Supplemental Information

Targeting acetylcholine signaling modulates persistent drug tolerance in EGFR-mutant lung cancer and impedes tumor relapse

Meng Nie, Na Chen, Huanhuan Pang, Tao Jiang, Wei Jiang, Panwen Tian, LiAng Yao, Yangzi Chen, Ralph J. DeBerardinis, Weimin Li, Qitao Yu, Caicun Zhou, and Zeping Hu

Supplemental Materials and Methods

Supplemental Figures 1-15

Supplemental Tables 1-4
Materials and methods

Cell culture and generation of drug-tolerant persister and acquired resistance cells. Human NSCLC cell lines PC9, HCC827, H1975 were obtained from ATCC and lung fibroblast cell line MRC-5 was obtained from Cell Bank of Chinese Academy of Sciences. PC9, HCC827 and H1975 were maintained in RPMI-1640 medium and MRC-5 was maintained in MEM medium, which were supplemented with penicillin/streptomycin, and 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere containing 5% CO₂ and 95% air. All cells were routinely screened for the absence of mycoplasma contamination and were validated by short tandem repeat (STR) DNA fingerprinting. DTP cells were generated through continuous treatment with 2 μM of Gefitinib, Erlotinib, Osimertinib or Rociletinib for 9 days, according to protocols described previously (1). Acquired resistant cells were generated by treating parental cells with increasing concentrations of Osimertinib starting at 10 nM, which was followed by a stepwise dose increase every 72 h up to 1 μM.

Chemicals. Osimertinib (S7297), Gefitinib (S1025), Erlotinib (S7786), Rociletinib (S7284), LGK974 (S7143), CHIR99021 (S2924) and ICG-001 (S2662) were purchased from Selleck Chemicals. D- (+)-Vesamicol hydrochloride (V104), Hemicholinium-3 (H108) and Acetylcholine chloride (A2661) were purchased from Sigma-Aldrich. Verteporfin (HY-B0146) was purchased from MedChemExpress. Compounds used for drug screen were listed below: Darifenacin HBr (S3144), Ipratropium Bromide (S1683), Oxybutynin (S1754), Irsogladine (S1929), Methscopolamine (S1978), Atropine sulfate monohydrate (S2130), Scopolamine HBr (S2508), Pancuronium dibromide (S2497) were purchased from Selleck Chemicals. Gallamine triethiodide (G8134), DL-Homatropine hydrobromide (H0126), p-Fluorohexahydro-siladifenidol hydrochloride (H127), Hexamethonium bromide (H0879), MG624 (M3184),
Mecamylamine hydrochloride (M9020), Methoctramine hydrate (M105), Orphenadrine hydrochloride (O3752), Pirenzepine dihydrochloride (P7412), Propantheline bromide (P8891), Succinylcholine chloride dihydrate (S8251), DL-Trihexyphenidyl hydrochloride (T1516), Tetraethylammonium chloride (T2265), Telenzepine dihydrochloride hydrate (T122), Tropicamide (T9778), Benztropine mesylate (B8262), Biperiden hydrochloride (B5311), and 4-DAMP methiodide (D104) were purchased from Sigma-Aldrich.

**Analysis of sequence variations.** DNA was extracted from parental cells and Osimertinib-tolerant cells from the PC9 cell line (TaKaRa) and sequenced by the Sanger method. DNA fragments containing EGFR exons 19 and exons 20 were amplified with primers EGFR-19 forward, 5′-CAATATCAGCCTAGGTGC-3′; EGFR-19 reverse, 5′-AACATTAGATGGAGATGAG-3′; EGFR-20 forward, 5′-ATGGCCAGCGTGGACAAC-3′; EGFR-20 reverse 5′-ATCTCCCTCCCTGATTAC-3′. PCR reaction was performed at 94°C for 5 min; 35 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 40 s; and 72°C for 7 min. The PCR products were bidirectional sequenced on ABI 3730 XL sequencers (Applied Biosystems) with ABI BigDye Terminator v3.1 Cycle Sequencing Kits and analyzed by Chromas Sequence Scanner Software.

**Cell cycle analysis.** PC9 cells were seeded at $3 \times 10^5$ cells / well in 6-well plastic culture plates at day 0. Then, 2 μM Osimertinib was added on the following day. Plates were incubated at 37°C in 5% CO$_2$ for 96 h. Following treatment, cells were stained with Propidium Iodide (Beyotime) following manufacturer instructions and analyzed by flow cytometry (BD Calibur).

**RNA sequencing.** Total RNA from approximately 1 million Osimertinib-tolerant PC9 cells or parental PC9 cells was isolated by Trizol (Life Technologies) following the manufacturer’s
instructions. cDNA libraries were sequenced on an Illumina HiSeqX-ten to obtain 150-bp pair-end sequence reads. Sequencing quality was evaluated by FastQC v.0.11.8. FASTQ files were aligned to hg38 (GRCh38) using STAR v.2.6.1b (default parameters). Gene level counts were computed using featureCounts v.1.6.3 with the same human genome reference used for mapping (Gencode v.27). Differential expression analysis was performed with DESeq2 v.1.22.2 in R, and a threshold of fold change > 2 and adjusted p value < 0.05 was used to call differentially expressed genes. KEGG pathway enrichment was analyzed using DAVID v.6.7 (https://david-d.ncifcrf.gov). Gene Ontology term enrichment was performed using Metascape (http://metascape.org). Only terms with p < 0.01 and with at least 3 enriched genes were considered as significant. Heatmaps were generated using pheatmap v.1.0.12 in R. For GSEA enrichment analysis, the Molecular Signatures Database (MSigDB) was used as a source of gene sets. The enrichment of signatures was assessed using the Gene Set Enrichment Analysis (GSEA) v.4.0.2. Genes were ranked by Diff_of_classes parameter. Gene sets used were listed in Supplemental Table 1. Enrichment scores were corrected for gene-set size (normalized enrichment score). The p value estimates the statistical significance of the enrichment score for a single gene set. The RNAseq data in our study have been deposited into the NCBI Gene Expression Omnibus (GEO) database under accession number GSE153183.

Metabolomics. PC9 and HCC827 derived-DTP cells were induced as protocols described above. Two hours before collection, cells were incubated with fresh medium. At the time of collection, cells were washed with ice-cold saline, lysed with 80% methanol in water and quickly scraped into Eppendorf tubes. The insoluble material was pelleted in a cooled centrifuge at 4°C and the supernatant was transferred to a new tube and evaporated to dryness using a SpeedVac concentrator (Thermo Scientific). Metabolites were reconstituted in LC-MS grade water with
0.03% formic acid, vortex-mixed and centrifuged to remove debris. For PC9 cell xenografts and NSCLC patient-derived xenografts metabolomics experiments, tumor samples were homogenized in 80% methanol in water and centrifuged at 15,000 rpm for 15 min at 4°C. The supernatant was transferred to a new tube and evaporated to dryness as described above for cell lysates. Samples were randomized and blinded before analyzing by LC-MS/MS. Chromatographic separation was performed on a Nexera Ultra High-Performance Liquid Chromatograph (UHPLC) system (Shimadzu), with a RP-UPLC column (HSS T3, 2.1 mm × 150 mm, 1.8 μm, Waters) and the following gradient: 0-3 min 99% mobile phase A; 3-15 min 99-1% A; 15-17 min 1% A; 17-17.1 min 1-99% A; 17.1-20 min 99% A. Mobile Phase A was 0.03% formic acid in water. Mobile Phase B was 0.03% formic acid in acetonitrile. The flow rate was 0.25 mL·min⁻¹, the column was at 35°C and the samples in the autosampler were at 4°C. Mass spectrometry was performed using an AB QTRAP 6500+ triple quadrupole mass spectrometer (Applied Biosystems SCIEX) in multiple reaction monitoring (MRM) mode for the detection of 256 unique endogenous water-soluble metabolites as we previously described, with some modifications (2, 3). Chromatogram review and peak area integration were performed using MultiQuant 3.0.2 (Applied Biosystems SCIEX), and the peak area of each detected metabolite was normalized to the sum of peak areas of all detected metabolites in that sample to correct for any variations introduced by sample handling through instrument analysis. The normalized data were used as variables for the multivariate and univariate statistical analysis. Metabolomics data analysis and hierarchical clustering were performed using Metaboanalyst v.3.0 (http://www.metaboanalyst.ca) and Simca v.14.1 (Umetrics). Univariate statistical differences of the metabolites between two groups were analyzed using a two-tailed Student’s t-test. t-SNE scatterplots were generated of metabolomics data of PC9 xenografts using R Rtsne v0.15.
package. Clustering for the control, MRD, and regrown tumors was performed using detected metabolites by R Mfuzz v2.44.0 package.

**Quantitative detection of ACh by mass spectrometry.** To measure ACh and choline in cells, cell culture medium, tumor tissues from xenografts and NSCLC patient-derived PDX as well as human plasma, Trimethyl-D9-choline was added to the 80% methanol in water as an internal standard (IS) to achieve the final concentration of 5 nM. A normal-phase UPLC (NP-UPLC) method was developed using the above-mentioned UHPLC system on a NP-UPLC column (BEH HILIC, 2.1 mm × 100 mm, 1.7 μm, Waters) with the following gradient: 0-3 min 99% mobile phase A; 3-15 min 99-1% A; 15-17 min 1% A; 17-17.1 min 1-99% A; 17.1-20 min 99% A. Mobile Phase A was 5 mM NH₄COOH in H₂O: ACN (5: 95). Mobile Phase B was 5 mM NH₄COOH in H₂O: ACN (50: 50). The flow rate was 0.5 mL·min⁻¹, the column was at 35°C and the samples in the autosampler were at 4°C. Mass spectrometry was performed using an AB QTRAP 6500+ triple quadrupole mass spectrometer (Applied Biosystems SCIEX) in MRM positive mode for the detection of ACh (146.0/87.0), choline (104.0/60.0), and IS (113.3/69.2). Chromatogram review and peak area integration were performed using MultiQuant software version 3.0.2. The concentrations of analytes were calculated by peak area ratios of the analytes to IS using standard curves generated with weighted linear regression analysis in MultiQuant 3.0.2. Statistical differences of the metabolites between the two groups were analyzed using a two-tailed Student’s t-test.

**Patient samples.** All human studies were conducted according to the principles of the Declaration of Helsinki, and approved by the Institutional Review Board and Biomedical Ethics Committee of Shanghai pulmonary hospital of Tongji University, Affiliated Tumor Hospital of Guangxi Medical University, and West China Hospital of Sichuan University. Pre-EGFR TKI
treatment plasma samples were obtained from Shanghai pulmonary hospital of Tongji University, Affiliated Tumor Hospital of Guangxi Medical University, and West China Hospital of Sichuan University. Tumor and normal adjacent tissues from NSCLC patients with or without EGFR mutation were obtained from Shanghai pulmonary hospital of Tongji University. Detailed patient clinical data are listed in Supplemental Table 2 and Supplemental Table 3.

**Cell viability and apoptosis assays.** Briefly, tumor cells (2-5 × 10³ cells / 100 μL / well) in RPMI 1640 medium supplemented with 3% or 10% FBS were plated in 96-well or 384-well plates and cultured with drugs. Cell viability was assessed using CellTiter Glo Luminescent Cell Viability assay (Promega). For DTP formation assays, the cells were treated with or without EGFR TKIs in combination with indicated compounds for 6 days. The remaining cell viability was measured for each parental and DTP cells model. For cell apoptosis assay, 1×10⁵ cells were stained with FITC Annexin V and PI from an apoptosis detection kit (Beyotime, C1062) and analyzed for apoptosis by flow cytometry on an LSRFortessa SORP (BD) according to the manufacturer’s recommendations.

**Drug screen.** We performed a drug screen using 26 small molecule compounds (detailed information of chemicals was list in the Supplemental Table 4) that targeting mAChR and nAChR. In brief, compounds were added in DMSO-containing (as parental cell group) or 2 μM EGFR TKI-containing medium (as DTP cell group). After 6 days of incubation, each well was measured using CellTiter Glo reagent (Promega). All compounds from the screen library were tested at six concentrations. The screens were performed in 6 replicates. The relative survival of parental PC9 cells and EGFR TKI-induced tolerant PC9 cells in the presence of compounds was normalized against negative control conditions (compounds-untreated cells).
Colony-formation assays. Cells were cultured and seeded into 6-well plates at a density of 3-5 × 10^4 cells / well and were cultured in medium containing the indicated drugs for 9 days. The medium was replenished every 3 days. Cells were fixed with 4% formaldehyde in PBS and stained with 0.5% crystal violet (Leagene). To evaluate the effect of exogenous ACh on DTPs formation, PC9 and HCC827 cells were pretreated with exogenous ACh for 3 and/or 9 days, followed by the combination with the 2 μM Osimertinib for 9 days. Images were analyzed using ImageJ with ColonyArea plugin.

RT-PCR and real-time PCR. Total RNA from cells and tumor samples was isolated using Trizol (Life Technologies) according to the manufacturer’s instructions. cDNA synthesis was performed using PrimeScript™ RT reagent Kit from TaKaRa. RT-PCR reactions were performed with 2x Taq Master Mix (Vazyme). Quantitative real-time PCR reactions were performed with iTaq Universal SYBR green Supermix with blended ROX and fluorescein passive reference dyes from Bio-Rad. The experiments were performed according to the manufacturer’s instructions. The sequences of the primers used for RT-PCR analyses were as follows:

common ChAT forward, 5’-GGAGATGTTCTGCTGCTATG-3’; common ChAT reverse, 5’-GGAGGTGAAACCTAGTGGCA-3’;
ChAT-212 transcript forward, 5’-GTTTCCCCCATGCTGTTCT-3’; ChAT-212 transcript reverse, 5’-TACAAGGCAGATGCAGCGC-3’.
GAPDH forward, 5’-GGAGCGAGATCCCTCCAAAAT-3’; GAPDH reverse, 5’-GGCTGTTGTCATACTTCTCATGG-3’.

The sequences of the primers used for quantitative real-time RT-PCR analyses were as follows:
common ChAT forward, 5’- CATCGTCTGGTGCAGTG -3’; common ChAT reverse, 5’-
GAAGTTTTTCTGCCAGAGAG -3’; ChAT-212 transcript forward, 5’-
GTTTCCCATGCTGGTCT -3’; ChAT-212 transcript reverse, 5’-
ATCGAATGGGAGTGTTCG-3’; CD133 forward, 5’-TTACGGCAGCTATTCACCT-3’;
CD133 reverse, 5’-TATTCACACAGCAAAA-3’; AXIN2 forward, 5’-
TACTCCTTATTGGGCGATCA-3’; AXIN2 reverse, 5’-
TTGGCTACTCGTAAAGTTTTGGT-3’; WNT3A forward, 5’-
TCAGCTGCCAGAGTGCAC-3’; WNT3A reverse, 5’-CGCCCCTCAGGGAGCAGCCTAC-3’;
WNT10A forward, 5’-CCAATGACATTCTGGACCT-3’; WNT10A reverse, 5’-
TAAGCGGGTGCAGCTTCCTAC-3’; WNT5A forward, 5’-ATGAACCTGCAACAACGA-3’;
WNT5A reverse, 5’-CTTCTCTCCCTCACGCGGCTAC-3’; WNT4 forward, 5’-
GCAGAGCCCCTCATGAACCT-3’; WNT4 reverse, 5’-CACCCGCATGTGTGTCAG-3’;
WNT8B forward, 5’-CCGACACCTTTCGCTCCATC-3’; WNT8B reverse, 5’-
CAGCCCTAGCGTTTTGTGCTTC-3’; WNT9A forward, 5’-AGCAGCAAGTTCCTGAGGAA-
-3’; WNT9A reverse, 5’-CCTTCACACCCAGAGGT-T3’; BCL2L1 forward, 5’-
GAGCTGTTGGACTTCTC-3’; BCL2L1 reverse, 5’-TCCATCTCCGATCCAGTCCCTCCT-3’;
S100A4 forward, 5’-AAGTAAAGGCTCGCTGAC-3’; S100A4 reverse, 5’-
TGTTGCTGAGGATGCTG-3’; WNT6 forward, 5’-AGAGTGCCAGTCCCTGAGTGG-3’;
WNT6 reverse, 5’-GAACAGCAGGAGCCGTGCTG-3’; GAPDH forward, 5’-
ACCCAGAAGACTGTGGATGG-3’; GAPDH reverse, 5’-TTCAGCTCAGGGATGACCTT-3’.

**RNA interference.** For siRNA transfection, lipofectamine RNAiMAX (Thermo) was used
according to the standard protocol. All siRNAs targeting the genes were obtained from
GenePharm Technologies. The two ChAT siRNA targeting sequences were 5’-
GGUAUGGUGCCUGCUACAA TT-3’ and 5’-AGAUGUUCAUGGAUGAAA CT TT-3’. pLV vectors expressing shRNAs targeting the mRNA sequences of human ChAT (shRNA1: pHS-ASR-0428; shRNA2: pHS-ASR-0429) and corresponding empty vector (shRNA: pHS-ASR-LW429) were purchased from Syngentech; pLKO vectors expressing shRNAs targeting M3R (shRNA1: TRCN0000011259, shRNA2: TRCN0000011260), VACHT (shRNA1: TRCN0000044606, shRNA2: TRCN0000044607), YAP (shRNA1: TRCN0000107265, shRNA2: TRCN0000107268) and control shRNA (SHC002) were purchased from the Centre of Biomedical Analysis, Tsinghua University, shRNA library. psPAX2 and pMD2.G were purchased from Addgene (Cambridge), Opti-MEM was purchased from Gibco, and Lipofectamine 3000 was purchased from Invitrogen. The ratios used were recombinant lentivirus plasmid: psPAX2: pMD2.G = 2:1:1 and plasmid: Lipofectamine = 1:1. Lentivirus production and viral transduction of 293T cells were performed as described above 8 h later, the medium was replaced with fresh medium. Virus-containing medium were collected after 48 h and 72 h transfection.

**ChAT overexpression.** Expression vectors for full-length of ChAT (pHS-AVC-LW2172) and corresponding empty vector (pHS-BVC-LW233) were purchased from Syngentech. Expression vectors for ChAT-212 short isoform (pHS-AVC-1609) and corresponding empty vector (pHS-B-0165) were purchased from Syngentech. All vectors were employed to construct the lentivirus according to previously described. PC-9 and HCC827 cells were transduced with lentivirus.

**Generation of ChAT CRISPR/Cas9 knockout in PC9 cells.** plentiCRISPR v2-ChAT sgRNA (pHS-ACR-0205/pHS-ACR-0206) and corresponding empty vector (pHS-ACR-LW1035) were purchased from Syngentech. All vectors were confirmed by DNA sequencing. Lentivirus were
produced also according to previously described. PC-9 cells were transduced with lentivirus for 72 h and then selected with puromycin. Selection of monoclonal cells were performed.

**Western blot analysis.** Protein lysates were prepared in RIPA buffer and quantified using the BCA protein assay kit (Beyotime). Proteins were separated on SDS-PAGE gels, transferred to PVDF membranes, and probed with antibodies against ChAT (1:1000, Proteintech, 20747-1-AP), GAPDH (1:3000, Proteintech, 60004-1-lg), α-Tubulin (1:3000, Proteintech, 11224-1-AP), HDAC3 (1:1000, Proteintech, 10255-1-AP), β-catenin (1:1000, Cell Signaling, 8480), Non-phospho (Active) β-catenin (1:1000, Cell Signaling, 8814), AChE (1:1000, Abcam, ab31276), VACHT (1:1000, Abcam, ab235201), M3R (1:1000, Abcam, ab126168), CHT1 (1:1000, Proteintech, 21848-1-AP), YAP (1:1000, ABclonal, A1002), FLAG M2 (1:1000, Sigma-Aldrich, F1804). After overnight incubation with primary antibody at 4°C, washing, and incubation with secondary antibodies, blots were performed using a chemiluminescence system (Pierce). To detect proteins with similar size, we did membrane stripping for re-probing.

**Immunohistochemistry.** For histology, samples were formalin-fixed, paraffin-embedded, sectioned at 5 μm and stained with H&E according to standard histopathological techniques. For immunohistochemical analysis, 5 μm paraffin sections were deparaffinized and rehydrated. The endogenous peroxidase was inactivated with a 3% hydrogen peroxide/methanol solution (ZSGB-BIO, PV-9001, reagent 1) and washed with PBS. After incubation for 15 min in a boiling water bath for antigen retrieval. Block in 10% normal serum with 1% BSA in TBS for 2 h at room temperature. Sections were incubated with primary antibodies diluted in blocking buffer overnight at 4°C. Primary antibodies used were the following: ChAT (1:100, Merck, AB143), WNT6 (1:25, Abcam, ab150588), WNT9A (1:50, Abcam, Ab125957), AChE (1:400, Abcam, Ab97299), M3R (1:100, Abcam, Ab87199), VACHT (1:50, Abcam, Ab235201), Ki-67 (1:200,
Abcam, Ab15580), pEGFR (1:50, CST, 3777), pERK (1:200, CST, 4370), pAKT (1:100, CST, 4060). The sections were washed with PBS for 3 times, and then incubated with antibody enhancing solution (ZSGB-BIO, PV-9001, reagent 2) for 20 min. The sections were washed with PBS for 3 times and then incubated with secondary antibody (ZSGB-BIO, PV-9001, reagent 3) for 20 min at room temperature. Samples were visualized with a scanning microscope (Pannoramic SCAN) and images were acquired in a bright field using the GS3-U3-51S5M-C system and processed using Case Viewer software (Pannoramic SCAN). Brightness and contrast for representative images were adjusted equally among groups. Images were captured using Case Viewer and immunoreactivity was evaluated with IHC Profiler as an image J plug-in in blinded manner. The evaluation was based on staining intensity and the extent of staining. Randomly selected fields were quantified cells staining positive values.

**Generation of patient-derived xenografts.** PDX-NSCLC645 and PDX-NSCLC868, derived from EGFR-mutant NSCLC patients, were conducted by LIDI Biotech Co. and CrownBio Co., respectively. The companies were responsible for any necessary IRB approvals and patient consents to allow the tumor tissue to be used in research. NSCLC645 was established from a 62-year-old woman with lung cancer containing exon EGFR 19 deletion & T790M and NSCLC868 was established from a woman with lung cancer containing EGFR L858R mutation & T790M. Tumor specimens were subcutaneously propagated in BALB/c nude mice. When tumors reached nearly 300 mm³ in volume, 5 mg/kg Osimertinib was administered daily by oral gavage. Tumor size was evaluated twice a week using a caliper, and the volume of the mass was calculated using the formula 0.5 × L × W², where L is the long diameter and W is the short diameter. To test the efficacy of the combination therapy in vitro, tumor cells derived from NSCLC645 were seeded
into 96-well plate and incubated with indicated drugs for 6 days. Cell viability was assessed using CellTiter Glo Luminescent Cell Viability assay.

**Luciferase assay.** Genomic region harboring ChAT enhancers (#1 and #3) was amplified by High-Fidelity PCR (NEB, M0492S) and cloned into pGL4.23[luc2-minP] vector (Miaolingbio, P0696). Briefly, ChAT enhancer reporter, control vector and Renilla (Addgene, 87121) were transfected into PC9 cells with shControl or shYAP using PEI transfection reagent (Proteintech, PR40001). Fresh media was replaced either with DMSO or with 2 μM Osimertinib post 12 h transfection. Cells were lysed 72 h later and detected for luciferase activity using the Dual-Luciferase Reporter Assay System (Promega, E1910). The firefly luciferase activity was normalized to Renilla.

**ChIP-qPCR assays.** The ChIP assay was performed using the SimpleChIP® Plus Sonication Chromatin IP Kit (CST, 56383), following the manufactures instructions. PC9 cells were cultured on 15 cm plates and treated either with DMSO or with 2 μM Osimertinib for 48 h. Then, cells were collected for further analysis according to the provided protocol. The following antibodies were used for ChIP: YAP (CST, 14074) or IgG (supplied with the kit).

All primer sequences used for ChIP-qPCR assay are as following:

ChAT-enhancer #1-forward: AGGGAGTCTGGAATCTGCTC, reverse: AGCCATTCCTCATTTGAACATCT;

ChAT-enhancer #2-forward: TAGCCAAGCAGAAGACGGG, reverse: TCAGTGCAAGGAGACAGGTT;
ChAT-enhancer #3-forward: GGCATGGGTGTAGAGGACAT, reverse:
CCTTCTTTTCTAAGGCTGCTT;

**Xenograft experiments.** All animal protocols related to mouse experiments were approved by the University of Tsinghua Institutional Animal Care and Use Committee. The animal protocols are compliant with all relevant ethical regulations. Female BALB/c nude mice aged 6–8 weeks were purchased from Charles River Laboratories Inc., Wilmington, MA. For experiments testing the effects of ChAT on MRD formation and formed MRD. Wild type PC9 or PC9 ChAT CRISPR/Cas9 knockout cells (3 × 10⁶ cells per mouse) were injected subcutaneously into the right flanks of mice. After tumor establishment, tumor-bearing mice with PC9 WT and ChAT knockout cells were all randomly assigned to receive either vehicle or Osimertinib (1 mg/kg/d), all agents were administered by oral gavage. Body weights were measured over the course of treatment, and tumor growth was monitored by caliper measurements twice weekly until endpoint was reached and mice were euthanized. In addition, PC9 cells (3 × 10⁶ cells per mouse) with shChAT or control shRNA, and HCC827 cells (3 × 10⁶ cells per mouse) with stably full-length ChAT overexpression or negative controls were injected subcutaneously into the right flanks of mice. After tumor establishment, mice were randomly placed into groups to receive either vehicle or Osimertinib. Mice were treated with drugs using doses for the indicated time described in the figure legends.

For the experiments testing the effects of Darifenacin on the MRD formation, PC9 cells (5 × 10⁶ cells per mouse) were injected subcutaneously into the right flanks of mice. After tumor establishment, mice were randomly placed into different groups. Groups were treated as follows: Group 1: vehicle daily to the end of the study. Group 2: Darifenacin (5 mg/kg/d, intra-peritoneal injection) alone to the end of the study. Group 3: Osimertinib (5 mg/kg/d, oral gavage) alone for
9 days + vehicle from day 9 to the end of the study. Group 4: combination of Osimertinib (5 mg/kg/d, oral gavage) with Darifenacin (5 mg/kg/d, intra-peritoneal injection) for 9 days + vehicle from day 9 to the end of the study. Group 5: combination of Osimertinib (5 mg/kg/d, oral gavage) with Darifenacin (5 mg/kg/d, intra-peritoneal injection) for 9 days + Darifenacin (5 mg/kg/d, intra-peritoneal injection) from day 9 to the end of the study. For the experiment testing the effects of Darifenacin on the formed MRD, PC9 xenografts were treated with Osimertinib (1 mg/kg/d, oral gavage) for 9 days and randomly assigned to treatment with Osimertinib (1 mg/kg/d, oral gavage) alone or a combination of Darifenacin (5 mg/kg/d, intra-peritoneal injection).

For the experiments testing the functional role of ChAT short isoform in driving drug tolerance, the xenograft models were established by injection of PC9 cells with stable overexpression of ChAT short isoform or negative controls. PC9 cells with stable overexpression of ChAT short isoform or negative controls cells (3 × 10^6 cells per mouse) were injected subcutaneously into the right flanks of mice. After tumor establishment, mice were randomly placed into different groups as indicated. Xenografts mice were treated with vehicle or Osimertinib (5 mg/kg/d, oral gavage) for 9 days, followed by drug withdrawal.

For the experiments exploring whether exogenous ACh supplementation can rescue the growth defect caused by ChAT knockout, we pretreated PC9 WT and ChAT knockout cells with or without exogenous ACh for 3 days, which were injected subcutaneously into the right flanks of mice (3 × 10^6 cells per mouse). After tumor establishment, the mice were pretreated with ACh (20 mg/kg/d, subcutaneous injection) for 7 days and then were randomly placed into groups to receive corresponding vehicle, Osimertinib (5 mg/kg/d, oral gavage) alone, ACh (20 mg/kg/d, subcutaneous injection) alone, or a combination of Osimertinib and ACh.
subcutaneous injection) alone, or the combination of Osimertinib and ACh for 9 days, followed by Osimertinib withdrawal and continuous ACh treatment.

Tumor size was measured using a caliper and tumor volume was calculated using the standard formula: \(0.5 \times L \times W^2\), where \(L\) is the long diameter and \(W\) is the short diameter. For survival analysis, Kaplan-Meier analysis was used for calculating percent survival using GraphPad Prism v.7.0. The percent survival was calculated when the tumors reached 1000 mm\(^3\).

1. Sharma SV, Lee DY, Li B, Quinlan MP, Takahashi F, Maheswaran S, et al. A chromatin-mediated reversible drug-tolerant state in cancer cell subpopulations. *Cell.* 2010;141(1):69-80.

2. Agathocleous M, Meacham CE, Burgess RJ, Piskounova E, Zhao Z, Crane GM, et al. Ascorbate regulates haematopoietic stem cell function and leukaemogenesis. *Nature.* 2017;549(7673):476-81.

3. Huang F, Ni M, Chalishazar MD, Huffman KE, Kim J, Cai L, et al. Inosine Monophosphate Dehydrogenase Dependence in a Subset of Small Cell Lung Cancers. *Cell Metab.* 2018;28(3):369-82 e5.
Supplemental Figure 1. Characterization of drug-tolerant persister cancer cells. (A) Analysis of EGFR exon 20 sequence variations of parental PC9 cell (top) and Osimertinib-tolerant PC9 cells (bottom). Repeated two times with similar results. (B and C) Distribution of parental PC9 and Osimertinib-tolerant PC9 cells in G1, S and G2 phases. (D and E) KEGG pathway enrichment for down-regulated genes and up-regulated genes identified by RNA-seq data of Osimertinib-tolerant PC9 cells compared to parental PC9 cells. (F and G) Heatmap of purine metabolism and pyrimidine metabolism related gene expression value in Osimertinib-tolerant PC9 cells compared to parental PC9 cells. Rows are z-scores calculated for each gene in both cell types. (H-J) Gene enrichment analysis of RNA-seq data using gene set enrichment analysis (GSEA). Normalized enrichment score (NES) and false-discovery rate (FDR) q value are shown.
Supplemental Figure 2. Metabolomics analysis of the EGFR-TKI induced DTP cells. (A-D) Relative abundance of metabolites extracted from Osimertinib-induced DTP cells, Gefitinib-induced DTP cells and parental cells of PC9 and HCC827. Peak area of each metabolite was normalized by the sum of peak areas of all detected metabolites in that sample and the heatmap displays the normalized data for each metabolite (n = 6). (E) Principal-component analysis of the metabolite detected from parental PC9 cells, Osimertinib-induced DTP cells (left) and Gefitinib-induced DTP cells (right) (n = 6). (F) Principal-component analysis of the metabolites detected from parental HCC827 cells, Osimertinib-induced DTP cells (left) and Gefitinib-induced DTP cells (right) (n = 6).
Supplemental Figure 3. Establishment of the Osi-regressed tumors in NSCLC PDX models.  

(A and B) Tumor volume of NSCLC645 PDX model and NSCLC868 PDX model exposed to 5
mg/kg/d Osimertinib \( (n = 3) \). (C and D) Representative image and quantification of immunohistochemical staining of Ki67 and pERK on the tumor sections from control and Osi-regressed tumors from NSCLC645 PDX model and NSCLC868 PDX model \( (n = 2 \text{ or } 3) \). Scale bar, 20 μm. Data are shown as mean ± s.e.m., two-tailed Student’s \( t \) test was used.
Supplemental Figure 4. Metabolomics analysis of the Osi-regressed tumors in NSCLC PDX models. (A and B) Principal-component analysis of the metabolites detected from NSCLC645 PDX model and NSCLC868 PDX model. (C and D) Relative abundance of metabolites extracted from control and Osi-regressed tumors from the NSCLC645 PDX model and the NSCLC868 PDX model. Peak area of each metabolite was normalized by the sum of peak areas of all detected metabolites in that sample and the heatmap displays the normalized data for each metabolite. (A-D) $n = 3$ or 4 mice, three fragments were harvested for analysis in each tumor of controls.
Supplemental Figure 5. Metabolomics analysis of the MRD and regrown tumors after drug withdrawal of PC9 xenografts. (A) Dose-response curves of PC9 or HCC827 parental, DTP, regrown and re-derived DTP cells incubated in increasing concentrations of Osimertinib for 72 h (n = 5). (B) Relative abundance of metabolites extracted from control (n = 9), MRD (n = 6) and regrown tumors (n = 9) from PC9 xenograft mouse models. Peak area of each metabolite was normalized by the sum of peak areas of all detected metabolites in that sample and the heatmap displays the normalized data for each metabolite. (C) Alterations of metabolites associated with choline metabolism. (A and C) Data are shown as mean ± s.e.m., two-way ANOVA adjusted by Bonferroni’s correction (A) and one-way ANOVA with Tukey’s test (C) was used.
Supplemental Figure 6. The expression of key proteins associated with ACh metabolism and signaling. (A and B) Western blot analysis of indicated proteins from PC9 and HCC827 DTP cells induced by 2 μM indicated drugs for 9 days or in the parental cells. (C) Different transcripts of ChAT gene via alternative splicing were explored by Ensembl database. (D) Western blot analysis of ChAT isoforms in mouse brain, EGFR-mutant NSCLC cell lines and normal lung fibroblast cell line. (E) RT-PCR of ChAT isoforms in EGFR-mutant NSCLC cell lines and normal lung fibroblast cell line. (F) Western blot analysis of short ChAT isoform (41 kDa) in tumor and normal adjacent tissues of NSCLC patients with or without EGFR mutation. (G) RT-PCR of ChAT isoforms in tumor and normal adjacent tissues of NSCLC patients with or without EGFR mutation.
Supplemental Figure 7. Immunohistochemistry analysis of PC9 xenograft mouse models.  
(A) Representative image and quantification of immunohistochemical staining of pEGFR, pAKT, pERK on the tumor sections from control, MRD and regrown tumors from PC9 xenograft mouse models. Scale bar, 50 μm. Data are shown as mean ± s.e.m., n = 3 mice per group, one-way ANOVA with Tukey’s test was used. (B) Kaplan-Meier curve showing the correlation between ChAT expression and overall survival. RNA-seq data and clinical information of EGFR-mutant NSCLC patients of TCGA dataset were obtained from the cbioportal website. Low expression of ChAT was defined as z-score ≤ -1. Log-rank test was used.
**Supplemental Figure 8.** The binding site of YAP/TEAD complex is located around the potential enhancer regions of ChAT gene. (A) Visualization of YAP/TEAD ChIP-seq signal around the potential enhancers of ChAT gene in PC9 cells from the public ChIP-seq data integrated by ChIP-Atlas. ChIP-seq data published in previous study (GSE131687) show increased ChIP-seq signal of YAP and H3K27ac around three potential enhancers (#1, #2 and #3) of ChAT gene in PC9 cells treated with the combination of 100 nM Osimertinib and 30 nM trametinib for 48 h compared to the cells treated with DMSO. (B and C) Relative mRNA and protein level of ChAT in PC9 cells treated with 2 μM Osimertinib with or without 2 μM
Verteporfin for 6 days ($n = 3$). Data are shown as mean ± s.e.m., one-way ANOVA with Tukey’s test.
Supplemental Figure 9. The role of ChAT in modulating DTP cells formation and residual tumor relapse. (A) ACh levels and short ChAT isoform expression in PC9 single cell clones \((n = 4)\). (B) Relative ACh levels and short ChAT isoform expression in cells with Flag-ChAT short isoform overexpression (ChAT-OE, 41 kDa) and negative control (NC) \((n = 6)\). (C) Western blot showing the overexpression of full-length ChAT. (D) Quantification of ACh levels in cells with overexpression of full-length ChAT (ChAT\textsubscript{FL}-OE) or negative control (NC) \((n = 5)\). (E) Colony formation assay of cells with ChAT\textsubscript{FL}-OE or NC exposed to the 2 \(\mu\)M Osimertinib for 9 days \((n = 3)\). (F) HCC827 xenograft mice injected with ChAT\textsubscript{FL}-OE or NC cells were treated with 5 mg/kg Osimertinib for 9 days, or treated with vehicle. \(n = 5\) or 8 per group. (G) Quantification of ACh levels in PC9 wild type (WT) and ChAT knockout cells with or without 2 \(\mu\)M Osimertinib for 6 days \((n = 4\) or 5\). ChAT sgRNA was confirmed by western blot in left. (H) Quantification of ACh levels in PC9 cells transfected with ChAT or control siRNA for 24 h and followed by 2 \(\mu\)M Osimertinib for 4 days \((n = 4)\). ChAT siRNA was confirmed by western blot. (I) Colony formation assay of cells transfected with ChAT or control siRNA for 24 h and followed by 2 \(\mu\)M Osimertinib for 9 days \((n = 3)\). (J) Western blot analysis of indicated proteins in cells with stably expressed control (shCtrl) and ChAT (shChAT) shRNAs. (K) Dose-response curves to Osimertinib in PC9 cells and Gefitinib in HCC827 cells expressing shCtrl or shChAT for 5 days \((n = 5-6)\). IC50 values were compared with one-way ANOVA. (L) PC9 xenograft mice injected with ChAT or control shRNA cells were treated with vehicle or 5 mg/kg Osimertinib for 9 days. \(n = 5\) or 6 per group. (A and B, D-I, K and L) Data are shown as mean \(\pm\) s.e.m., two-tailed Student’s \(t\) test (B, D and E), one-way ANOVA with Tukey’s test (A, G and H), one-way ANOVA with Dunnett’s test (I), two-way ANOVA adjusted by Bonferroni’s correction (F and L) were used.
### A

|          | PC9          | HCC827       |
|----------|--------------|--------------|
|          | shCtrl       | shM3R        | kDa | 70 | shCtrl       | shM3R        |
|          | shHVR #1     | shHVR #2     |     | 35 | shHVR #1     | shHVR #2     |
| MG53     | 0.91         | 1.24         |     |    | 1.16         | 1.31         |
| GAPDH    | 0.71         | 0.87         |     |    | 0.88         | 0.98         |

### B

#### Relative cell viability

- **PC9**
  - shCtrl IC50 = 15.45 nM, P = 0.0003
  - shM3R #1 IC50 = 5.56 nM, P = 0.0488

- **HCC827**
  - shCtrl IC50 = 7.99 nM, P = 0.0200
  - shM3R #1 IC50 = 7.99 nM, P = 0.0301
  - shM3R #2 IC50 = 7.39 nM, P = 0.0190

### C

#### Relative cell viability

- **PC9**
  - shCtrl IC50 = 20.53 nM, P < 0.0001
  - shM3R #1 IC50 = 12.47 nM, P = 0.0303

- **HCC827**
  - shCtrl IC50 = 20.53 nM, P = 0.0048
  - shM3R #1 IC50 = 10.15 nM, P = 0.0022
  - shM3R #2 IC50 = 14.43 nM, P = 0.0092

### D

#### Treatment of 2 μM Osimertinib for 9 d

- **PC9**
  - shControl
  - shM3R
  - shVACHT

- **HCC827**
  - shControl
  - shM3R
  - shVACHT

### E

- **Osimertinib**
  - Relative viability
  - Darifenacin (μM)
  - Bendetopine mesylate (μM)
  - Irinotecan (μM)

- **PC9**
  - MGC24 (μM)
  - Mecamylamine (μM)
  - Pancuronium dibromide (μM)

- **HCC827**
  - Relative viability
  - Rottcitib (μM)
  - Benetopine mesylate (μM)
  - MGC24 (μM)

### F

- **Rottcitib**
  - Relative viability
  - Darifenacin (μM)
  - MGC24 (μM)
  - Pancuronium dibromide (μM)

- **HCC827**
  - Relative viability
  - Irinotecan (μM)
  - Mecamylamine (μM)
  - Pancuronium dibromide (μM)
Supplemental Figure 10. Blockade of ACh metabolism and signaling enhanced EGFR-TKI sensitivity of cancer cells and inhibited DTP formation. (A) Western blot analysis of indicated proteins in PC9 and HCC827 cells with stably expressed control (shCtrl), M3R (shM3R) or VAChT (shVAChT) shRNAs. (B) Dose-response curves to Osimertinib in PC9 cells expressing shCtrl, shM3R or shVAChT for 5 days. (C) Dose-response curves to Gefitinib in HCC827 cells expressing shCtrl, shM3R or shVAChT for 5 days. For B and C, IC50 values were compared with one-way ANOVA (n = 4-6). (D) Colony-formation assays were performed in PC9 and HCC827 cells with stably expressed shCtrl, shM3R or shVAChT and cultured with 2 μM Osimertinib for 9 days (n = 3). (E and F) Dose-response curves of PC9 parental, Osimertinib-tolerant, Rociletinib-tolerant and Erlotinib-tolerant cells incubated in increasing concentrations of indicated compounds for 6 days (n = 6). (G) Normalized apoptosis of PC9 and HCC827 cells treated with indicated drugs for 6 days (n = 3). (B-G) Data are shown as mean ± s.e.m., one-way ANOVA with Dunnett’s test (D) and one-way ANOVA with Tukey’s test (G) was used.
Supplemental Figure 11. The effect of ACh metabolism and signaling inhibition on the drug sensitivity of single cell-derived PC9 clones. (A) Relative cell viability of single cell-derived PC9 clones transfected with ChAT or control siRNA for 24 h and followed by indicated concentrations of Osimertinib for 72 h (n = 5). (B) Relative cell viability of single cell-derived PC9 clones co-treated with indicated concentrations of Osimertinib and Darifenacin for 6 days (n = 5). (A and B) Data are shown as mean ± s.e.m., two-way ANOVA with Tukey’s test was used.
Supplemental Figure 12. The effect of ACh metabolism and signaling inhibition on de novo resistance and acquired resistance. (A) Relative cell viability of PC9 and HCC827 cells co-treated with indicated concentrations of Osimertinib and 100 µM Hemicholinium-3, 50 µM Vesamicol and 10 µM Darifenacin for 48 h and 72 h (n = 4-6). (B) Relative cell viability of PC9 cells with short ChAT isoform overexpression (ChAT-OE) and negative control (NC) treated with indicated concentrations of Osimertinib for 48 h and 72 h (n = 6). (C) Relative cell viability of PC9 cells with shCtrl, shChAT, shVACHT or shM3R treated with indicated concentrations of Osimertinib for 48 h and 72 h (n = 6). (D) Western blot analysis of short ChAT isoform in PC9
and HCC827 parental, DTP and Osi-resistant cells. (E) Relative abundance of ACh in PC9 and HCC827 parental and Osi-resistant cells ($n = 6$). (F) Dose-response curves to Osimertinib combined with 100 µM Hemicholinium-3, 50 µM Vesamicol and 10 µM Darifenacin in PC9 parental and Osi-resistant cells for 72 h ($n = 6$). (G) Dose-response curves to Osimertinib in PC9 parental and Osi-resistant cells with shCtrl, shChAT, shVACht or shM3R for 72 h ($n = 6$). (A-C and E-G) Data are shown as mean ± s.e.m., two-way ANOVA with Tukey’s test (A) and two-tailed Student’s $t$ test (E) was used.
Supplemental Figure 13. Alterations of the key metabolites and proteins in PC9 xenografts exposed to different treatment strategies. (A) Relative abundance of ACh and choline in tumors sections from PC9 xenografts at the end point with pharmacological inhibition. $n = 5$ mice for Vehicle, $n = 4$ mice for Darifenacin, $n = 7$ mice for Osimertinib → Vehicle, $n = 8$ mice for Combination → Vehicle, $n = 8$ mice for Combination → Darifenacin. (B and C) Representative image and quantification of immunohistochemical staining of ChAT, pERK, pEGFR, pAKT on the tumor sections after indicated treatment. Scale bar, 50 μm. $n = 4$ mice per group. (A and C) Data are shown as mean ± s.e.m., one-way ANOVA with Tukey’s test was used.
Supplemental Figure 14. The modulatory role of ACh metabolism and signaling on WNT signaling. (A) Relative mRNA levels of indicated genes related to WNT signaling in PC9 cells transfected with ChAT or control siRNA and treated with 2 μM Osimertinib for 6 days (n = 3). (B-C) Relative mRNA levels of indicated genes related to WNT signaling in PC9 (B) and HCC827 (C) cells with full-length ChAT overexpression (ChAT_{FL}-OE) or negative control (NC) (n = 3). (D) Western blot analysis of β-catenin and non-phospho β-catenin in nuclear extract (NE) and cytosol extract (CE) in PC9 cells treated with indicated concentrations of ACh or Darifenacin alone or in combination for 6 days. (E) Western blot analysis of β-catenin in nuclear
extract (NE) and cytosol extract (CE) in PC9 cells with ChATFL-OE or NC. (A-C) Data are shown as mean ± s.e.m., one-way ANOVA with Tukey’s test (A) and two-tailed Student’s t test (B and C) were used.
Supplemental Figure 15. The modulatory role of YAP on WNT signaling. (A) Relative mRNA levels of indicated genes related to WNT signaling in PC9 cells with shCtrl or shYAP and treated with or without the 2 μM Osimertinib and 10 μM ACh for 6 days (n = 3). (B) Relative mRNA levels of indicated genes related to WNT signaling in PC9 cells treated with or without 2 μM Osimertinib, 2 μM Verteporfin and 10 μM ACh for 6 days (n = 3). (C) ACh levels in culture medium of parental PC9 and PC9-derived DTP cells. Neo, Neostigmine, an acetylcholinesterase inhibitor (n = 5 or 6). (A-C) Data are shown as mean ± s.e.m., one-way ANOVA with Tukey’s test (A and B) and two-way ANOVA with Tukey’s test (C) was used.
| #cell cycle | #purine metabolism | #pyrimidine metabolism |
|-------------|-------------------|------------------------|
| CDC16       | TFDP1             | ADCY1                  |
| CDC7        | ZBTB17            | NPR1                   |
| CDC45       | CDK1              | GUCY2D                 |
| GADD45B     | ESPL1             | GUCY1A2                |
| DBF4        | ANAPC10           | PDE4C                  |
| ANAPC1      | RAD21             | PDE8B                  |
| CREBBP      | BUB1B             | PDE5A                  |
| MDM2        | ANAPC11           | PDE6G                  |
| ABL1        | RB1               | PDE6C                  |
| SMC1B       | SKP2              | PDE7B                  |
| LOC728622   | CUL1              | ADCY10                 |
| GADD45G     | SMAD3             | PDE6A                  |
| ATM         | SMAD4             | ADSSSL1                |
| ATR         | ANAPC2            | ENTPD8                 |
| ANAPC7      | TFDP2             | ADCY9                  |
| RBL2        | PRKDC             | PDE4B                  |
| ANAPC5      | MAD2L1            | GMPR                   |
| RBL1        | ANAPC4            | ENPP1                  |
| MYC         | SMAD2             | NT5E                   |
| CDC14B      | YWHAQ             | ENPP4                  |
| SMC1A       | CHEK2             | PAPSS2                 |
| CDC14A      | CDC23             | PDE9A                  |
| LOC731751   | EP300             | POLR3K                 |
| SKP1        | GSK3B             | PRPS2                  |
| TGFB2       | CDKN2A            | TWISTNB                |
| TGFB1       | CDKN1C            | POLR1C                 |
| GADD45A     | CDKN1B            | PFAS                   |
| STAG1       | CDKN1A            | PPAT                   |
| LOC650621   | CDKN2D            | ADSSL                  |
| PLK1        | CCNA1             | POLD2                  |
| RBX1        | CDKN2B            | ADCY3                  |
| STAG2       | CDKN2C            | POLE3                  |
| TGFB3       | FZR1              | POLA2                  |
| MCM4        | SMC3              | AK2                    |
| ORC6        | ANAPC13           | PRIM2                  |
| CCND1       | PCNA              | POLR2D                 |
| MAD1L1      | TTK               | POLR1B                 |
| MCM3        | PKMYT1            | DCK                    |
| MCM6        | CDK2              | POLD3                  |
| MCM5        | CDC26             | GDA                    |
| YWHAH       | E2F5              | GART                   |
| CCNA2       | CDK4              | GMPS                   |
| MCM7        | WEE1              | POLA1                  |
| BUB1        | E2F4              | POLR2G                 |
| CHEK1       | E2F3              | PAICS                  |
| WEE2        | TP53              | PNPT1                  |
| MCM2        | E2F2              | RRM1                   |
| PTTG1       | ORC1              | AK5                    |
| CDC27       | ORC2              | XDH                    |
| CDC25B      | CCNE2             | AK6                    |
| CDC25C      | CDK6              | HPRT1                  |
| Gene 1      | Gene 2 | Gene 3 |
|------------|--------|--------|
| LOC651610  | ORC4   | PRIM1  |
| CDC25A     | CCNB2  | NME1   |
| CDC6       | CDK7   | POLE2  |
| CDC20      | MAD2L2 | PNP    |
| BUB3       | ORC5   | POLR3G |
| YWHAZ      | HDAC1  | RRM2   |
| CCND2      | HDAC2  | PDE11A |
| YWHAH      | CCNH   | PDE2A  |
| CCNB1      | CCNB3  | AK4    |
| YWHAG      |        |        |
| YWHAE      |        |        |
| CCNE1      |        |        |
| ORC3       |        |        |
| CCND3      |        |        |
| PTTG2      |        |        |
| SFN        |        |        |
| E2F1       |        |        |
## Supplemental Table 2. Clinical information of NSCLC patients for plasma sample collection

| Patient ID | stage     | EGFR mutation | EGFR TKI     | Response rate | PFS | PFS status |
|------------|-----------|---------------|--------------|---------------|-----|------------|
| P01        | T4N2M1B-IV| 19del         | Osimertinib  | PR            | 4.5 | 1          |
| P02        | T4N2M1B-IV| L858R         | Osimertinib  | SD            | 4.2 | 1          |
| P03        | T4N1M1A-IV| 19DEL+T790M   | Osimertinib  | PR            | 14  | 1          |
| P04        | T4N2M1B-IV| 19del         | Osimertinib  | PR            | 10.4| 1          |
| P05        | T4N2M1A-IV| L858R         | Osimertinib  | PR            | 7   | 1          |
| P06        | T4N3M1B-IV| L858R         | Osimertinib  | PR            | 21.5| 0          |
| P07        | T4N2M1B-IV| L858R+T790M   | Osimertinib  | PR            | 9.9 | 1          |
| P08        | T4N3M1B-IV| 19del         | Osimertinib  | SD            | 32.4| 1          |
| P09        | T1AN3MO-IIIB| 19del     | Osimertinib  | PR            | 5.6 | 0          |
| P10        | T3N3M1B-IV| L858R         | Gefitinib    | PD            | 1.2 | 1          |
| P11        | cT3N2M1c-IVB| 19del      | Icotinib     | PR            | 17.4| 0          |
| P12        | cT4N3M1c-IVB| L858R    | Gefitinib    | PR            | 9.9 | 1          |
| P13        | cT2bN3M1c-IVB| 19del    | Gefitinib    | PR            | 18.5| 0          |
| P14        | cT4N3M1c-IVB| 19del      | Icotinib     | PR            | 20.5| 1          |
| P15        | cT2aN0M1b-IVA| 19del       | Gefitinib    | PR            | 10  | 0          |
| P16        | cT2N1M1a-IVA| L858R      | Gefitinib    | PR            | 16.1| 1          |
| P17        | cT1cN2M1c-IVB| 19del      | Gefitinib    | PR            | 17.4| 0          |
| P18        | cT4N3M1c-IVB| L858R      | Gefitinib    | PR            | 6.1 | 1          |
| P19        | cT1cN3M0-IIIIB| 19del    | Icotinib     | PR            | 18.6| 1          |
| P20        | cT4N3M1c-IVB| L858R      | Gefitinib    | PR            | 11  | 0          |
| P21        | cT3N3M1a-IVA| 19del      | Icotinib     | PR            | 14.3| 1          |
| P22        | cT2aN3M1a-IVA| L858R     | Icotinib     | PR            | 8.3 | 1          |
| P23        | cT4N1M1a-IVA| 19del      | Icotinib     | PR            | 16  | 0          |
| P24        | T4N3M1a-IVA| L858R         | Gefitinib    | PR            | 9   | 1          |
| P25        | cT1bN3M1a-IVA| 19del      | Icotinib     | PD            | 1.3 | 1          |
| P26        | cT2aN2M1b-IVA| 19del      | Gefitinib    | PR            | 8.5 | 0          |
| P27        | cT4N2M1c-IVB| 19del      | Gefitinib    | PR            | 14.2| 1          |
| P28        | cT1cN3M0-IIIIB| 19del   | Gefitinib    | PR            | 9.1 | 0          |
| P29        | cT3N3M1b-IVA| 19del      | Icotinib     | PR            | 18  | 0          |
| P30        | cT4N2M0-IIIIB| 19del      | Gefitinib    | PR            | 9.3 | 0          |
| P31        | cTxN3M1c-IVB| L858R     | Icotinib     | PR            | 6.3 | 1          |
| P32        | cT4N3M1c-IVB| G719X     | Afatinib     | SD            | 2.1 | 1          |
| P33        | IVB        | L858R         | Icotinib     | SD            | 12.7| 1          |
| P34        | IVA        | 19del         | Gefitinib    | PR            | 13.9| 1          |
| P35        | IVA        | 19del+L768I   | Afatinib     | PR            | 6.6 | 1          |
| P36        | IVB        | L858R         | Icotinib     | SD            | 6.6 | 1          |
| P37        | IIIB       | 19del         | Afatinib     | PR            | 7.5 | 1          |
| P38        | IVA        | 19del         | Icotinib     | PR            | 8.9 | 1          |
| P39        | IIIB       | 19del         | Icotinib     | PR            | 30.4| 1          |
| P40        | IVB        | 19del         | Gefitinib    | PR            | 7.3 | 1          |
| P41        | IIIB       | L858R         | Gefitinib    | PD            | 1.5 | 1          |
| P42        | IVB        | L858R         | Gefitinib    | SD            | 20  | 1          |
| P43        | IVA        | Icotinib     | SD            | 11.7 | 1          |
| P44        | IVA        | L858R         | Gefitinib    | SD            | 28.2| 1          |
| P45        | IVA        | 19del         | Gefitinib    | PR            | 2.8 | 1          |
| P46        | IVB        | L858R         | Icotinib     | SD            | 4.4 | 1          |
| P47        | IVB        | 19del         | Gefitinib    | PR            | 30  | 1          |
| P48        | IVB        | L858R         | Erlotinib    | PD            | 3.9 | 1          |
| P49        | IVA        | 19del         | Gefitinib    | PR            | 12  | 1          |
| P50        | IVB        | 19del         | Gefitinib    | SD            | 5.4 | 1          |
| P51        | IVB        | L858R         | Gefitinib    | PR            | 4   | 1          |
| Patient | Mutation | Drug | Status | Response | Progression Free Survival (months) |
|---------|----------|------|--------|----------|----------------------------------|
| P52     | IIIIC    | L858R| Gefitinib | PR       | 16.1                             |
| P53     | IB       | L858R| Icotinib  | PR       | 23.5                             |
| P54     | IVB      | 19del| Gefitinib  | SD       | 4.6                              |
| P55     | IVA      | 19del| Gefitinib  | PR       | 10.9                             |
| P56     | IVA      | 19del| Icotinib   | PR       | 25.9                             |
| P57     | IVA      | 19del| Gefitinib  | PR       | 21.3                             |
| P58     | IVB      |      | Gefitinib  | PR       | 19.7                             |
| P59     | IVA      | L858R| Erlotinib  | PR       | 6.3                              |
| P60     | IVB      | 19del| Icotinib   | SD       | 12.8                             |
| P61     | IVB      | L858R| Gefitinib  | SD       | 5.7                              |
| P62     | IVA      | 19del| Gefitinib  | SD       | 4.1                              |
| P63     | IVA      | L858R| Gefitinib  | SD       | 6.5                              |
| P64     | IIIB     | L858R| Gefitinib  | PR       | 28.5                             |
| P65     | IVA      | L858R| Icotinib   | PR       | 13.8                             |
| P66     | IVA      | 19del| Icotinib   | PR       | 22.4                             |
| P67     | IVA      | 19del| Gefitinib  | PR       | 13.2                             |
| P68     | IVB      | L858R| Gefitinib  | SD       | 7.2                              |
| P69     | IVB      | L858R| Gefitinib  | PR       | 17.2                             |
| P70     | IVA      | L861Q| Afatinib   | SD       | 28.6                             |
| P71     | IVB      | 19del| Gefitinib  | PR       | 26.9                             |
| P72     | IVA      | 19del| Gefitinib  | SD       | 10.3                             |
| P73     | IVA      | 19del| Gefitinib  | PR       | 18.5                             |
| P74     | IIIIC    | L858R| Gefitinib  | SD       | 6.1                              |
| P75     | IVA      |      | Erlotinib  | SD       | 12.6                             |
| P76     | IVA      | L858R| Gefitinib  | PR       | 20.8                             |
| P77     | IVB      | 19del| Gefitinib  | SD       | 11.5                             |
| P78     | IVB      | L858R| Erlotinib  | PR       | 9.1                              |
Supplemental Table 3. Clinical information of NSCLC patients for paired tumor tissue

| Patient ID | Stage | EGFR mutation | EGFR TKI | Response rate |
|------------|-------|---------------|----------|---------------|
| T01        | IIIC  | L861Q         | afatinib | PR            |
| T02        | IVB   | L858R         | gefitinib| PD            |
| T03        | IVB   | 19DEL         | afatinib | PR            |
| T04        | IVA   | L858R         | icotinib | SD            |
| T05        | IVB   | L858R         | gefitinib| SD            |
| T06        | IIIA  | 19DEL         | icotinib | SD            |
| T07        | IVB   | L858R         | gefitinib| PR            |
| T08        | IVA   | 19DEL         | gefitinib| PR            |
| T09        | IVB   | L858R         | gefitinib| PR            |
| T10        | IVB   | 19DEL         | erlotinib| PR            |
| T11        | IVA   | L858R         | gefitinib| PR            |
| T12        | IIIIB | 19DEL         | gefitinib| PR            |
| T13        | IVA   | 19DEL         | erlotinib| PR            |
| T14        | IVB   | 19DEL         | gefitinib| PR            |
| T15        | IVA   | 19DEL         | gefitinib| PR            |
| T16        | IVB   | 19DEL         | gefitinib| PR            |
| T17        | IVA   | 19DEL+L861Q   | gefitinib| PR            |
| T18        | IVB   | L858R         | icotinib | PD            |
| T19        | IVB   | G719X         | afatinib | PR            |
| T20        | IVB   | L858R         | gefitinib| PR            |
### Supplemental Table 4. Small molecule compound screen

| Category          | Parameter                              | Description                                                                                                                                                                                                 |
|-------------------|----------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| **Assay**         | Type of assay                          | ATP- measurement in vitro                                                                                                                                                                                   |
|                   | Target                                 | mAChR and/or nAChR                                                                                                                                                                                         |
|                   | Primary measurement                    | Luminescent detection of ATP                                                                                                                                                                                 |
|                   | Key reagents                           | CellTiter-Glo Luminescent Cell Viability Assay kit (Promega), osimertinib (Selleck), rociletinib (Selleck), erlotinib (Selleck) and small molecule libraries.                                                                 |
|                   | Assay protocol                         | Detailed method is described in the ‘Drug Screen’ section in Methods.                                                                                                                                       |
| **Library**       | Library size                           | 26 compounds                                                                                                                                                                                                 |
|                   | Library composition                    | Darienacin HBr: Selleck (S3144)                                                                                                                                                                                |
|                   |                                        | Ipratropium Bromide: Selleck (S1683)                                                                                                                                                                          |
|                   |                                        | Oxybutynin: Selleck (S1754)                                                                                                                                                                                    |
|                   |                                        | Irsogludine: Selleck (S1929)                                                                                                                                                                                   |
|                   |                                        | Methscopolamine: Selleck (S1978)                                                                                                                                                                               |
|                   |                                        | Atropine sulfate monohydrate: Selleck (S2130)                                                                                                                                                                |
|                   |                                        | Scopolamine HBr: Selleck (S2508)                                                                                                                                                                               |
|                   |                                        | Pancuronium dibromide: Selleck (S2497)                                                                                                                                                                         |
|                   |                                        | Gallamine triethiodide: Sigma-Aldrich (G8134)                                                                                                                                                                |
|                   |                                        | DL-Homatropine hydrobromide: Sigma-Aldrich (H0126)                                                                                                                                                           |
|                   |                                        | p-Fluorohexahydro-sila-difenidol hydrochloride: Sigma-Aldrich (H127)                                                                                                                                         |
|                   |                                        | Hexamethonium bromide: Sigma-Aldrich (H0879)                                                                                                                                                                 |
|                   |                                        | MG624: Sigma-Aldrich (M3184)                                                                                                                                                                                   |
|                   |                                        | Mecamylamine hydrochloride: Sigma-Aldrich (M9020)                                                                                                                                                             |
|                   |                                        | Methoctramine hydrate: Sigma-Aldrich (M105)                                                                                                                                                                  |
|                   |                                        | Orphenadrine hydrochloride: Sigma-Aldrich (O3752)                                                                                                                                                             |
|                   |                                        | Pirenzepine dihydrochloride: Sigma-Aldrich (P7412)                                                                                                                                                           |
|                   |                                        | Propantheline bromide: Sigma-Aldrich (P8891)                                                                                                                                                                 |
|                   |                                        | Succinylcholine chloride dihydrate: Sigma-Aldrich (S8251)                                                                                                                                                   |
|                   |                                        | DL-Tetraethylammonium chloride: Sigma-Aldrich (T2265)                                                                                                                                                         |
|                   |                                        | Telenzepine dihydrochloride hydrate: Sigma-Aldrich (T122)                                                                                                                                                   |
|                   |                                        | Tropicamide: Sigma-Aldrich (T9778)                                                                                                                                                                             |
|                   |                                        | Benztrapine mesylate: Sigma-Aldrich (B8262)                                                                                                                                                                  |
|                   |                                        | Biperiden hydrochloride: Sigma-Aldrich (B5311)                                                                                                                                                               |
|                   |                                        | 4-DAMP methiodide: Sigma-Aldrich (D104)                                                                                                                                                                      |
| Source            | Original sources are listed above.     |                                                                                                                                                    |
| **Screen**        | Format                                 | 384 well                                                                                                                                                                                                    |
|                   | Concentration(s) tested                | 1.25, 2.5, 5, 10, 20, 50 μM compound, < 0.5% DMSO                                                                                                                                                           |
|                   | Plate controls                         | DMSO-containing (as parental cell group) and 2 μM EGFR TKI-containing medium (as DTP cell group) represented 100% cell viability (0% inhibition)                                                                 |
|                   | Reagent/ compound dispensing system    | Labcyte-Echo 550                                                                                                                                                                                             |
|                   | Detection instrument and software      | BioTek Cytation3                                                                                                                                                                                             |
|                   | Normalization                          | The relative survival of parental PC9 cells and EGFR TKI-induced tolerant PC9 cells in the presence of compounds was normalized against respectively negative control conditions. The wells with DMSO or 2 μM EGFR TKI were averaged to determine the average signal without a compound. The signal for each compound on each
plate was divided by the average negative control signal to give a % cell viability value. \[
\frac{\text{signal with compound}}{\text{average signal with negative control}} \times 100\% = \% \text{ cell viability.}
\]