Multiomics Reveals Ectopic ATP Synthase Blockade Induces Cancer Cell Death via a lncRNA-mediated Phospho-signaling Network

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In Brief
Ectopic ATP synthase (ecto-ATP synthase), located on the plasma membrane of lung cancer cells, served as an indicator of gefitinib’s efficacy and an alternative therapeutic target for gefitinib-resistant NSCLC. Multi-omics revealed that ecto-ATP synthase inhibitor (eATPi) mediated CK2-dependent phosphorylation of DNA topoisomerase II alpha (topo IIα) on Ser1106, and subsequently increased the expression of lncRNA-GAS5 and induced the p53-mediated cell death. Furthermore, elevated GAS5 interacted with topo IIα and enhanced its phosphorylation to reinforce eATPi-triggered anticancer effects.

Highlights
- Ectopic ATP synthase as a therapeutic target for gefitinib-resistant NSCLC.
- Multimics uncovers the dynamic network in response to ecto-ATP synthase blockade.
- Ecto-ATP synthase blockade induces cytotoxicity by CK2/phospho-topo IIα/GAS5 axis.
- A positive feedforward circuit between phospho-topo IIα and lncRNA-GAS5.
Multiomics Reveals Ectopic ATP Synthase Blockade Induces Cancer Cell Death via an IncRNA-mediated Phospho-signaling Network

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The EGFR tyrosine kinase inhibitor gefitinib is commonly used for lung cancer patients. However, some patients eventually become resistant to gefitinib and develop progressive disease. Here, we indicate that ecto-ATP synthase, which ectopically translocated from mitochondrial inner membrane to plasma membrane, is considered as a potential therapeutic target for drug-resistant cells. Quantitative multi-omics profiling reveals that ecto-ATP synthase inhibitor mediates CK2-dependent phosphorylation of DNA topoisomerase IIα (topo IIα) at serine 1106 and subsequently increases the expression of long noncoding RNA, GAS5. Additionally, we also determine that downstream of GAS5, p53 pathway, is activated by ecto-ATP synthase inhibitor for regulation of programmed cell death. Interestingly, GAS5-proteins interactomic profiling elucidates that GAS5 associates with topo IIα and subsequently enhancing the phosphorylation level of topo IIα. Taken together, our findings suggest that ecto-ATP synthase blockade is an effective therapeutic strategy via regulation of CK2/phospho-topo IIα/GAS5 network in gefitinib-resistant lung cancer cells.

Lung cancer ranks as the leading cause of cancer-related mortalities and is responsible for an estimated 18.4% of total cancer deaths worldwide (1). There are two major types of diagnosed lung cancer: small-cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC). NSCLC, which accounts for approximately 85% of all lung cancers, is further divided into several subcategories by histological characteristics (2, 3). In addition to the histological diversity, accumulating evidence indicates that molecularly heterogeneous subsets are present within NSCLC (4–6). For instance, activating mutations, including exon 19 deletion and L858R point mutation on exon 21, in epidermal growth factor receptor (EGFR) are frequently observed in NSCLC and ultimately lead to constitutive activation of downstream signaling, causing aberrant growth and survival of cancer cells (7–10). Therefore, EGFR tyrosine kinase inhibitors (EGFR-TKI), such as gefitinib (Iressa) and erlotinib (Tarceva), are commonly used clinically for patients with these activating EGFR mutations (11, 12). Unfortunately, most of the EGFR-TKIs-responsive patients have dramatic response initially, but would eventually acquire resistance and develop progressive disease within about one year of EGFR-TKIs treatment (13, 14). EGFR T790M secondary mutation, which leads to TKIs-binding deficiency, generally occurs in NSCLC patients who acquired drug resistance to EGFR-TKIs (15–17). In addition to the acquired resistance to gefitinib by reactivating EGFR, Kirsten Rat Sarcoma Viral Oncogene Homolog (KRAS) mutations result in the intrinsic resistance to gefitinib in NSCLC (18, 19). KRAS encodes a small guanosine triphosphate (GTP)-binding protein, which is involved in downstream signaling of EGFR and has been frequently observed activating mutations at residues G12, G13, or Q61 in patients with gefitinib refractory (20, 21). The presence of KRAS mutations is associated with poor response to gefitinib, so KRAS mutations are considered as biomarkers for the therapeutic decision to give gefitinib in patients with NSCLC (22). Therefore, the development of an alternative therapeutic approach for gefitinib-resistant NSCLC with either EGFR or KRAS mutation is necessary. In this study, two cell lines with EGFR mutation/KRAS WT and two cell lines with EGFR WT/KRAS mutation were used to investigate a new therapeutic target for gefitinib-resistant cells.

Adenosine-5′-triphosphate (ATP) synthase locates in mitochondrial to produce ATP (23). Recent studies indicate that ATP synthase is present on the cell surface in many cell types, called as ectopic ATP synthase (ecto-ATP synthase) (24–26). Ecto-ATP synthase not only participates in numerous cellular functions (27, 28). An increasing number of ectopic ATP synthase inhibitors (eATP), which specifically block the
catalytic subunits of ectopic ATP synthase, have been found to be useful as therapies for various diseases (29–31). Our previous findings demonstrate that ecto-ATP synthase is highly expressed on the cell surface of NSCLC cells. Furthermore, one such eATPi, citreoviridin, induces cytotoxic effect on NSCLC cells both in vitro and in vivo xenograft models via the disruption of several biological processes, including the unfolded protein response, endothelial reticulum stress, and metabolism (32–35). However, few studies have referred to the relationship between ectopic ATP synthase and EGRF-TKIs resistance in tumor cells.

Recently, advanced transcriptome technologies have provided new insights that noncoding RNAs (ncRNAs) account for up to 90% of the whole human genome (36). The ncRNAs that have more than 200 nucleotides, which are usually transcribed by RNA polymerase II and are typically polyadenylated, are classified as long noncoding RNAs (lncRNAs) (37–39). Emerging evidence has implicated lncRNAs as pivotal regulators of tumor progression such as migration and angiogenesis (40, 41). In this study, we first clarify whether ectopic ATP synthase is a potential therapeutic target of NSCLCs with acquired gefitinib resistance. To further understand the molecular mechanism of ecto-ATP synthase in gefitinib-resistant cells, we reveal both quantitative phosphoproteomic and transcriptomic profiles after short-term and long-term treatments with eATPi, respectively, as well as IncRNA binding proteomics, establishing a comprehensive eATPi-responsive phospho-incRNA network. Consequently, we provide a crucial axis in gefitinib resistant NSCNC regulated by ecto-ATP synthase.

**EXPERIMENTAL PROCEDURES**

**Reagents and Antibodies**—The tyrosine kinase inhibitor gefitinib (Cat# 184475-35-2, Cayman Chemical, Ann Arbor, Michigan), ecto-ATP synthase inhibitor citreoviridin (Cat#ALX-630-118, Enzo Life Sciences, Farmingdale, NY), and CK2 inhibitor 4,5,6,7-tetrabromo-benzimidazol (TBB; Cat#T0826, Sigma-Aldrich, St Louis, MO) were solubilized in dimethyl sulfoxide (DMSO; Cat#D2650, Sigma-Aldrich). For the multi-omics profiling and functional assay, cells were treated with the IC50 of eATPi or the equivalent volume of DMSO as a control. To inhibit CK2-dependent phosphorylation, cells were pretreated with 50 μM of TBB or the equivalent volume of DMSO for 8 h. All the drug preparation and treatment procedures were performed in the dark. Antibodies against the following proteins were used in this study: ATP synthase complex (Cat#ab109867, Abcam, San Diego, CA), topo IIα (Cat#ab74715, Abcam), pS1106-topo IIα (Cat#ab75765, Abcam), pS139-H2AX (Cat#9718, Cell Signaling Technology, Danvers, MA), HA-tag (Cat#ab01501, BioLegend, San Diego, CA), and β-actin (Cat#ab81501, Sigma-Aldrich).

**Cell Lines and Culture**—Human lung cancer cell line A549 was purchased from American Type Culture Collection (ATCC, Manassas, VA). Human lung cancer cell lines: H3255, H1975 and H460 were the gifts from Dr. P. C. Yang (Department of Internal Medicine, National Taiwan University College of Medicine). All cell lines were cultured in Dulbecco’s modified Eagle’s medium (DME-M; #12800-017, Thermo Fisher Scientific) supplemented with 10% Fetal Bovine Serum (FBS; #10437-028, Thermo Fisher Scientific) at 37°C with 5% CO2 and routinely passaged at 80–90% confluence. All cell lines were authenticated by matching the STR profile to the ATCC public STR Database.

**Identification of ecto-ATP Synthase-Associated Genes**—Firstly, we quantified ecto-ATP synthase expression levels on the plasma membrane by flow cytometry in a panel of NSCLC cell lines (H3255, H1975, A549, H460, H1299, CL1-0, and CL1-5) (supplemental Fig. S1A). The mean fluorescent intensity (MFI) values of ecto-ATP synthase were further used to segregated cell lines into low (<400), intermediate (400-800), or high (>800) level of ecto-ATP synthase expressing- subtype. The gene expression data for these seven cell lines were downloaded from the Gene Expression Omnibus (GEO) repository to establish the ecto-ATP synthase-associated genes (supplemental Table S1). To avoid the data imbalance, H3255 cell line with low level of ecto-ATP expression was grouped with intermediately-expressed H1975 cell line, whereas the remaining cell lines with high level of ecto-ATP synthase expression (CL1-5, A549, H1299, CL1-0, and H460) are grouped in another (supplemental Fig. S1B).

**Constructs, siRNAs, and Transfection**—The pGL3-B-pGAS5 plasmid, containing the promoter region of GAS5 within the pGL3-Basic vector, was a gift from Rolf Stahel (Cat#ab74715, Addgene plasmid, Watertown, MA) (45). The coding region of TOP2A was amplified via PCR and then cloned to the pcMV-HA-N vector (Cat#635690, CLONTECH, Mountain View, CA). The mutant TOP2A (TOP2A-S1106A) was generated by site-directed mutagenesis using the WT TOP2A. Full-length GAS5 was cloned to the pcDNA3.1(+) vector (Cat#V79020, Invitrogen, Carlsbad, CA). The SP6 promoter region was first introduced onto the 5’ end of the GASS sequence and then the amplified 5’-SP6 promoter-GASS-3’ cDNA fragment was cloned into the pBlueScript SK (+) vector, which was a gift from Prof. J. D. Wen (Institute of Molecular and Cell Biology, National Taiwan University). The primers used for this construction are described in supplemental Table S2. All clones were verified by sequencing. The siGENOME SMARTpool siRNA against GAS5 was purchased from Dharmacon (Lafayette, CO) (Cat#0-00310-10). Plasmids and siRNAs were transfected into cells using jetPrime (Cat#114-15, Polyplus transfection, New York City, NY) according to the manufacturer’s instructions.

**Experimental Design and Statistical Rationale**—For quantitative phosphoproteomics, H1975 cells were treated with citreoviridin (eATPi) at IC50 concentration or with the same volume of DMSO for 1 min, 10 min, and 60 min. The DMSO-treated cells were served as the control. To inhibit CK2-dependent phosphorylation, cells were pretreated with 50 μM of TBB or the equivalent volume of DMSO for 8 h. All the drug preparation and treatment procedures were performed in the dark. Antibodies against the following proteins were used in this study: ATP synthase complex (Cat#ab109867, Abcam, San Diego, CA), topo IIα (Cat#ab74715, Abcam), pS1106-topo IIα (Cat#ab75765, Abcam), pS139-H2AX (Cat#9718, Cell Signaling Technology, Danvers, MA), HA-tag (Cat#ab01501, BioLegend, San Diego, CA), and β-actin (Cat#ab81501, Sigma-Aldrich).

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was used to determine differentially expressed genes. For LncRNA–protein interactomics, A549 or H1975 gefitinib-resistant cells were harvested and incubated with biotin-labeled GASS transcripts, respectively. For each cell line, two independent batches of biological samples were prepared.

**Gene Expression Microarray Analysis**—A total of 0.2 μg of total RNA was amplified by a Low Input Quick-Amp WT Labeling Kit (#5190-2943, Agilent Technologies) and labeled with Cy3 during the in vitro transcription process. A total of 0.6 μg of Cy3-labeled cRNA was fragmented to an average size of about 50–100 nucleotides by incubating with fragmentation buffer at 60 °C for 30 min. Correspondingly fragmented labeled cRNA was then pooled and hybridized to Agilent SurePrint G3 Human V2 GE 8 × 60K Microarray (#G4851A, Agilent Technologies) at 65 °C for 17 h. After washing and drying by nitrogen gun blowing, microarrays were scanned with an Agilent microarray scanner (Agilent Technologies, Santa Clara, CA) at 535 nm for Cy3. Scanned images are analyzed by Feature extraction 10.5.1.1 software (Agilent Technologies).

**Quantitative Phosphoproteomics**—Experiment procedures of quantitative phosphoproteomics were modified from our previous study (35). Cells were harvested in triethylammonium bicarbonate (TEAB; #T4704, Sigma-Aldrich) buffer with sodium deoxycholate (SDC; #D7570, Sigma-Aldrich) and sodium lauroyl sarcosine (SLS; #194009, MP Biomedicals, Santa Ana, CA) Protease inhibitor mixture (#PIC002, BioShop) and phosphatase inhibitor cocktails (#PIC008 and #PIC009, BioShop) were added for preventing proteins and phosphoproteins degradation. Cells were homogenized and the supernatant was collected by centrifugation. The concentration of proteins in the supernatant were measured by Pierce™ BCA Protein Assay Kit (#23225, Thermo Fisher Scientific) according to the manufacturer’s instructions. A total of 500 μg proteins were reduced with DTT (DTT; #DTT001, BioShop) and the reduced cysteines were then alkylated by iodoacetamide (IAM; #IOD500, BioShop). Alkylated proteins were digested with endopeptidase Lys-C (1:100 w/w; #129-02541, WAKO) and the reduced cysteines were then alkylated with DTT (DTT; #DTT001, BioShop) and the reduced cysteines were then alkylated by iodoacetamide (IAM; #IOD500, BioShop). Alkylated proteins were digested with endopeptidase Lys-C (1:100 w/w; #129-02541, WAKO) for 3 h followed by trypsin (1:10 w/w; #90305, Thermo Fisher Scientific) for overnight at 37 °C. After digestion, ethyl acetate (#928001, J. T. Baker, Phillipsburg, NJ) was added into the digested peptides and mixed well by vortexed for detergent removal (48). The digested peptides were collected by centrifugation and subjected to desalting using ZipTip Pipette Tips (#ZTC18S960, Millipore).

**NanoLC-MS/MS Analysis**—The LTQ-Orbitrap XL (Thermo Scientific, Bremen, Germany) connected to a nanoACQUITY UPLC system (Waters Corp., Milford, MA) was used for NanoLC-MS/MS analyses as previously described (35). Peptide mixtures were injected onto a capillary trap column and then separated in nanoACQUITY Ethylene Bridged Hybrid (BEH) C18 column. The flow rate was 300 nL/min. The mobile phases consisted of solution A (0.1% FA) and solution B (0.1% FA and 80% ACN). The Mass spectra survey scan range was m/z 350-1500. The top ten precursor ions were selected from each scan for MS/MS analysis. The mobile phases consisted of solution A (0.1% FA) and solution B (0.1% FA and 80% ACN). The Mass spectra survey scan range was m/z 350-1500. The top ten precursor ions were selected from each MS scan by ion trap in the automated gain control (AGC) mode. For each biological experiment, technical duplicate of nanoLC-MS/MS analyses were performed.

**Mass Spectrometric Data Analysis**—The raw MS data were processed and analyzed by MaxQuant software (46) (version 1.5.2.8 for phosphoproteomics and version 1.5.5.1 for RNA-protein interactome) with the following criteria described as previous study (35): trypsin specificity allowing for up to two missed cleavage sites; carbamidomethylation on cysteine (C) was set as fixed modification; oxidation on methionine (M) and phosphorylation on serine, threonine or tyrosine residues (STY) (only used in phosphopeptide identification) were selected as variable modifications. The first search peptide tolerance was 20 ppm, whereas the main search peptide tolerance was 4.5 ppm. FTMS MS/MS match tolerance was set as 20 ppm. Andromeda search engine was performed against UniProtKB/Swiss-Prot human database version canonical (53). 20,196 UniProtKB/Swiss-Prot human protein entries published on Nov-11, 2015 was used for phosphoproteomics, whereas 20,316 UniProtKB/Swiss-Prot human protein entries published on January 18, 2017 was used for RNA-protein interactomics. Samples were searched as duplex (multiplicity as 2) with the corresponding dimethyl labels selected (light: DimethLys0, DimethNterm0, heavy: DIMETHYL LYS6, DIMETHYL NTERM6). The re-quantity option was selected and the match between runs function was enabled with a match time window of 1805–1825.
0.7 min. A minimum of six amino acids forming the peptide chain was required. Peptide, protein, and site identifications were filtered at a false discovery rate (FDR) of 1% searching against a randomized decoy database created by the MaxQuant. For multiple phosphopeptides, the localization probability of all putative phosphosites was determined using the MaxQuant PTM score algorithm (54). A site localization probability of 0.75 was set as the threshold for localization of phosphorylation sites. The ratios between dimethyl-labeled and unlabeled localization probability of all putative phosphosites was required. Peptide, protein, and site identifications were further calculated as the final ratios.

Functional Network Analysis—The Gene Ontology (GO) over-representation analysis was performed by the R package clusterProfiler (55), and we only focused on biological process category. The enriched GO terms were constructed into a network by enrichmentMap (56) with the combined score >0.5 and then visualized by Cytoscape (v 3.6.1). For gene expression datasets, the pathway activity was assessed by GSEA with default parameters (57), and the transcription factor activity analysis was performed by ISMARA with default parameters (58). For phosphoproteomics datasets, the kinase activity was assessed by DynaPho (59).

Immunocytochemistry—Cells were seeded on a glass coverslip and allowed to adhere for 48 h, then fixed with 3.7% paraformaldehyde (Cat#P6148, Sigma-Aldrich) in PBS for 20 min. The cells were incubated in 5% BSA (Cat#ALB001, BioShop) to block nonspecific binding and permeabilized with 0.1% Triton X-100 (Cat#T8787, Sigma-Aldrich). After washing with PBS, cells were incubated with specific primary antibodies at 4 °C overnight. Alexa-488 conjugated to goat anti-mouse or anti-rabbit (Cat#ab6721 and Cat#ab6789, Abcam). Protein bands were detected using ECL detection kit (#WBKLO500, Millipore) and captured using FluorChem M (ProteinSimple, San Jose, CA). Immunoblots quantifications were analyzed by ImageJ. Protein relative levels were normalized to the expression of β-actin served as loading control.

RNA Extraction and RT-qPCR Analysis—Total RNA was prepared as described above and the concentration was determined by NanoDrop ND-1000 (NanoDrop Technologies, Montchanin, DE). RevertAid H Minus First Strand cDNA Synthesis Kit (#21132, Thermo Fisher Scientific) was used with 1 μg of total RNA for reverse transcription. Quantitative real-time PCR (qRT-PCR) was performed using iQTM SYBR® Green Supermix (#1708882, Bio-Rad) with CFX96 system (Bio-Rad, Hercules, CA). The relative expression levels of studied genes were normalized to GAPDH and by Bio-Rad CFX Manager software. The primers used for qRT-PCR were listed in supplemental Table S2.

Cell Counting Assay—Cells were counted using a trypan blue exclusion method.

Clonogenic Assay—Cells were seeded in 6-well plates and adhered for 24 h. Cells were treated with indicated drugs and RT-qPCR was performed to measure the promoter activity of GAS5 according to the manufacturer’s instructions. Data were normalized to Renilla luciferase activity.

RNA Immunoprecipitation—Protein-interacting RNAs were precipitated by Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit (#MAGNARIP01, Millipore) according to the manufacturer’s instructions using topo IIα antibody (ab274715, Abcam) and subsequently detected via RT-qPCR using the primers as follows: GASS-F, 5' CGACTCTCTGTGAGGTATGGT-3' and GASS-R, 5'-GGAGACACTGTT TTAATCTTCTGT-3'. The PCR products were analyzed by 1.7% agarose gel electrophoresis for 110V/30 min.

RESULTS

The Expression Level of ecto-ATP Synthase is Associated with Gefitinib Sensitivity and Tumor Prognosis—To investigate whether
the ectopic translocation of ATP synthase into the plasma membrane can be considered as a new therapeutic target in gefitinib-resistant NSCLCs, we clarified the correlation between the expression levels of ecto-ATP synthase and gefitinib sensitivity. At first, MTS cell viability assay was performed to characterize the sensitivities to gefitinib in two cell lines with EGFR mutations, and two cell lines that express WT EGFR but mutated KRAS. The results of this experiment indicated that only the H3255 cells harboring the activating EGFR mutation (EGFR\(^{L858R,T790M}\)) were sensitive to gefitinib treatment, with a IC_{50} value of 9.5 \(\mu\)M. In contrast, H1975 cells containing the secondary EGFR mutation (EGFR\(^{L858R,T790M}\)), A549 cells (KRAS\(^{G12S}\)) and H460 cells (KRAS\(^{G12S}\)) that have a KRAS point mutation had relatively higher IC_{50} values and displayed gefitinib resistance (Fig. 1A). We also measured the expression levels of ecto-ATP synthase on the cell surface. Immunocytochemistry using nonpermeabilized cells revealed that the gefitinib-resistant cell lines (H1975, A549, and H460) had more ecto-ATP synthase on their plasma membranes than the gefitinib-sensitive cell line (H3255) (Fig. 1B). However, the expression levels and distributions of intracellular ATP synthase were similar in all four cell lines (supplemental Fig. S2A). These results indicate that the abundance of ecto-ATP synthase on the cell surface is independent of the amount of ATP synthase located on the mitochondrial inner membrane. Flow cytometry showed consistent results regarding the expression of ectopic ATP synthase in these cell lines (Fig. 1C).

We then investigated whether primary tumors with a high abundance of ectopic ATP synthase resembled the cell lines from the perspective of gefitinib-resistance. Hence, we adopted a transcriptome-based method to evaluate the expression level of ecto-ATP synthase. In comparison of the expression profiles of cells with differential expression levels of ecto-ATP synthase, we identified 438 ecto-ATP synthase-associated genes (Fig. 1D and supplemental Table S3), and these genes were used to calculate the ecto-ATP synthase scores for tumor samples by the single-sample gene set enrichment analysis (ssGSEA). Validation using an independent data set revealed that ecto-ATP synthase scores successfully discriminate between cells with differential levels of ecto-ATP synthase (supplemental Fig. S2B) and associated with gefitinib sensitivity (Fig. 1E). Samples from The Cancer Genome Atlas Lung Adenocarcinoma (TCGA-LUAD) study were classified into two ecto-ATP synthase groups based on their ecto-ATP synthase scores (supplemental Fig. S2C), and we found that higher ecto-ATP synthase scores were associated with poorer prognosis (Fig. 1F). These lines of evidence imply that plasma-membrane ecto-ATP synthase is associated with gefitinib resistance and tumor progression in lung cancer.

**Ecto-ATP Synthase is a Novel Drug Target for Gefitinib Resistance**—To explore whether ecto-ATP synthase is a potential therapeutic target for gefitinib-resistant cells in lung cancer, we examined cell viability in both gefitinib-sensitive and gefitinib-resistant cell lines after treatment with citreoviridin, which serves as an ecto-ATP synthase inhibitor (eATPI). Both MTS assays and cell counting by hemocytometer indicated that eATPI robustly suppressed proliferation in gefitinib-resistant cell lines (Fig. 2A and 2B). In addition, we demonstrated that eATPI strongly inhibited the colony forming ability of resistant cells (Fig. 2C). Thus, ecto-ATP synthase may be regarded not only as a marker of accelerated tumor progression but also as a novel drug target for cells with gefitinib resistance.

**Integrative Omics Data Identify Comprehensive Functional Networks Regulated via ecto-ATP Synthase Blockade**—Although we had confirmed that eATPI certainly suppresses the proliferation of gefitinib-resistant cells, the eATPI-induced network had not yet been thoroughly elucidated. Next, therefore, we performed an analysis integrating multiple omics approaches to comprehensively delineate the molecular mechanism that is regulated by eATPI. We applied quantitative phosphoproteomics to investigate the temporal dynamics of the phosphorylation modification after short-term treatment with eATPI (1 min, 10 min, and 60 min) and microarray-based transcriptomics to determine the changes in gene expression in response to treatment for 24 h. These omics data were further analyzed via bioinformatics approaches to unveil the eATPI-responsive networks (Fig. 3A). In our time-series phosphoproteomics analysis, we identified 1222 phosphopeptides corresponding to 1977 phosphorylation sites (supplemental Table S4) on 828 phosphoproteins (supplemental Table S5). Most of the phosphopeptides had single- or double-site phosphorylation, although a few exhibited triple or quadruple phosphosites. (supplemental Fig. S3A). Of the 1977 phosphosites, 1323 (67%) had a high localization probability (at least 0.75; supplemental Fig. S3B) and were used for downstream analysis. The distribution of phosphorylated residues was 84% phosphoserine, 14% phosphothreonine, and 2% phosphotyrosine (supplemental Fig. S3C). To further explore the time-dependent dynamic patterns of eATPI-regulated phosphorylation events, all identified phosphosites were classified into six different clusters based on their phosphorylated changes at each time point using our developed phosphoproteomics tool, DynaPho (59). Approximate 43% of phosphorylation events (clusters 1 and 2) showed rapidly up-regulated within 10 min but then decreased after eATPI treatment for 10 min (supplemental Fig. S3D). The phosphoproteins displaying rapid but transient phosphorylation were involved in several biological processes related to chromosome organization, cell cycle, and apoptosis. To more specifically reveal eATPI-mediated phosphorylation, we defined 284 phosphosites with significant changes in phosphorylation levels that were induced by eATPI based on a p-value of \(<0.05\) (Fig. 3B and supplemental Table S6). We also used microarray analysis on A549 cells that had been treated with eATPI for 24 h, and a total of 290 and 227 genes were identified as up-regulated and down-regulated, respectively, using 2-fold change and an adjusted p-value of \(<0.001\) as the threshold (Fig. 3C and supplemental Table S7). The gene ontology (GO)
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A

H3255

IC\textsubscript{50}=9.5 \textmu M

H1975

IC\textsubscript{50}>40 \textmu M

A549

IC\textsubscript{50}>40 \textmu M

H460

IC\textsubscript{50}=39.7 \textmu M

Relative cell viability (%)

24hr 48hr 72hr

0 1 2 5 10 20 40 Gefitinib concentration (\textmu M)

B

Gefitinib-sensitive

Gefitinib-resistant

H3255

H1975

A549

H460

ecto-ATP synthase

ecto-ATP synthase / DAPI

C

H3255

MFI=82.9

H1975

MFI=764.1

A549

MFI=1454.8

H460

MFI=2050.8

% of Max

0 20 40 60 80 100

ATP synthase-FITC

D

355 genes

83 genes

-\log_{10}(\text{p-value})

\log_{2}(\text{High/Low ecto-ATP synthase})

E

Gefitinib

\text{Cell viability (AUC)}

ecto-ATP synthase score

F

Survival probability

Years

ecto-ATP synthase score

High (n = 149)

Low (n = 206)

HR = 1.79 (95% CI: 1.25-2.57)

p = 0.0012

r = 0.24

p = 0.007
enrichment analysis of proteins exhibiting differential phosphorylation status and genes with differential expression revealed that terms relating to the cell cycle, DNA-damage response (DDR), apoptosis, and DNA-conformation change were significantly enriched under eATPi treatment (Fig. 3D and supplemental Table S8). Combined, these data suggest that eATPi may be involved in the regulation of the DDR.

**Ecto-ATP Synthase Blockade Leads to DNA Damage, Cell Cycle Arrest at G0/G1 Phase and Programmed Cell Death**—To verify whether blocking ecto-ATP synthase induces DDR, both immunofluorescence and western blotting were performed to detect the phosphorylation level of histone γ-H2AX at Ser 139, which is regarded as a surrogate marker for DNA double-strand breaks (DSBs). The phosphorylation level of this histone was increased in gefitinib-resistant cells after eATPi treatment (Fig. 4A–4C). Cell-cycle arrest and apoptosis are known to be cellular responses to DNA damage (60), consistent with the enriched function of perturbed genes and phosphosites in response to eATPi (Fig. 3D). Therefore, we further investigated whether eATPi caused cell death via a series of cellular responses comprising DNA damage, cell-cycle perturbation, and apoptosis. Flow cytometry analysis was used to assess the changes in DNA content distribution in gefitinib-resistant cell lines following eATPi or control treatment. As expected, the results indicated that eATPi treatment led to arrest during the G0/G1 phase (Fig. 4D). In addition, the proportion of cells undergoing apoptosis was also increased in gefitinib-resistant cells after eATPi treatment (Fig. 4E). Hence, our findings demonstrate that ecto-ATP synthase blockade induces both DNA damage and cell cycle arrest, which then leads to programed proogram cell death.

**CK2-Dependent Phosphorylation of DNA Topoisomerase IIα at Ser1106 plays a critical role in the early response to ecto-ATP synthase blockade**—As mentioned above, functional studies gefitinib-resistant cells had clearly identified the key biological processes affected by ecto-ATP synthase; however, a detailed exploration of the molecular mechanisms was still needed. To further clarify the potential kinase accountable for the ecto-ATP synthase-regulated network, we extracted the phosphosites that showed alterations after eATPi treatment (1.5-fold change) to construct a CK2-centered kinase regulated network. Of the substrates of CK2α, DNA topoisomerase IIα (topo IIα) undergoes the greatest alteration in phosphorylation after eATPi treatment. Topo IIα has been reported to be involved in the functions such as apoptotic chromosome condensation, cell response to DNA damage stimuli, and cell cycle (62–64), which supports our observed results of ecto-ATP synthase blockade (Fig. 4). Furthermore, in the time-series phosphoproteomic data set, the phosphorylation of topo IIα at Ser1106 was up-regulated approximately 3-fold in eATPi-treated cells at all-time points (supplemental Fig. S4A). The sequence pattern topo IIα at serine 1106 in our tandem MS (MS/MS) findings also matches the consensus phosphorylation motifs of CK2α and CK2α′ (supplemental Fig. S4B and S4C). Therefore, we considered CK2α as the kinase that potentially phosphorylates topoisomerase IIα at serine 1106. The degree of phosphorylation of Ser1106 in topo IIα was further validated by western blotting to confirming the accuracy of the MS-

**Fig. 1. Expression of ecto-ATP synthase was correlated with gefitinib sensitivity and cell survival.** A, Cell viabilities with increasing concentrations of gefitinib throughout the time course (24, 48, and 72 h) were detected in lung cancer cell lines with different EGFR mutations via MTS assay. The relative cell viabilities are presented as the mean ± S.D., n = 3. The average IC50 for each cell line at 48 h is shown. B, The expression levels of ecto-ATP synthase were observed via immunocytochemistry with ATP-synthase–complex antibodies in nonpermeablized lung cancer cells. DAPI was used to stain nuclei. Scale bars, 10 μm. C, Expression of the ATP synthase complex on the cell surface was analyzed via flow cytometry using antibodies for the ATP synthase complex, or control mouse IgG, in nonpermeablized lung cancer cells. MFI, represents the mean fluorescent intensity values of ecto-ATP synthase. D, Volcano plot depicting the gene expression differences between cells with high and low levels of ecto-ATP synthase. The orange points represent the significantly differentially expressed genes (q < 10^{-6} and fold-change > 4). E, Scatter plot displaying the positive correlation between the abundance of ecto-ATP synthase and cell viability under gefitinib treatment in lung cancer cells. Cell viability is represented as the area under the receiver operating characteristic (ROC) curve (AUC). Large AUCs indicate greater resistance to gefitinib. F, Kaplan–Meier plot showing overall survival stratified according to ecto-ATP synthase levels in lung cancer patients. P: log-rank test.
Multiomics Reveals the CK2/Phospho-Topo IIα/GAS5 Axis

As shown in Fig. 5E, the phosphorylation level at Ser1106 in topo IIα was higher in treatment groups than in control groups, although the protein expression level of topo IIα did not differ between these two groups.

Next, we aimed to elucidate whether topo IIα is phosphorylated by a CK2 kinase reaction. After cotreatment with CK2 inhibitor, the Ser1106 phosphorylation level of topo IIα in eATPi-treated cells was greatly reduced (Fig. 5F). In addition,
to assess whether Ser1106 of topo IIα is functionally significant, WT and dephosphorylation mutations of topo IIα (TOP2A-WT and TOP2A-S1106A) were transfected into gefitinib-resistant lung cancer cells. Western blotting demonstrated that the phosphorylation of topo IIα at Ser1106 was decreased in TOP2A-S1106A mutant-transfected cells relative to TOP2A-WT-transfected cells, whereas the expression levels of topo IIα protein were similar in both (Fig. 5G). As expected, eATPi suppressed the proliferation of TOP2A-WT-overexpressing cells, but not of TOP2A-S1106A mutant-overexpressing cells (Fig. 5H). These results illustrate that ecto-ATP synthase inhibitor induces cell death through CK2-dependent phosphorylation of topo IIα at Ser1106.

**Ecto-ATP Synthase Inhibition Activates the Genes in p53 Pathway**—Next, we further investigated which regulators are recruited in the transcriptional response to ecto-ATP synthase inhibition. To identify the transcription factors with differential transcriptional activity after eATPi treatment, the integrated system for motif activity response analysis (iSMARA) (65) was applied to the gene expression profiles with and without eATPi treatment. This analysis revealed that the p53 motif was more active in eATPi-treated cells than in control cells (Fig. 6A). In addition, gene-set enrichment analysis (GSEA) demonstrated that the p53 pathway was obviously activated in eATPi-treated cells (Fig. 6B). p53 is known to be a key transcriptional regulator for programmed cell death in various cancers (66). Therefore, we speculated that the eATPi drove programmed cell death via regulation of p53 transcriptional activity. Indeed, real-time quantitative PCR (RT-qPCR) also found that the p53 and p53-related genes that are involved in eATPi-mediated biological functions, such as DNA damage, cell-cycle arrest, and apoptosis, were up-regulated in response to eATPi treatment (Fig. 6C and 6D). This confirms that p53 is the key transcriptional factor involved in eATPi-induced programmed cell death after long-term stimulation.

**Transcriptomics Identified IncRNA GAS5 Involved in the Cell Death After Inhibition of ecto-ATP Synthase**—Recently, studies have revealed that long noncoding RNAs (lncRNAs) can regulate the expression of protein-coding genes by controlling transcription, post-transcription, and even chromatin remodeling (37, 67). Interestingly, in addition to the protein-coding genes, plenty of noncoding genes were also regulated in response to long-term treatment with eATPi (supplemental Fig. S5A). The top six differentially expressed IncRNAs in eATPi-treated cells, VLDLR-AS1, GAS5, ZFAS1, MEG3, LINCO00662, and LOC728730, are listed in supplemental Fig. S5B. The changes in their expression were further confirmed using RT-qPCR analysis (supplemental Fig. S5C). Because one of the IncRNAs, GAS5, has been reported to crucially regulate the cell cycle and apoptosis via activation of the p53 pathway (68, 69), we therefore investigated whether eATPi could not only trigger p53-mediated programmed cell death but also modulate the level of GAS5 in gefitinib-resistant cells. The RT-qPCR analysis revealed increased expression levels of GAS5 in eATPi-treated cells compared with control cells (Fig. 6E).

To confirm whether the elevated GAS5 RNA levels observed in eATPi-treated cells were consistent with increased GAS5 promoter activity, we transfected gefitinib-resistant cells with a luciferase reporter plasmid into which GAS5 promoter sequences had been inserted (pG3L3-B-pGAS5). This luciferase reporter assay indicated that GAS5 promoter activity was higher in cells treated with eATPi than in control cells (Fig. 6F). Functionally, a clonogenic assay revealed that knockdown of GAS5 relieved the eATPi-mediated suppression of proliferation in gefitinib-resistant cells (Fig. 6G).

Recent studies have revealed that a variety of lncRNAs are regulated in response to DNA damage (70). Our findings showed that the disruption of topo IIα phosphorylation by a CK2 inhibitor depresses the expression level of GAS5 therefore imply that eATPi might regulate GAS5 expression by enhancing the CK2-dependent phosphorylation of topo IIα (Fig. 6H). By combining the early-response network mentioned above, ecto-ATP synthase blockade is thus able to orchestrate cell death via regulating the CK2/phospho-topo IIα/GAS5 axis.

**RNA-Binding Proteomics Identifies Topoisomerase IIα is One of GAS5-Interacting Proteins and Phosphorylated in CK2/Topo IIα/GAS5 Axis**—LncRNAs modulate various cellular functions by forming complexes with their protein partners. To investigate potential GAS5-interacting proteins, we developed an RNA-binding proteomics approach, which combined in vitro transcription with an RNA-pull down assay followed by a liquid chromatography (LC)-MS/MS analysis. A schematic diagram displaying the detailed experimental procedures is given in supplemental Fig. S6A. The proteins that bound with biotin-labeled GAS5 transcripts and subsequently pulled down by biotin-streptavidin interaction were considered as GAS5-interacting partners. Pulldown experiment with the unlabeled GAS5 transcripts was used as the negative control to identify the proteins that had nonspecific binding with streptavidin-coupled beads. supplemental Fig. S6B showed a comparison among GAS5-interacting proteins and negative control. In total, 226 and 125 GAS5-interacting proteins that were generally present in the two biological replicates were identified in the A549 and H1975 cell lines, respectively. We selected 107 candidates from these proteins that were common to both replicates of both cell line (Fig. 7A). Detailed information about the GAS5-interacting proteins that were expressed in each cell lines is given in supplemental Table S9. The selected GAS5-binding partners were functionally enriched in terms related to DNA damage, apoptosis, and p53 signal transduction, which was consistent with functions that emerge in response to eATPi treatment (Fig. 7B and supplemental Table S10). Interestingly, topo IIα was one of the potential GAS5-interacting proteins. To validate our GAS5-binding proteomics results, GAS5 pulldown complexes were
Identification of an eATPi-induced dynamic molecular network.

A. Schematic representation of the multi-omics approach used with gefitinib-resistant lung cancer cells under control or eATPi treatment. Quantitative phosphoproteomic profiling of cells under treatment for 1 min, 10 min, and 60 min and transcriptomic profiling of cells under treatment for 24 h were performed to identify the early and late responses, respectively. For quantitative phosphoproteomic profiling, protein extracts obtained from the cells treated with citreoviridin (eATPi) at IC_{50} concentration or with the same volume of DMSO were enzymatic digested and differentially stable isotope dimethyl-labeled. L represents light stable isotopes (CH_{3}), whereas H represents heavy stable isotopes (C^{13}D_{2}H). All omics data were analyzed using bioinformatics platforms to establish a global eATPi-responsive network.

B. Dot plot depicting the changes in the phosphorylation level of the proteins between eATPi-treated and control cells. Orange points represent the phosphosites with significant change (p < 0.05, derived from significance B).

C. Volcano plot depicting the gene expression differences between eATPi-treated and control cells. The orange points represent the significantly differentially expressed genes (q < 10^{-3} and fold-change > 2).

D. Enrichment map of eATPi-responsive genes and proteins. Nodes depict gene sets that were statistically over-represented in the genes with differential expression or phosphosites with phosphorylation changes. Links connecting nodes indicate the relatedness between nodes.
detected using western blotting with the specific antibody against topo IIα. The result showed that topo IIα was indeed associated with GAS5 transcripts in both gefitinib-resistant lung cancer cell lines (Fig. 7C). An RNA immunoprecipitation (RIP) assay further clarified the interaction between GAS5 and topo IIα (Fig. 7D).

According to our findings shown above, the CK2/phospho-topo IIα/GAS5 axis plays an essential role in the eATPi response. Thus, we investigated whether the interaction between GAS5 and topo IIα had any effect on this eATPi-stimulated network. First, we verified the mRNA expression level of topo IIα in GAS5-overexpressing cells. The qPCR

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**Fig. 4.** The inhibitor of eATP induced cell death by promoting DNA damage, G1 cell-cycle arrest, and apoptosis. A, Cells were treated with control or the IC₅₀ of eATPi for 24 h and subjected to western blotting to detect the presence of phospho-S139-γH₂AX. B, Relative expression levels of phospho-S139-γH₂AX in control and eATPi-treated cells of each independent biological replicates were quantified and shown as the bar charts. C, Cells treated with control or the IC₅₀ of eATPi for 24 h were immunostained for phospho-S139-γH₂AX antibody (green) and counterstained with DAPI to visualize nuclei. Representative images were taken by confocal laser microscopy. Scale bars, 5 µM. D, Cell-cycle distributions of gefitinib-resistant cells treated with control or the IC₅₀ of eATPi for 24 h, detected via flow cytometry. The percentages of G0–G1 phase, S phase, and G2–M phase in each cell line were analyzed using FlowJo software. E, The percentages of apoptotic cells after eATPi treatment for 24 h were determined via Annexin V-FITC/PI double staining assay and quantified using flow cytometry. Data in D and E are illustrated using representative images, and quantitative results are reported as mean ± S.D. in the histograms, n = 3. *p < 0.05; **p < 0.01; ***p < 0.001 (two-tailed t-test).
FIG. 5. Ecto-ATP synthase blockade triggered cell death via CK2-mediated phosphorylation of topoisomerase IIα at Ser1106. A, Kinases with significant activity changes in response to eATPi, as inferred using DynaPho software. Kinases are ranked according to p-value, shown on the x-axis. B, Cells were treated with or without the IC50 of eATPi for 48 h in the presence or absence of CK2 inhibitor. Cells were counted via a hemocytometer cell counting assay. Scale bars, 50 μM. Images are representative of triplicate experiments. C, Quantitative results are presented as the bar charts. D, Phosphoproteins significantly regulated by eATPi were extracted to establish the CK2-substrates network. The color of squares indicates the relative levels of phosphoproteins (eATPi treatment/control treatment). E, Phosphorylation of topoisomerase IIα at serine 1106 was validated via western blotting using a topo IIα-pS1106 site-specific antibody. F, Cells were treated with or without the IC50 of eATPi in the presence or absence of CK2 inhibitor. The phosphorylation levels of topoisomerase IIα at serine 1106 were detected via western blotting. The intensities of the bands in d and e are shown relative to β-actin, which was used as a loading control. G, Whole-cell lysates were prepared from cells transfected with HA-tagged plasmids encoding WT or S1106A-mutant topoisomerase IIα. The levels of overexpressed proteins were detected using western blotting. H, Cells were transfected with HA-tagged plasmids encoding WT or S1106A-mutated topoisomerase IIα prior to eATPi treatment. An MTS assay was performed to measure cell viability. Error bars show the mean ± S.D., n = 3. * p < 0.05; ** p < 0.01; *** p < 0.001 (two-tailed t-test).
| Accession Number | Protein Name                      | Phosphosite | Modified Peptide Sequence | Normalized Ratio (Treatment/Control) | Local Prob. | PEP Score | Predicated Kinase |
|------------------|----------------------------------|-------------|--------------------------|--------------------------------------|-------------|-----------|------------------|
| P07900           | Heat shock protein HSP 90-alpha (HS90A) | S231        | _YPITLFEKERDEKVEpSD-DEAEKEDKEEEEKE_ | 1.57 1.20 5.24 | 1.000 | 3.0E-10 | CK2α             |
| P08238           | Heat shock protein HSP 90-beta (HS90B) | S226        | _YPITLYLEKEREKEipSD-DEAEEEKGEKEEED_ | 0.82 0.62 1.35 | 1.000 | 7.3E-17 | CK2α             |
| P08238           | Heat shock protein HSP 90-beta (HS90B) | S255        | _EDKpDEEKPKIEDVgpSpS-DEEEDSGKpKpKKGTK_ | 1.24 1.84 1.71 | 1.000 | 1.3E-211 | CK2α             |
| Q13547           | Histone deacetylase 1 (HDAC1) | S421        | _SICSSDKRACEEEFpSD-pSEEEEGGRKNNSN_ | 1.21 1.58 0.93 | 1.000 | 1.2E-15 | CK2α             |
| Q13547           | Histone deacetylase 1 (HDAC1) | S423        | _CSSDKRACEEEFpSD-pSEEEEGGRKNSSNFK_ | 1.21 1.58 0.93 | 1.000 | 1.2E-15 | CK2α             |
| P11388           | DNA topoisomerase 2-alpha (TOP2A) | S1106       | _KEAQKVPEDEEENpSD-NEKETEKDSVTDS_ | 3.67 3.54 3.05 | 1.000 | 3.1E-11 | CK2α             |
| P23588           | Eukaryotic translation initiation factor 4B (IF-4B) | S409 | _PKLERPpPRERHPpSWRp-pSEEpTQpPERSRpTQpGpSES_ | 0.82 1.00 0.71 | 0.994 | 2.2E-06 | CK2α             |
| P18858           | DNA ligase 1 (DNLI1) | S51         | _KAALKpEWNGVSgpSpSP-VKRPRGpKAARpVLGS_ | NA 0.57 1.27 | 0.997 | 2.7E-06 | CK2α             |

*Unique identifier for proteins in Uniprot/Swiss-Prot database.
*The name of identified proteins this peptide is associated with.
*The position of the phosphorylated residues within this protein.
*The sequence of matched peptide including the locations of modified amino acid.
*Normalized ratio of the raw intensities.
*The best probability of phosphorylation localization at the position in this peptide.
*The posterior error probability of the best identified modified peptide containing this phosphosite.
*The score for the best associated MS/MS spectrum in Andromeda.
*The kinases for the phosphorylation sites predicated by PhosphoNetworks database.
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Figure A: TP53

- Graph showing transcriptional activity
- Comparison of eATPi and Control

Figure B: Enrichment plot: PD_P53_DOWNSTREAM_PATHWAY

- Bar graph indicating enrichment levels
- Comparison of experimental conditions

Figure C: Relative expression level

- Graph comparing Control and eATPi

Figure D: Relative gene expression level

- Bar graph showing gene expression levels for DNA damage, Cell cycle, and Apoptosis
- Comparison of Control and eATPi

Figure E: GAS5 relative expression level

- Bar graph showing GAS5 expression levels for A549 and H1975
- Comparison of eATPi conditions

Figure F: Relative luciferase activity

- Graph comparing Control and eATPi

Figure G: si-NTC and si-GAS5

- Images showing results of si-GAS5 treatment
- Comparison of Control, eATPi, and si-GAS5 conditions

Figure H: GAS5 relative expression levels

- Bar graph showing GAS5 expression levels for eATPi and CK2i
- Comparison of A549 and H1975

Absorbance at 595 nm

- Graph showing absorbance levels for eATPi, si-GAS5, and Control
- Comparison of A549 and H1975
analysis suggested that the mRNA level of GAS5 was significantly elevated (Fig. 7E), but the mRNA level of topo IIα was not affected (Fig. 7F). Similarly, the mRNA expression level of topo IIα changed only slightly when GAS5 was depleted using siRNA (Fig. 7G and 7H). Neither the mRNA nor the protein levels of topo IIα had any effect in GAS5-overexpressing or GAS5-knockdown cells (Fig. 7I–7L). Therefore, we suspected that GAS5 might be involved in the eATPi-stimulated network via post-translational modification rather than via regulation of transcription or translation. Surprisingly, we found that the phosphorylation level of topo IIα at Ser1106 was increased in GAS5-overexpressing cells (Fig. 7I and 7K), whereas it was reduced in cells with GAS5 knockdown (Fig. 7J and 7L). Combined, our data indicate that GAS5 plays a crucial role in the phosphorylation of topo IIα and consequently generates a positive feedback loop to sustain the eATPi-induced CK2/phospho-topo IIα/GAS5 axis.

**DISCUSSION**

Increasing evidence hints at the existence of ecto-ATP synthase in many different cell types (71). Several studies have also shown that increasing expression levels of ecto-ATP synthase are related to more-invasive phenotypes, larger tumor sizes, and more-advanced tumor stages (72, 73). Despite this, the connection between ecto-ATP synthase and chemoresistance, which is the leading cause of tumor recurrence, remains to be identified. A better understanding of the pathogenetic mechanisms of ecto-ATP synthase may thereby offer novel strategies for improving both the diagnosis and the treatment of cancer. In this work, we provide the evidence that gefitinib-resistant cells display elevated levels of ecto-ATP synthase relative to gefitinib-sensitive cells. Further experiments showed that ecto-ATP synthase blockade indeed resulted in cytotoxicity in gefitinib-resistant cells. Other studies have demonstrated that ecto-ATP synthase is involved in the establishment of pH homeostasis across the plasma membrane, which is crucial for cellular functions such as proliferation and angiogenesis (74).

Type II topoisomerases, topoisomerase IIα (topo IIα) and -β (topo IIβ), play vital roles in the maintenance of genomic integrity by regulating DNA topology through the creation of transient DSBs during DNA replication, transcription, and chromosome segregation (75, 76). These topo II poisons, such as daunorubicin, doxorubicin and etoposide, interfere with the DNA cleavage/replication cycle via stabilization of the topo II-DNA cleavable complex and eventually trigger cell death following the DDR (77). With other types of DNA damage, post-translational modification of topo II has been reported to cause irreversible DNA DSBs by modulating its enzymatic activity (78–80). For instance, phosphorylation of serine residue 1106 located in the catalytic domain of topo IIα abrogates its catalytic activity during mitosis (62, 81). We have previously revealed that pyrvinium pamoate, which was discovered via intensity-based similarity metric, acts as an anti-cancer drug by elevating the phosphorylation of topo IIα at Ser1106 in lung cancer cells (82). Consistently, in this study, we showed that eATPi induced cytotoxic effects through upregulating the phosphorylation of topo IIα at Ser1106 in gefitinib-resistant cells. Moreover, the kinase-substrate network suggests that CK2 is the major kinase involved in this site-specific phosphorylation of topo IIα at Ser1106. The cytotoxic assay showed that reducing the phosphorylation of topo IIα at Ser1106 via both a CK2 inhibitor and a point-mutation of serine 1106 to alanine (S1106A) rescued eATPi-induced cell death. These results demonstrate that ecto-ATP synthase inhibition causes cytotoxic effects by triggering CK2-dependent phosphorylation of topo IIα at Ser1106. In addition to the increased phosphorylation levels of topo IIα, we also found that eATPi enhanced DNA damage responses, including elevated the phosphorylation of histone H2AX at Ser139 the expression level of p53 level, similar to the response triggered by the known topo IIα inhibitor-pyrvinium pamoate. Interestingly, pyrvinium pamoate displays toxicity to both gefitinib-sensitive and gefitinib-resistant cancer cells (supplemental Fig. S7), whereas ecto-ATP synthase blockade selectively killed gefitinib-resistant cancer cells. For this reason, ecto-ATP synthase may be served as a specific surface target for cancer cells with acquired gefitinib resistance.

In response to cellular stress, such as DNA damage, p53 accumulates and triggers its target genes to induce cell cycle arrest and apoptosis (83–85). Although as an essential guardian of the genome, p53 is frequently dysfunctional in a growing number of human cancers. Therefore, restoring p53 activation becomes a potential goal for cancer therapy (86, 87).
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Our transcriptomics analysis that ecto-ATP synthase blockade successfully increased the expression levels of p53 and its related genes that are involved in DNA damage, cell-cycle arrest, and apoptosis. Moreover, increasing evidence has suggested that post-translational modifications, including phosphorylation, acetylation, and ubiquitination are able to regulate the transcriptional activity, stability, and subcellular localization of p53. For instance, phosphorylation at the carboxyl-terminal region regulate tetramerization of p53 to elevate its binding affinities for the promoters of target genes (88, 89). Herein, phosphoproteomic profile indicated that the C-terminal Ser314 and Ser315 residues were phosphorylated upon eATPi stimulation. Combining these results mentioned above, ecto-ATP synthase blockade induces programmed cell death by modulating both expression level and phosphorylation of p53 in response to the accumulation of genotoxic stress caused via CK2-dependent phosphorylation of topo IIα.

Growth arrest-specific transcript 5 (GASS), a well-studied tumor-suppressing lncRNA, is positively correlated with prognosis and serves as a diagnostic biomarker for a variety of tumor types (90). Moreover, for several chemotherapeutic drugs, levels of GASS are directly related to drug-induced cell death (91, 92). Our findings provide the new insight that extracellular stimuli, ecto-ATP synthase inhibition, triggers anti-cancer effects through GASS regulation in gefitinib-resistant lung cancer cells. We also found that the disruption of topo IIα phosphorylation by a CK2 inhibitor depresses the expression level of GASS therefore imply that eATPi might regulate GASS expression by enhancing the CK2-dependent phosphorylation of topo IIα. Accumulating recent studies have demonstrated that, by directly binding to its interacting partners or serving as a molecular decoy for its regulatory targets, GASS contributes to the regulation of the cell cycle and apoptosis (93). Notably, we revealed that GASS interacts with topo IIα and increases its phosphorylation, although it has no effect on either the RNA or protein levels of topo IIα. Surprisingly, our GASS-binding proteomics discovered that several kinases, including casein kinase I isoform alpha, are recruited into the GASS-topo IIα complex. Casein kinase I has been recognized as the major kinase involving in the phosphorylation of topo IIα at the C-terminal region (64). Therefore, we hypothesize that GASS serves as a molecular scaffold for the assembly of topo IIα and kinases that are responsible for topo IIα phosphorylation. Sharma et al. have reported that the IncRNA NRON forms a complex comprising NFAT and several regulatory kinases that carries out the phosphorylation of NFAT (94). On the other hand, several studies have provided the contrasting insight that the IncRNA Inc-DC enhances STAT3 phosphorylation by masking the phosphatase-binding motif of STAT3 (95). Therefore, whether GASS prevents topo IIα dephosphorylation by occupying the phosphatase-binding motif of topo IIα will need to be further identified. Understanding how GASS regulates the post-translational modification of its interacting proteins may have a bearing on various aspects of cancer progression. Interestingly, topoisomerase II has been found to interact with the C-terminal region of p53 (96). Moreover, one of GASS-interacting partners, DDX5, has been shown to be the transcriptional co-activator of p53 and capable of modulating p53-induced apoptosis and cell cycle arrest (97, 98). Hence, the cross-talk between GASS and p53 pathway in response to topolalpha-mediated genomic damage and their subsequent programed cell death should be further clarified.

In summary, our investigations define ecto-ATP synthase not only as an indicator of gefitinib’s efficacy but also as an alternative therapeutic target in gefitinib resistance lung cancer by regulating DNA damage, cell-cycle arrest, and apoptosis. Additionally, a multi-omics analysis was performed, leading to the identification of a novel CK2/phospho-topo IIα/GASS axis, which was induced in response to eATPi treatment and subsequently promoted p53-mediated programed cell death. Finally, the results of an RNA-binding proteomics approach indicated that GASS binds to topo IIα, and further led to increased topo IIα phosphorylation, which reinforced the eATPi-mediated CK2/phospho-topo IIα/GASS axis.

DATA AVAILABILITY

The datasets supporting the conclusions of this article are available in the publicly available repositories. Gene expression...
profile with eATPi treatment in A549 lung cancer cells data are available through GEO with identifier GSE112988. All of the information about Mass spectra have been deposited to ProteomeXchange via the PRIDE partner repository with the data set identifier PXD013139 and PXD010767.

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Conflict of interest—Authors declare no competing interests.

Abbreviations—The abbreviations used are: CK2, casein kinase II; eATPi, ectopic adenosine triphosphate synthase; eATPi, ectopic adenosine triphosphatase synthase inhibitor; MTS, [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt.

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