds true in persister cells from patient tumors. Single-cell RNA sequencing (scRNA-seq) has emerged as the leading technology to interrogate rare subpopulations such as persister cells due to its ability to capture the transcriptome at individual-cell resolution (Aissa et al., 2021). However, practical limitations exist: post-treatment biopsies are often difficult to obtain due to tumor shrinkage; recovered tissue may contain very few cancer cells; and persister cells may be highly sensitive to dissociation and processing steps. New fixation and preservation protocols such as those provided by 10x Genomics may mitigate some of these challenges.

Several international initiatives—including the Human Tumor Atlas Network (<https://humantumoratlas.org/>) and Persist-seq (<https://persist-seq.org/>)—are attempting to generate comprehensive single-cell atlases capturing cancer progression and therapeutic resistance. Based on our findings, we propose that genome-wide CRISPR screens offer a complementary framework to interpret such transcriptomic data, potentially uncovering novel dependencies and therapeutic vulnerabilities in DTP cells.

Declarations

AUTHOR CONTRIBUTIONS

Conceptualization: Muhammed Yusuf. Experimental Investigation: Muhammed Yusuf Data Analysis: Muhammed Yusuf Manuscript Preparation – Original Draft: Muhammed Yusuf Manuscript Review and Editing: Muhammed Yusuf

CONFLICT OF INTEREST

The authors declare no competing interests.

DATA AVAILABILITY

The datasets generated and/or analyzed during this study are available from the corresponding author upon reasonable request.

Ethics, Consent to Participate, and Consent to Publish declarations: not applicable.

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Figures

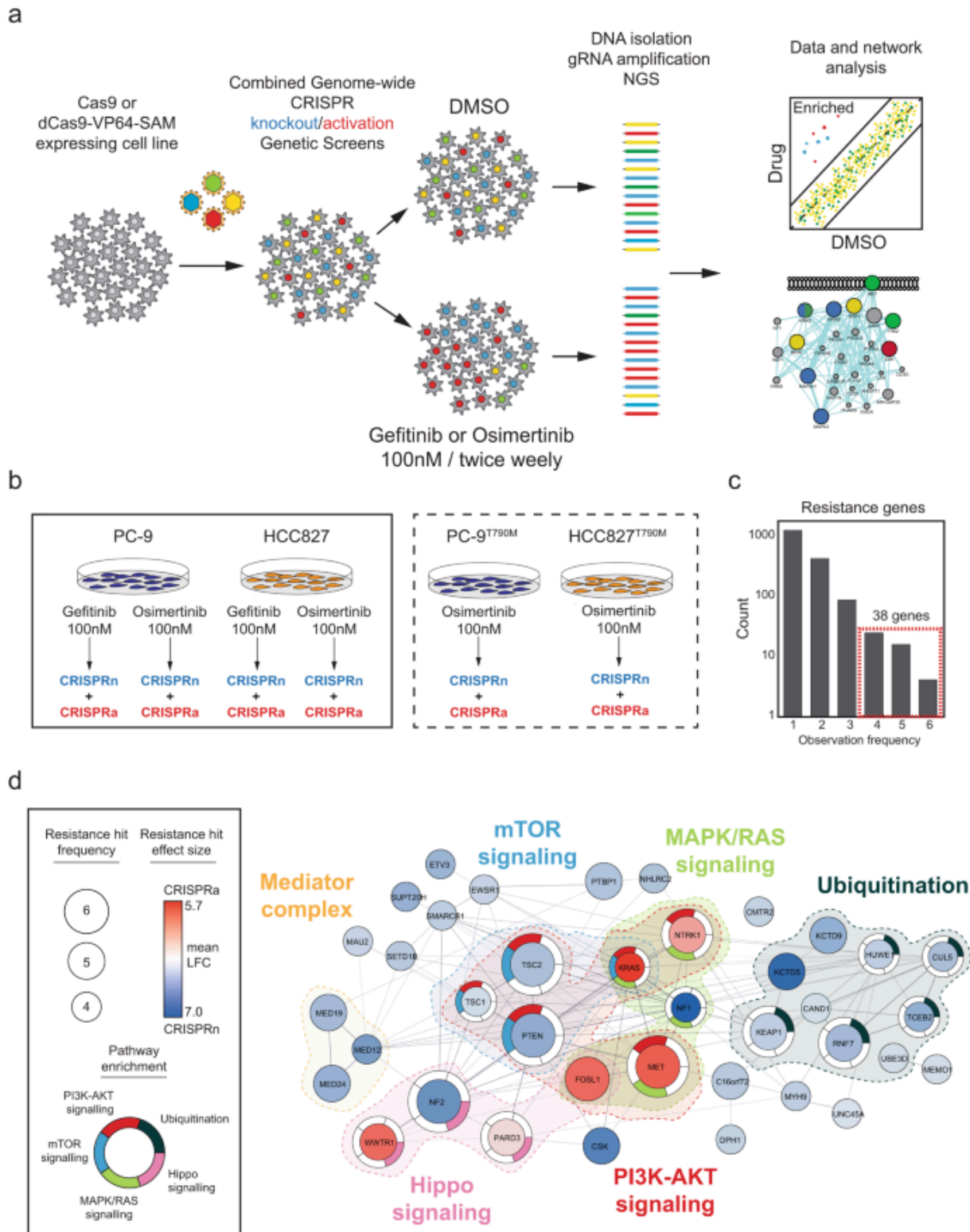


Figure 1

Genome-wide CRISPRn and CRISPRa screens reveal resistance mechanisms in EGFR-mutant lung cancer. **a, b** Overview of the screening strategy, including the EGFR-mutant cell lines and treatments applied. Cells transduced with genome-wide CRISPRn or CRISPRa libraries were cultured for 14 days under gefitinib or osimertinib treatment (IC80–90, 100 nM, administered twice weekly), or vehicle control. gRNA representation was quantified by next-generation sequencing to identify genes whose perturbation

conferred resistance. **b** Bar plot showing recurrence frequency of resistance-associated genes (defined by fold change >2 and $p < 0.005$) across six screens. Thirty-eight genes were identified in at least four screens and designated as core resistance genes. **d** Enrichment analysis of pathways associated with resistance genes. Genes are color-coded based on the type of perturbation conferring resistance: blue indicates loss-of-function (CRISPRn), and red indicates gain-of-function (CRISPRa)

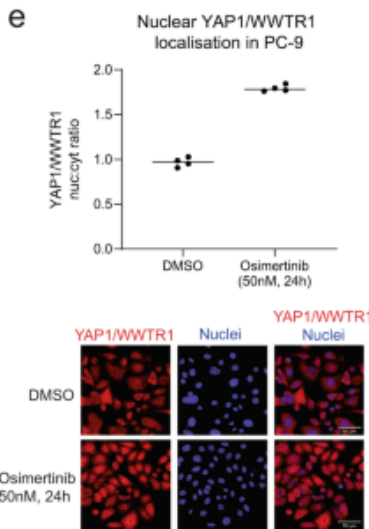
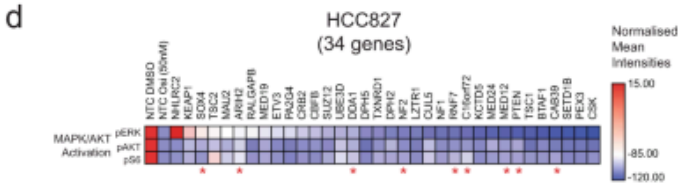
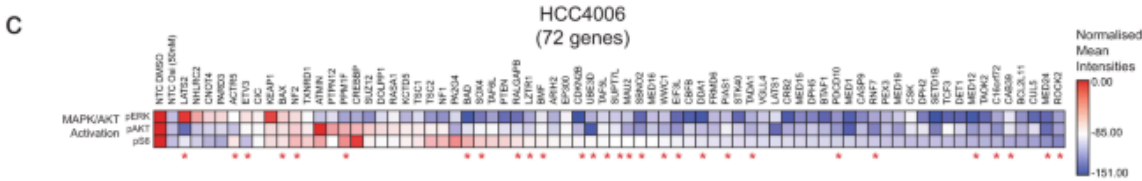
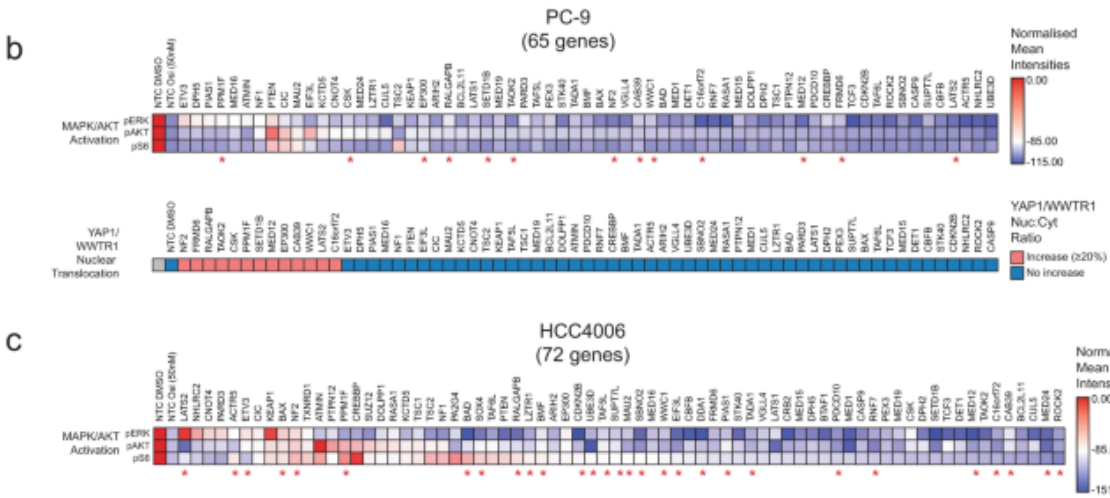
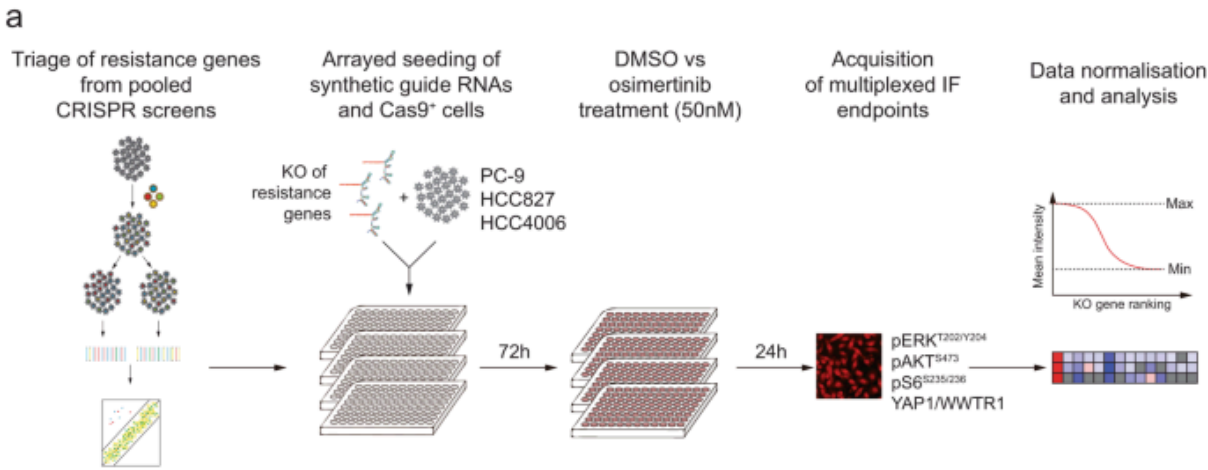


Figure 2

High-content imaging reveals Hippo pathway activation as a key feature of osimertinib resistance. a Schematic of the arrayed CRISPR knockout screening assay coupled with high-content microscopy. Cas9-expressing PC-9, HCC827, and HCC4006 cells were transfected with pools of synthetic sgRNAs targeting candidate resistance genes identified in genome-wide screens. After 72 h, cells were treated with either DMSO or 50 nM osimertinib for 24 h. A multiplexed immunofluorescence approach was used to assess expression of pathway markers: phospho-AKT (S473), phospho-ERK1/2 (T202/Y204), phospho-S6 (S235/236), and nuclear localization of YAP1/WWTR1, a readout of Hippo pathway activity. Data were normalized using a max/min scaling approach for pAKT, pERK1/2, and pS6, and by nuclear-to-cytoplasmic ratio for YAP1/WWTR1.

–d Heatmaps showing normalized effects of gene knockouts on signaling pathway reactivation in each cell line. For each, the first two columns reflect the baseline impact of osimertinib alone on pAKT, pERK, and pS6, serving as reference points (0 = DMSO, –100 = osimertinib). Remaining columns display the extent to which specific gene knockouts reverse or maintain inhibition. Red asterisks mark genes whose knockout also led to increased nuclear localization of YAP1/WWTR1. Data represent at least two biological replicates per condition.

–e Osimertinib treatment for 24 h induces elevated nuclear YAP1/WWTR1 in PC-9 cells compared to cytoplasmic levels, as quantified by the nuclear:cytoplasmic ratio. Quantification method is described in the Methods section.

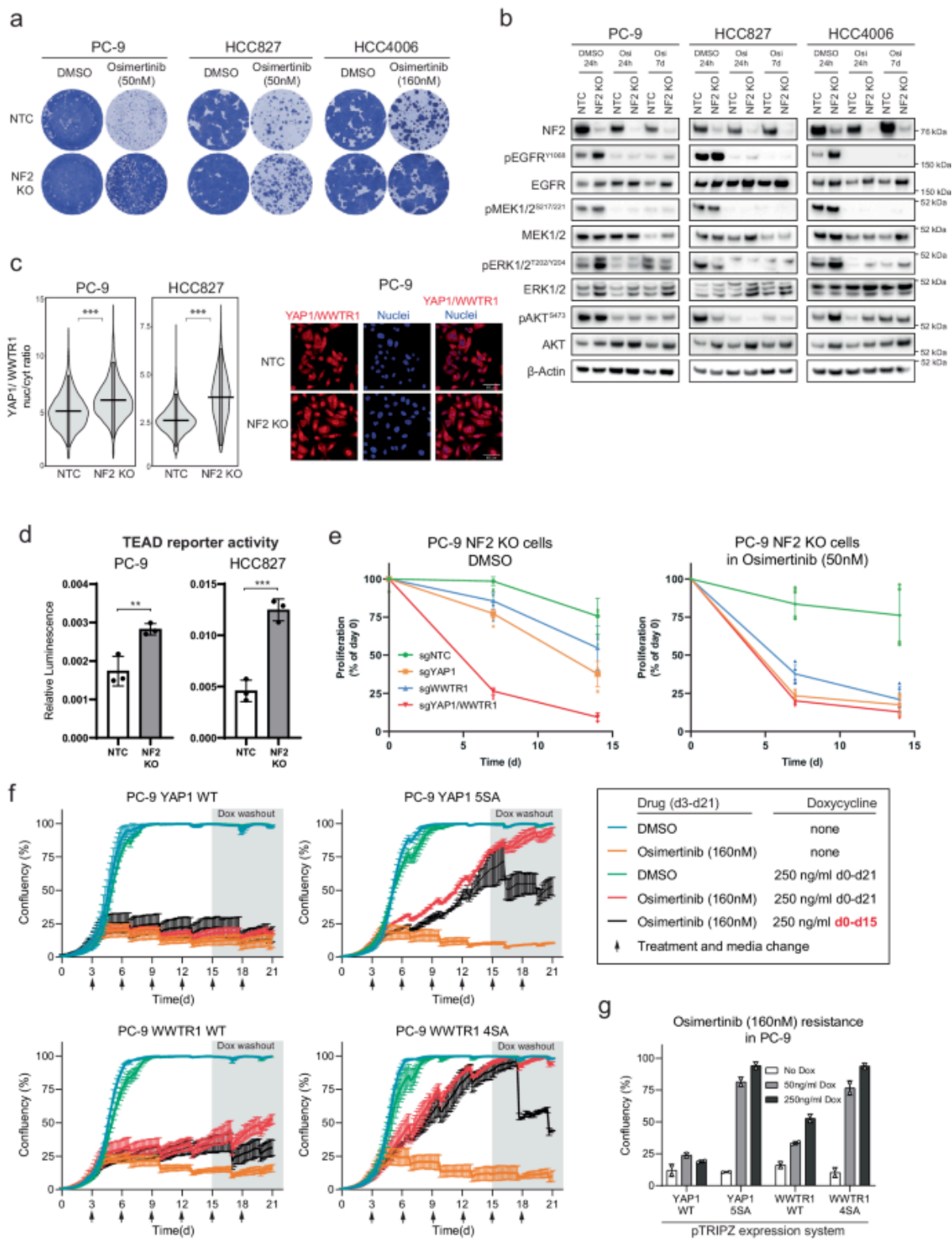


Figure 3

Hippo signaling influences osimertinib sensitivity in EGFR-mutant lung cancer. **a** Long-term clonogenic assays (21 days) in isogenic PC-9, HCC827, and HCC4006 cell lines show that NF2 knockout promotes resistance to osimertinib. **b** Western blot analysis of downstream EGFR signaling 24 hours post-osimertinib treatment indicates that NF2 loss does not restore PI3K or MAPK activity but is associated with increased basal EGFR phosphorylation. **c** Quantification of nuclear YAP1/WWTR1 levels in NF2

knockout versus control cells reveals significant nuclear enrichment following NF2 loss (Wilcoxon rank-sum test; *** $p < 0.001$). Representative immunofluorescence images illustrate enhanced nuclear localization of YAP1/WWTR1. d TEAD reporter assay demonstrates elevated transcriptional activity in NF2-deficient PC-9 and HCC827 cells compared to controls (Student's t-test; ** $p < 0.01$, *** $p < 0.001$). e Viability assays over 15 days in NF2-deficient PC-9 cells following silencing of YAP1, WWTR1, or both, and treatment with DMSO or osimertinib. Combined YAP1/WWTR1 silencing resensitizes cells to osimertinib and reduces viability. f Effects of doxycycline-induced expression of wild-type or mutant (constitutively active) YAP1 or WWTR1 on osimertinib response over 21 days in PC-9 cells. g Dose-dependent impact of doxycycline induction on osimertinib resistance in PC-9 cells expressing wild-type or mutant YAP1/WWTR1 forms, assessed after 21 days of treatment.

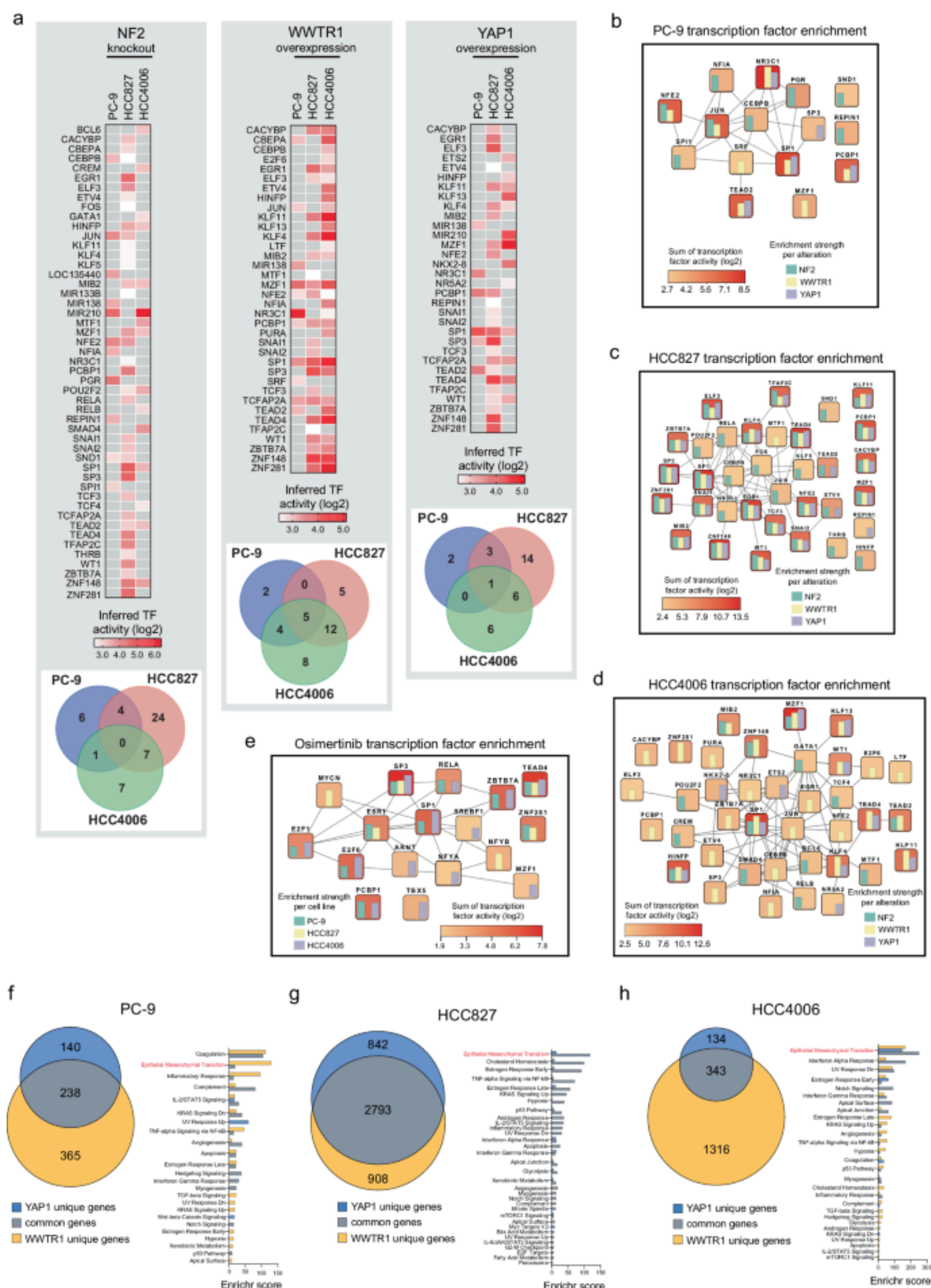


Figure 4

Transcriptional regulatory landscape following Hippo pathway activation in EGFR-mutant lung cancer cells. a Inferred transcription factor (TF) activity in PC-9, HCC827, and HCC4006 cells following activation of the Hippo pathway via NF2 knockout or overexpression of YAP1 or WWTR1. TF activity was computed using Enrichr ($p < 0.01$ threshold), and results are presented as log₂-normalized values. Each heatmap is scaled individually from its minimum to maximum values. b–e STRING interaction networks

illustrating TF connectivity and activation in response to (i) NF2 loss, (ii) WWTR1 or YAP1 overexpression, and (iii) osimertinib treatment (160 nM, 48 h). Mean TF activity scores are indicated by color shading within each node, with internal bar plots reflecting contributions from individual TF targets. f–h Bar plots displaying the number of significantly deregulated genes ($p < 0.01$, fold change ± 1.5) following Hippo effector activation and corresponding enrichment of Hallmark pathways in (f) PC-9, (g) HCC827, and (h) HCC4006 cells. Pathway significance was assessed via Enrichr, and scores represent a combined metric of enrichment significance and gene set size.

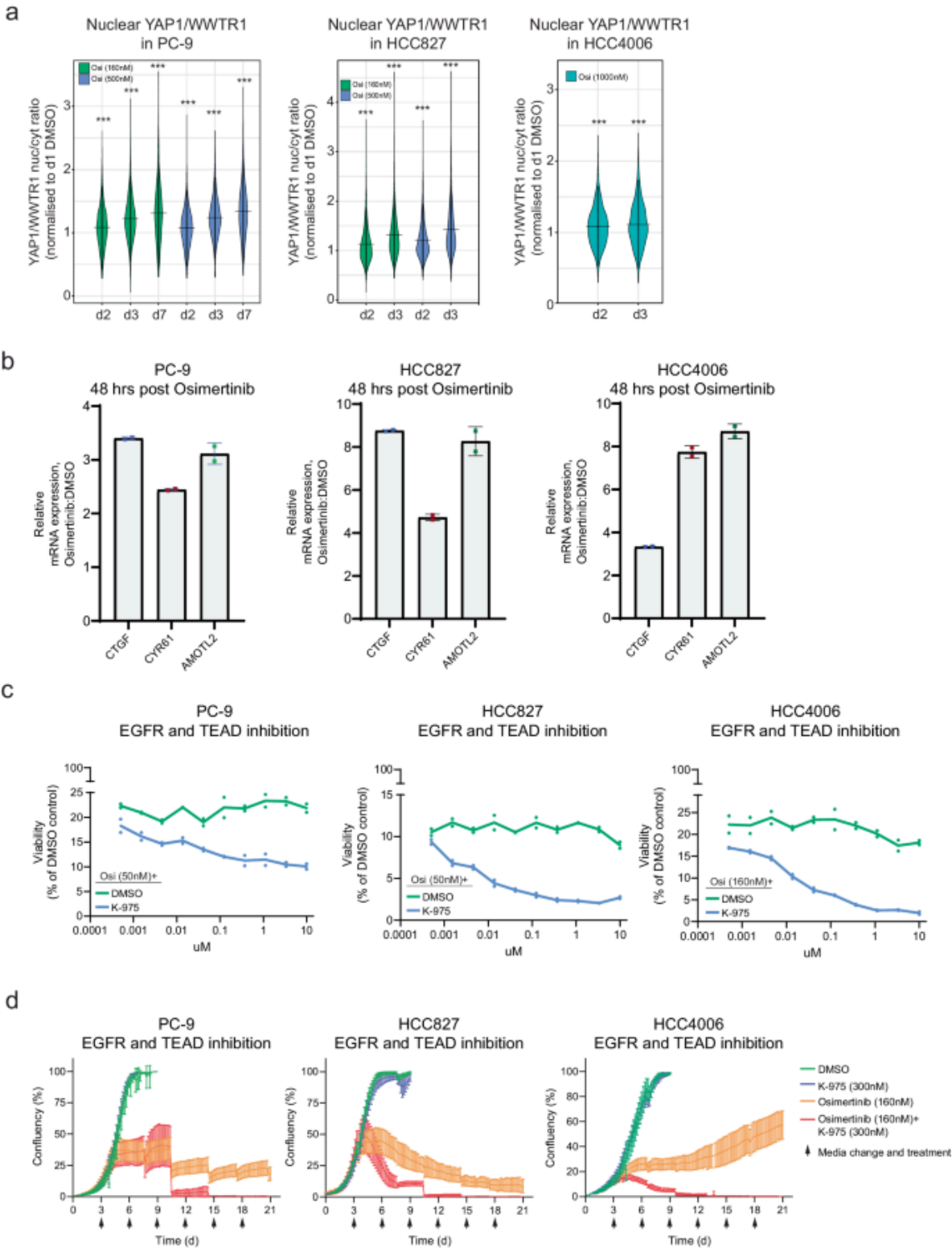


Figure 5

Hippo pathway activation promotes resistance in osimertinib-tolerant persister cells in EGFR-mutant lung cancer. a Time-course analysis of the nuclear-to-cytoplasmic ratio of YAP1 and WWTR1 expression, assessed by immunofluorescence in PC-9, HCC827, and HCC4006 cells treated with osimertinib (normalized to DMSO-treated cells at day 1). Significant increases in nuclear localization were observed at 2, 3, and 7 days post-treatment (Wilcoxon rank sum test, *** $P < 0.001$). b Quantitative PCR analysis of canonical Hippo pathway target transcripts (CTGF, CYR61, and AMOTL2) at 48 hours following osimertinib treatment, indicating transcriptional activation. c Short-term (5-day) cell viability assays in EGFR-mutant cell lines exposed to a dose range of TEAD inhibitors (K-975 or MYF-01-037) in combination with a fixed concentration of osimertinib, showing enhanced efficacy with combination therapy. d Long-term (21-day) viability assays demonstrating that co-treatment with osimertinib (160 nM) and K-975 (300 nM) leads to a marked reduction in cell viability compared to either agent alone in PC-9, HCC827, and HCC4006 cells.

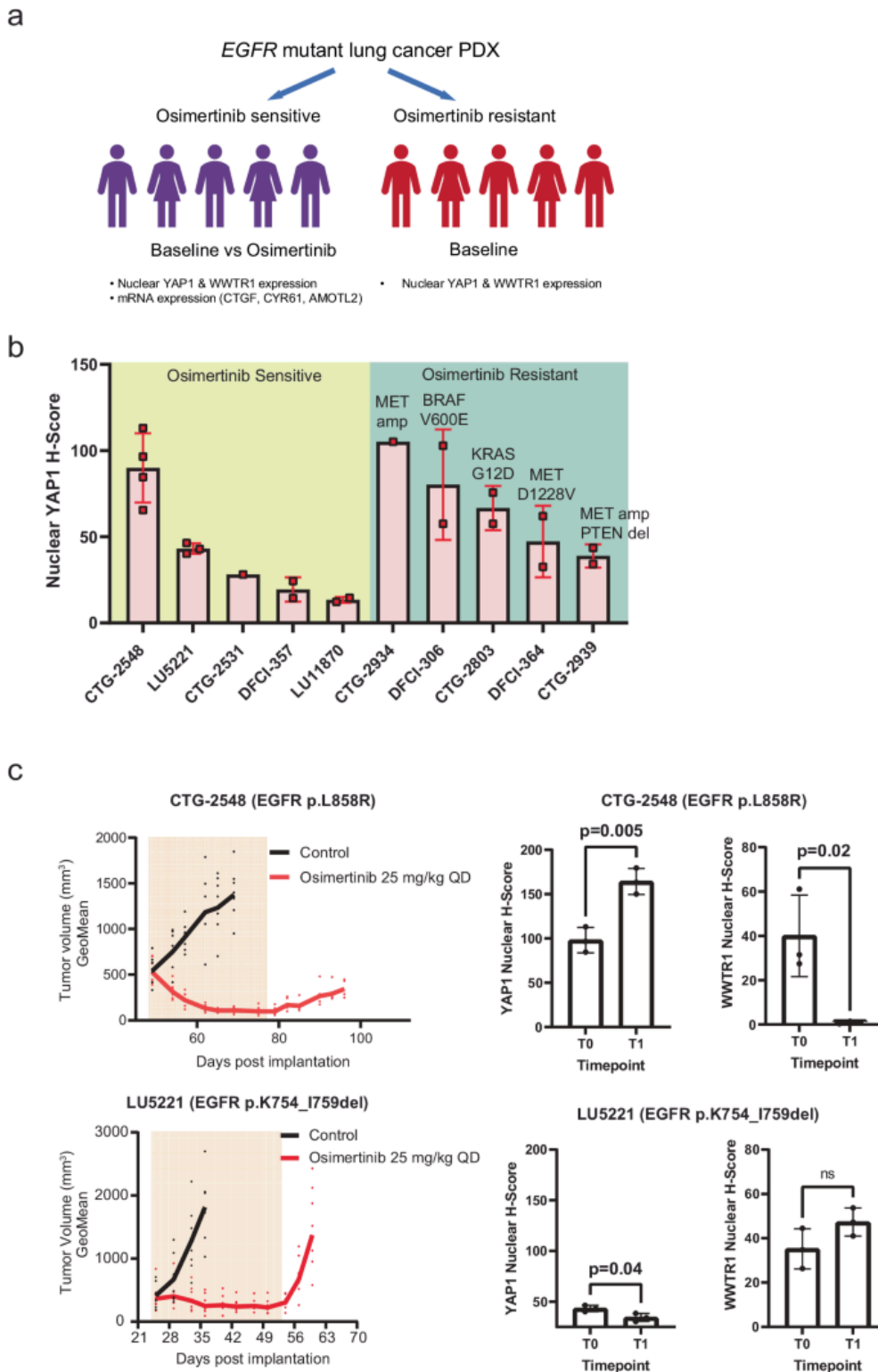


Figure 6

Acute activation of Hippo signaling in patient-derived xenograft (PDX) models treated with osimertinib. a Schematic overview of the PDX models used, their response classification to osimertinib (sensitive vs. resistant), and the assays applied. b Quantification of nuclear YAP1 expression across a panel of 10 *EGFR*-mutant lung cancer PDX models. Samples are stratified by sensitivity or resistance to osimertinib, with each point representing data from an individual mouse. The likely acquired resistance alteration

(mutation or copy number gain) is annotated for each resistant model. c (Left) Tumor growth dynamics in two osimertinib-sensitive PDX models (CTG-2548 and LU5221) over 28 days of treatment. (Right) Nuclear localization of YAP1 and WWTR1 assessed by immunofluorescence at baseline (t0) and after 28 days of treatment (t1), illustrating treatment-induced changes in Hippo pathway activity.

Supplementary Files

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