

## Research Paper

# N6-methyladenosine-induced ERRγ triggers chemoresistance of cancer cells through upregulation of ABCB1 and metabolic reprogramming

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## Abstract

**Background:** Drug resistance severely reduces treatment efficiency of chemotherapy and leads to poor prognosis. However, regulatory factors of chemoresistant cancer cells are largely unknown.

**Methods:** The expression of estrogen receptor related receptors (ERRs) in chemoresistant cancer cells are checked. The roles of ERRγ in chemoresistance are confirmed by *in vitro* and *in vivo* studies. The mechanisms responsible for ERRγ-regulated expression of *ABCB1* and *CPT1B* are investigated.

**Results:** The expression of ERRγ is upregulated in chemoresistant cancer cells. Targeted inhibition of ERRγ restores the chemosensitivity. ERRγ can directly bind to the promoter of *ABCB1* to increase its transcription. An elevated interaction between ERRγ and p65 in chemoresistant cells further strengthens transcription of *ABCB1*. Further, ERRγ can increase the fatty acid oxidation (FAO) in chemoresistant cells via regulation of *CPT1B*, the rate-limiting enzyme of FAO. The upregulated ERRγ in chemoresistant cancer cells might be due to increased levels of N6-methyladenosine (m<sup>6</sup>A) can trigger the splicing of precursor *ESRRG* mRNA.

**Conclusions:** m<sup>6</sup>A induced ERRγ confers chemoresistance of cancer cells through upregulation of *ABCB1* and *CPT1B*.

Key words: Chemoresistance; ERRγ; *ABCB1*; *CPT1B*; FAO

## Introduction

Chemotherapy using one or more anticancer drugs is the major strategy for cancer treatment, particularly for patients with advanced and/or metastatic tumors that cannot undergo surgery [1]. It is generally preferred in developing countries due to

high costs of targeted therapies [2]. However, many patients gradually develop resistance to progressive chemotherapy, resulting in treatment failure that has become a serious clinical problem in cancer therapy. One important feature of chemoresistance is that

cancer cells often become resistant to not just one drug, but also to different drugs. This is known as multidrug resistance (MDR) and will seriously affect the treatment efficiency.

Several mechanisms involved in chemoresistance have been identified in the last few decades [3-5]. ATP-binding cassette (ABC) transporters with a family of 48 human members can regulate the absorption, disposition and elimination of drugs to mediate chemoresistance [6]. ATP binding cassette subfamily B member 1 (*ABCB1*), which encodes multidrug resistance protein-1 (MDR-1)/P-glycoprotein (P-gp), is one of the best studied molecules in drug resistance [3]. It can confer cancer cell resistance to numerous anticancer agents such as doxorubicin (Dox), taxol (Tax), colchicine, vincristine and even tyrosine kinase inhibitors [7].

Recently, emerging evidence indicates that metabolic properties of chemoresistant cancer cells diverge significantly from those of their parental cells [8-10]. Dysregulation of glucose metabolism, fatty acid synthesis and glutaminolysis have been linked to therapeutic resistance in cancer treatment [11]. Fatty acids (FAs) are important cellular energy resources utilized through FA oxidation (FAO), which has been shown to be involved in cancer stem cell self-renewal and chemoresistance of breast [10] and gastric [12] cancers. Inhibition of FAO can repress stemness and *in vivo* growth of cancer cells [13, 14]. However, the roles and regulatory factors of FAO in chemoresistant cancer cells are largely unknown.

Estrogen receptor related receptors (ERRs), which include  $ERR\alpha/\beta/\gamma$ , are orphan nuclear receptors and share sequence homology with estrogen receptor  $\alpha$  ( $ER\alpha$ ) [15]. It has been reported that  $ERR\alpha$  is involved in chemotherapy resistance of osteosarcoma cells [16].  $ERR\gamma$  is a crucial mediator of multiple endocrine and metabolic signals [17] and mediates tamoxifen (TAM) resistance of invasive lobular breast cancer, in which knockdown of  $ERR\gamma$  restores TAM sensitivity [18]. Androgen-dependent repression of  $ERR\gamma$  reprograms metabolic properties of prostate cancer [19], whereas miR-378 can inhibit the expression of  $ERR\gamma$  to suppress tricarboxylic acid cycle (TCA) gene expression and oxygen consumption as well as an increase in lactate production [20]. All these data suggest that ERRs are likely involved in chemoresistance of cancer cells.

We found that  $ERR\gamma$  was significantly upregulated in chemoresistant cancer cells, with knockdown of  $ERR\gamma$  restoring the chemosensitivity. Mechanistically,  $ERR\gamma$  can mediate the chemoresistance of cancer cells via upregulation of *ABCB1* and facilitation of FAO. Our results identify a new macromolecule that may serve as a predictive

marker of chemotherapy and as an effective target for overcoming chemoresistance.

## Results

### **$ERR\gamma$ is upregulated in chemoresistant cancer cells**

The chemoresistance of breast (MCF-7/ADR) and liver (HepG2/ADR) cancer cells was confirmed by evaluation of Dox sensitivity and compared with that of their corresponding parental cells (Figure S1A and S1B). A potential role of ERR signals in chemoresistance was then assessed by quantifying the mRNA levels of  $ERR\alpha$  (*ESRRA*) and  $ERR\gamma$  (*ESRRG*). Quantitative (q) RT-PCR showed enhanced mRNA levels of  $ERR\gamma$  in MCF-7/ADR cells as compared to that in MCF-7 cells (Figure 1A). Increased expression of  $ERR\gamma$  was also observed in HepG2/ADR cells as compared to that in HepG2 cells (Figure 1B). An elevated protein level of  $ERR\gamma$  was also observed in MCF-7/ADR and HepG2/ADR cells by Western blot analysis (Figure 1C). In contrast, the protein levels of  $ERR\alpha$  had no clear difference between chemoresistant and parental cells (Figure S1C). An increased mRNA level of  $ERR\gamma$  was also observed in other chemoresistant lines, including MCF-7/Tax (resistance to Tax), MDA-MB-231/Tax, and A549/Tax cells as compared to their corresponding parental cells (Figure S1D). Western blot analysis further confirmed that the protein level of  $ERR\gamma$  was increased in MCF-7/Tax and MDA-MB-231/Tax as compared to their controls (Figure S1E). Confocal microscopy showed that the expression of  $ERR\gamma$  was mainly located within the cytoplasm of HepG2 cells, while the expression and nuclear accumulation of  $ERR\gamma$  were increased in HepG2/ADR cells (Figure 1D). An increased nuclear localization of  $ERR\gamma$  in HepG2/ADR cells was confirmed by subcellular fractionation and Western blot analysis (Figure 1E) and further validated in MCF-7/ADR cells (Figure S1F). The expression levels of  $ERR\alpha/\gamma$  in cancer cells were also examined following Dox treatment. Western blot analysis showed Dox treatment rapidly increased the expression of  $ERR\gamma$  (Figure 1F), but not  $ERR\alpha$  (Figure S1G), in both HepG2 and MCF-7 cells. This might be due to that Dox treatment can increase the mRNA of  $ERR\gamma$  expression in cancer cells (Figure 1G). Collectively, these data showed that  $ERR\gamma$  is upregulated in chemoresistant cancer cells.

### **$ERR\gamma$ regulates chemoresistance of cancer cells**

To determine whether  $ERR\gamma$  is involved in chemoresistance of cancer cells, we knocked down the expression of  $ERR\gamma$  by siRNA transfection (Figure

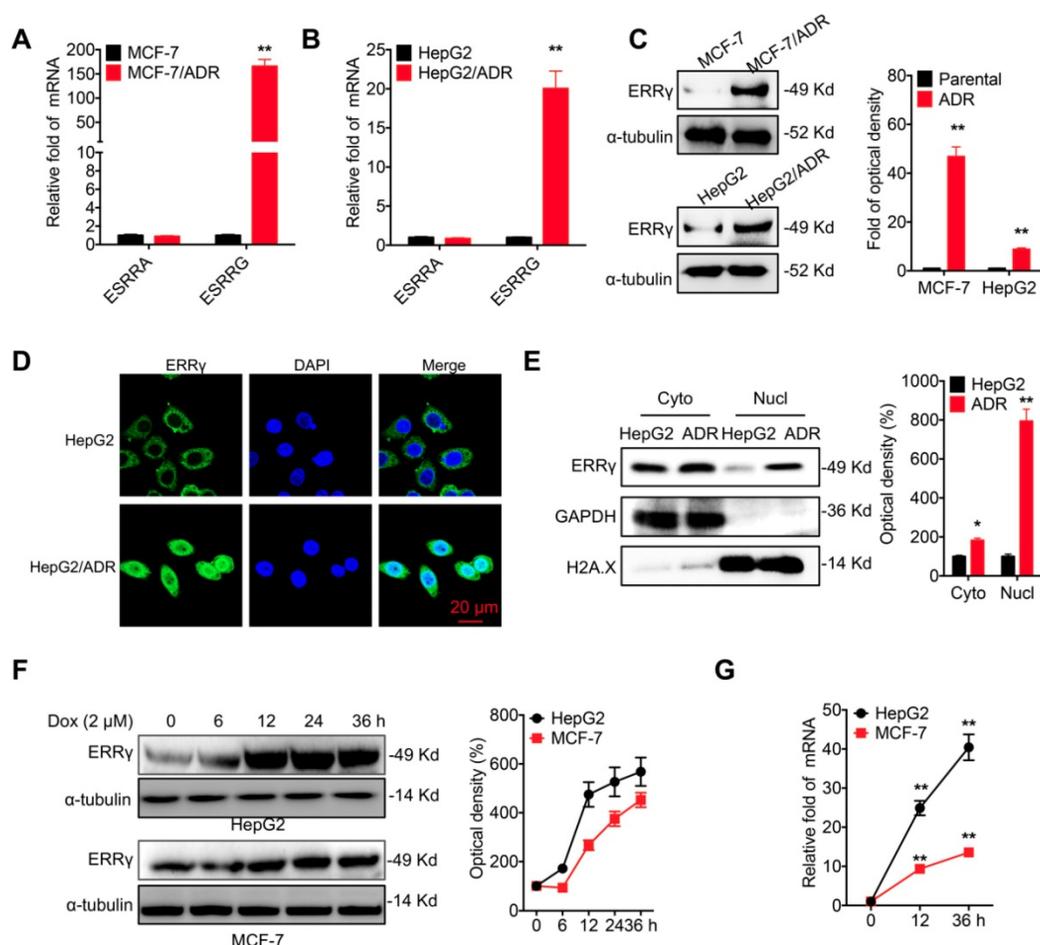
S2A). Knockdown of ERR $\gamma$  significantly restored Dox and Tax sensitivity in HepG2/ADR cells, with IC<sub>50</sub> values of Dox for si-NC and si-ERR $\gamma$  being 173 and 15.9  $\mu$ M (Figure 2A) and of Tax for si-NC and si-ERR $\gamma$  being 29.2 and 2.36  $\mu$ M, respectively (Figure 2B). As to MCF-7/ADR cells, the IC<sub>50</sub> values of Dox for si-NC and si-ERR $\gamma$  were 30.2 and 4.96  $\mu$ M (Figure 2C) and of Tax for si-NC and si-ERR $\gamma$  were 23.4 and 2.76  $\mu$ M, respectively (Figure 2D). Colony formation assay showed that knockdown of ERR $\gamma$  significantly inhibited colonization of both HepG2/ADR (Figure 2E) and MCF-7/ADR (Figure 2F) cells. In contrast, overexpression of ERR $\gamma$  in HepG2 and MCF-7 cells by transfection with pcDNA/ERR $\gamma$  (Figure S2B) decreased the sensitivity of both cells to the treatment of Dox and Tax (Figure S2 C-F).

To evaluate whether ERR $\gamma$  is essential for *in vivo* chemoresistance of cancer cells, we established mouse xenograft tumors by using sh-ERR $\gamma$ -transfected HepG2/ADR cells (Figure S2G). The tumor growth

rate and tumor size at the end of the experiments were significantly decreased in the sh-ERR $\gamma$  group relative to the scrambled group (Figure 2G). When treated with Dox, the scrambled group showed no obvious decrease in tumor size as compared with that of the control group. As expected, sh-ERR $\gamma$  increased *in vivo* Dox sensitivity of HepG2/Dox cells (Figure 2G). Subsequent IHC analysis confirmed the *in vivo* knockdown efficiency of sh-ERR $\gamma$  (Figure 2H). Further, Dox treatment obviously reduced the expression of the proliferation marker Ki-67 in the sh-ERR $\gamma$  group (50%) than in the scrambled group (77%) (Figure 2H). These data suggested that ERR $\gamma$  regulates both *in vitro* and *in vivo* chemoresistance of cancer cells.

### P-gp is involved in ERR $\gamma$ -regulated chemoresistance of cancer cells

ABC transporters are critical for chemoresistance of cancer cells [6]. Expression of the major ABC



**Figure 1. ERR $\gamma$  is upregulated in chemoresistant cancer cells.** (A&B) Expression of ERR $\alpha$  (ESRRA) and ERR $\gamma$  (ESRRG) measured in MCF-7/ADR (A), HepG2/ADR (B), and their corresponding parental cells by qRT-PCR; (C) Protein levels of ERR $\gamma$  in MCF-7/ADR, HepG2/ADR, and their corresponding parental cells measured by Western blot analysis (left) and quantitatively analyzed (right); (D) Subcellular expression of ERR $\gamma$  in HepG2/ADR and HepG2 cells visualized by confocal imaging; (E) The subcellular expression of ERR $\gamma$  in HepG2/ADR and HepG2 cells was checked by Western blot analysis (left) and quantitatively analyzed (right); (F) Cells were treated with Dox (2  $\mu$ M) for the indicated times, then the protein expression of ERR $\gamma$  was checked by Western blot analysis (left) and quantitatively analyzed (right); (G) Cells were treated with Dox (2  $\mu$ M) for the indicated times, then the mRNA expression of ERR $\gamma$  was checked by qRT-PCR. Data were presented as means  $\pm$  SD from three independent experiments. \* $p$ <0.05, \*\* $p$ <0.01 compared with control.

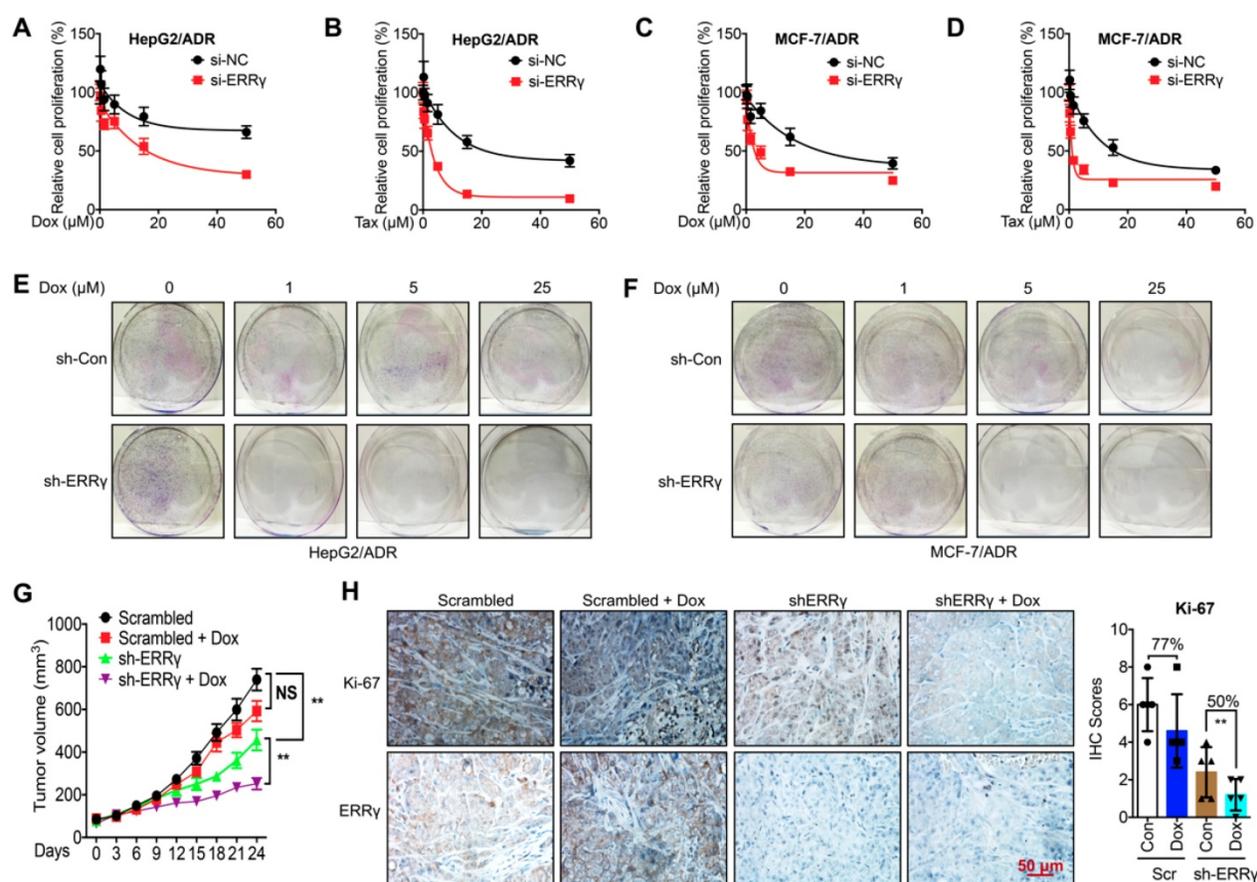
transporters, including *ABCA1*, *ABCB1*, *ABCC1*, *ABCC2*, *ABCC3* and *ABCG2*, was assessed in chemoresistant cancer cells transfected with si-ERR $\gamma$ . qRT-PCR showed that si-ERR $\gamma$  significantly decreased the expression of *ABCB1*, but not others, in both HepG2/ADR (Figure 3A) and MCF-7/ADR (Figure 3B) cells. Both si-ERR $\gamma$ -1 and si-ERR $\gamma$ -2 decreased *ABCB1* mRNA levels in MCF-7/Tax and MDA-MB-231/Tax cells (Figure S3A). Decreased protein expression of P-gp (encoded by *ABCB1*) was observed in both HepG2/ADR and MCF-7/ADR cells transfected with si-ERR $\gamma$ -1 and si-ERR $\gamma$ -2 (Figure 3C), while overexpression of ERR $\gamma$  increased the expression of P-gp in both HepG2 and MCF-7 cells (Figure 3D).

Although P-gp is known to mediate tumor cell chemoresistance [6], its role in ERR $\gamma$ -regulated chemosensitivity was further investigated. Our data confirmed that si-ERR $\gamma$  increased cellular accumulation of Rh123, a well-known fluorescent

P-gp substrate, in both HepG2/ADR and MCF-7/ADR cells (Figure 3E). Overexpression of P-gp (Figure S3B) restored si-ERR $\gamma$ -induced upregulation of Dox sensitivity in both HepG2/ADR and MCF-7/ADR cells (Figure 3F). These data indicated that P-gp is indeed involved in ERR $\gamma$ -regulated chemoresistance of cancer cells.

### ERR $\gamma$ interacts with p65 to regulate the transcription of *ABCB1*

The mechanism responsible for ERR $\gamma$ -regulated transcription of *ABCB1* was further investigated. Computer-assisted searches of potential ERR $\gamma$ -binding sites (ERR response element, ERRE, TNAAGGTCA) within the *ABCB1* promoter region (-1 kb) was conducted by using the TESS database, which predicts transcription factor-binding sites. Two putative ERREs located at -454 and -256 bp upstream of the transcription start site of the *ABCB1* promoter were identified (Figure 4A). ChIP-PCR confirmed that



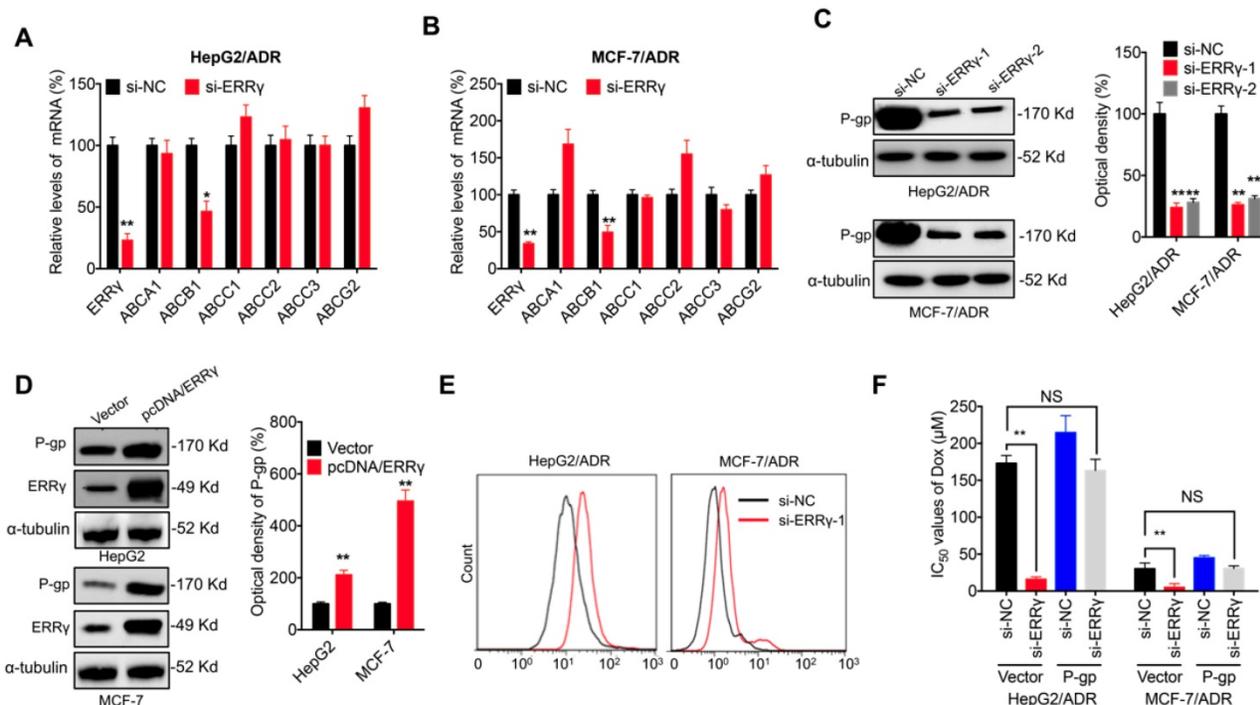
**Figure 2. ERR $\gamma$  regulates chemoresistance of cancer cells.** (A&B) Cell proliferation rate in si-NC- or si-ERR $\gamma$ -1-transfected HepG2/ADR cells for 24 h and followed by treatment with increasing concentrations of Dox (A) or Tax (B) for 48 h; (C&D) Cell proliferation rate in si-NC- or si-ERR $\gamma$ -1-transfected MCF-7/ADR cells for 24 h and followed by treatment with increasing concentrations of Dox (C) or Tax (D) for 48 h; (E&F) HepG2/ADR (E) or MCF-7/ADR (F) cells transfected with scrambled shRNA or sh-ERR $\gamma$  were split and cultured in fresh medium for the next 15 days. The colonies were fixed with methanol/glacial acetic acid (7:1) and stained with 0.1% of crystal violet; (G) Tumor volume measurement in mouse xenografts. HepG2/ADR cells stably transfected with scrambled shRNA or sh-ERR $\gamma$  were subcutaneously inoculated in nude mice. We randomly divided the mice into Scramble, sh-ERR $\gamma$ , Dox + Scramble and Dox + sh-ERR $\gamma$  groups and treated them as described in the Methods. Tumor growth curves were constructed based on the tumor volumes measured in the mice; (H) IHC analysis of mouse xenograft tissues. Expression of ERR $\gamma$  and proliferation marker Ki-67 was determined in tumor tissue sections from the xenografts using IHC (scale bar, 50  $\mu$ m) and quantitatively analyzed; Data were presented as means  $\pm$  SD from three independent experiments. \*\* $p$  < 0.01. NS, no significant.

ERR $\gamma$  binds to these two ERREs (Figure 4B). Next, an *ABCB1* promoter (-1024 to -1)-driven luciferase reporter assay was conducted with reporters containing wild-type (WT) or mutated (Mut) ERR $\gamma$ -binding sites (Figure 4A and 4C). As shown in Figure 4D, ERR $\gamma$  induced robust luciferase expression in pGL3-*ABCB1*-WT, whereas mutation at ERRE1 and/or ERRE2 significantly decreased ERR $\gamma$ -induced luciferase activity, suggesting that ERR $\gamma$  binds directly to the *ABCB1* promoter to regulate its transcription.

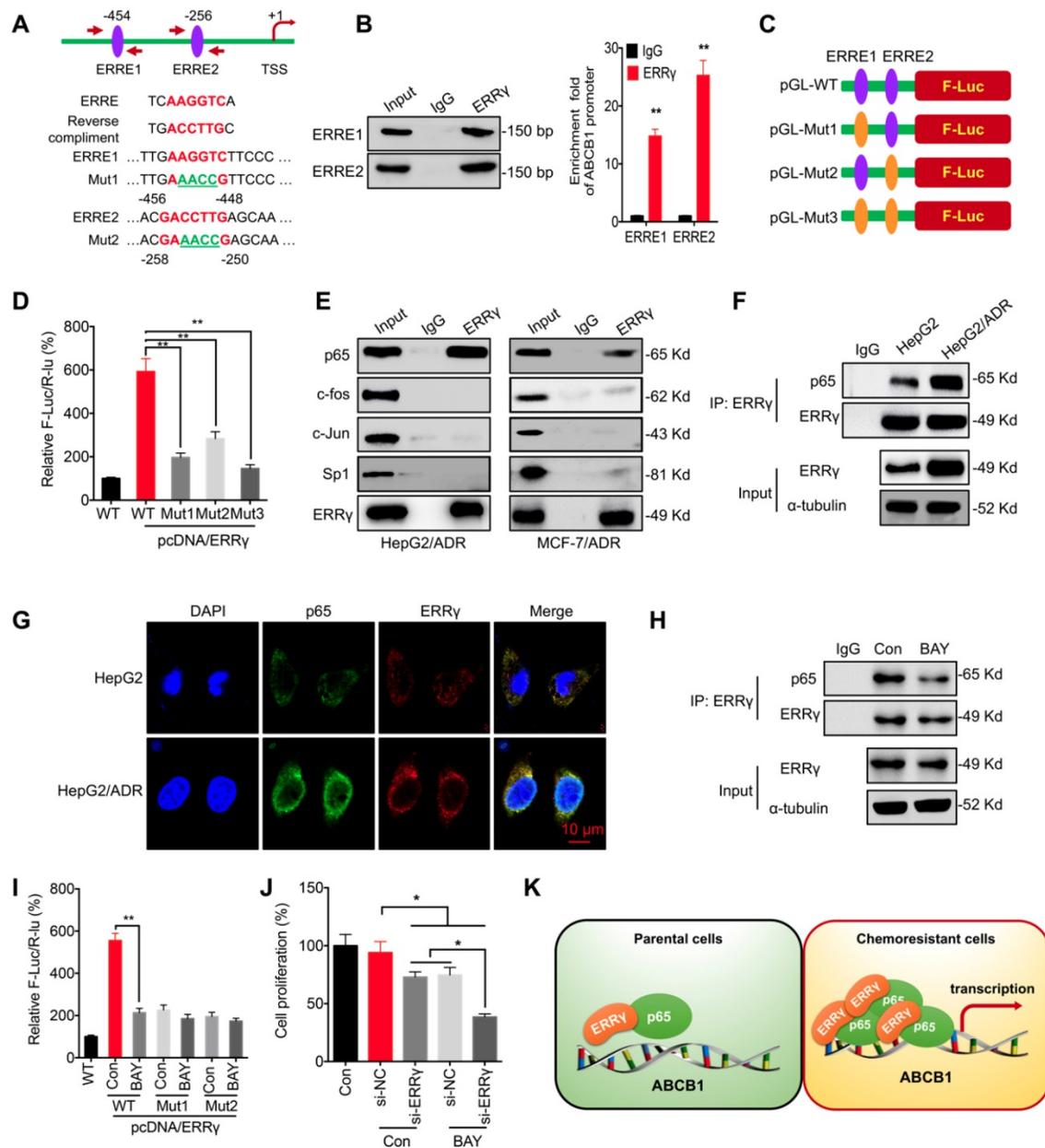
ERR $\gamma$  can form homodimers and heterodimers via its ligand-binding domain (LBD) [21, 22]. We hypothesized that transcription factors regulating *ABCB1* expression, including c-Jun, c-Fos, NF- $\kappa$ B/p65, and Sp1 [23, 24], might interact with ERR $\gamma$  to increase its activity. Co-IP showed that ERR $\gamma$  associated with endogenous p65, but not the other transcription factors, in HepG2/ADR and MCF-7/ADR cells (Figure 4E). Consistently, reciprocal co-IP showed that ERR $\gamma$  was pulled down in HepG2/ADR and MCF-7/ADR cell lysates by anti-p65 antibody (Figure S4A). To compare the binding between ERR $\gamma$  and p65 in chemoresistant and parental cells, an equal amount of ERR $\gamma$  after immunoprecipitation by use of its antibody was loaded for normalization according to a pre-Western blot since the endogenous ERR $\gamma$  was increased in

HepG2/ADR cells. The data showed that the binding between ERR $\gamma$  and p65 was increased in HepG2/ADR cells as compared with that in HepG2 cells (Figure 4F). Confocal imaging showed enhanced expression of ERR $\gamma$  in HepG2/ADR cells and colocalized with p65 in both HepG2 and HepG2/ADR cells (Figure 4G). These results suggest that the interaction between ERR $\gamma$  and p65 was upregulated in chemoresistant cells.

We further investigated whether p65 was involved in ERR $\gamma$ -regulated transcription of *ABCB1*. An inhibitor of NF- $\kappa$ B, BAY 11-7082, suppressed the mRNA (Figure S4B) and protein (Figure S4C) expression of P-gp in HepG2/ADR and MCF-7/ADR cells. BAY 11-7082 also decreased the interaction between p65 and ERR $\gamma$  in HepG2/ADR cells (Figure 4H). Moreover, BAY 11-7082 could decrease the promoter activity of pGL3-*ABCB1*-WT, while had no significant effect on the relative F-Luc/R-Luc for pGL-*ABCB1*-Mut-1/2 (Figure 4I), suggesting that ERR $\gamma$  was involved in p65-regulated transcription of *ABCB1*. Further, our data showed that BAY 11-7082 can significantly elevate the si-ERR $\gamma$ -increased Dox sensitivity of HepG2/ADR cells (Figure 4J). Our data suggest that ERR $\gamma$  can interact with p65 to promote *ABCB1* transcription in chemoresistant cells (Figure 4K).



**Figure 3. P-gp is involved in ERR $\gamma$ -regulated chemoresistance of cancer cells.** (A&B) mRNA expression of ABC transporters measured in HepG2/ADR (A) or MCF-7/ADR (B) cells 24 h post transfection with si-NC or si-ERR $\gamma$ -1; (C) Expression of P-gp protein measured by Western blot analysis (left) and quantitatively analyzed (right) in HepG2/ADR or MCF-7/ADR cells 24 h post transfection with si-NC or si-ERR $\gamma$ -1/2; (D) Expression of P-gp protein measured by Western blot analysis (left) and quantitatively analyzed (right) in HepG2 or MCF-7 cells 24 h post transfection with vector control or pcDNA/ERR $\gamma$ ; (E) P-gp function analyzed by flow cytometric measurement of the intracellular accumulation of Rh123 in HepG2/ADR or MCF-7/ADR cells 24 h post transfection with scrambled siRNA or si-ERR $\gamma$ -1; (F) IC<sub>50</sub> values of Dox in HepG2/ADR or MCF-7/ADR cells co-transfected with si-ERR $\gamma$  and P-gp construct. Data were presented as means  $\pm$  SD from three independent experiments. \* $p$  < 0.05, \*\* $p$  < 0.01. NS, no significant.



**Figure 4. ERRγ interacts with p65 to regulate ABCB1 transcription.** (A) Schematic representation of ERREs in the promoter region of ABCB1 with changes of nucleotides in ERRE1 and ERRE2 shown as indicated; (B) ChIP-PCR assay showing ERRγ binding to ERRE1 and ERRE2 in ABCB1 promoter. The input (5%), binding between ERRγ and the promoter of ABCB1 at the potential binding site ERRE1/2, was amplified by qPCR (right) and confirmed by 2% agarose gel electrophoresis (left); (C) Schematic representation of mutated ERRE positions in pGL-ABCB1 vector; (D) Reporter gene assay performed in HepG2 cells 24 h post transfection with pGL-ABCB1-WT or pGL-ABCB1-Mut1/2/3 by dual-luciferase analysis; (E) Examination of ERRγ interaction with different transcription factors in HepG2/ADR and MCF-7/ADR cells following immunoprecipitation with ERRγ or control antibody and analyzed by Western blot analysis; (F) Interaction between ERRγ and p65 in HepG2 and HepG2/ADR cells monitored by immunoprecipitation using anti-ERRγ antibody; After ERRγ was immunoprecipitated, the binding between ERRγ and p65 was examined by Western blot analysis. An equal amount of ERRγ was loaded for normalization according to a pre-Western blot; (G) Expression and localization of p65 (green) and ERRγ (red) in HepG2 and HepG2/ADR cells visualized by confocal imaging; (H) Interaction between ERRγ and p65 in HepG2/ADR cells treated with or without BAY 11-7082 for 12 h and then analyzed by immunoprecipitation using an antibody against ERRγ; (I) Dual-luciferase reporter gene assay performed in HepG2 cells transfected with pGL-ABCB1-WT or pGL-ABCB1-Mut1/2, with or without pcDNA/ERRγ, for 12 h and then further treated with or without BAY 11-7082 for 12 h; (J) HepG2/ADR cells were treated with si-RNA or si-ERRγ combined with or without BAY 11-7082 for 12 h and then further treated with 5 μM Dox for 48 h; (K) Model for ERRγ/p65-promoted transcription of ABCB1 in chemoresistant cancer cells. Data were presented as means ± SD from three independent experiments. \*\*p < 0.01. NS, no significant.

### ERRγ dictates metabolic reprogramming in chemoresistant cancer cells

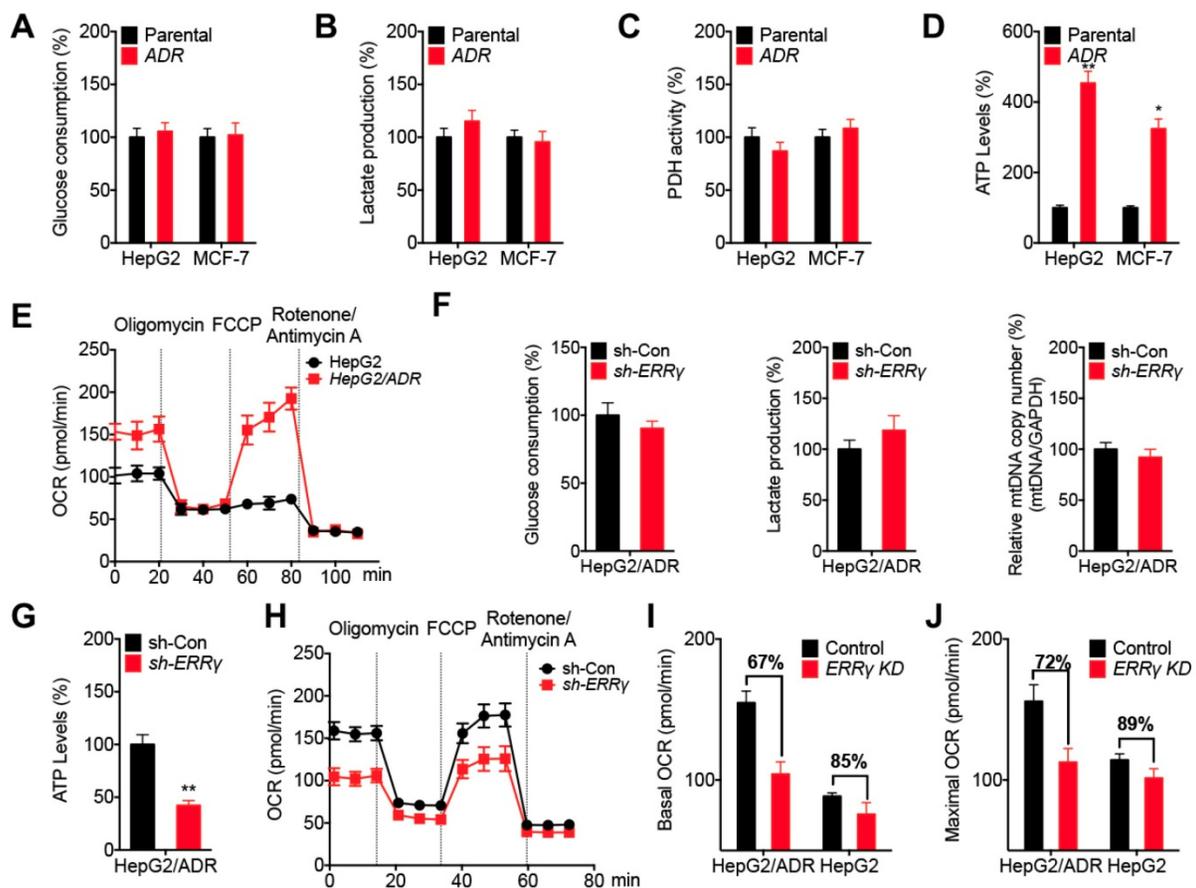
Chemoresistant cancer cells diverge metabolic properties such as aerobic glycolysis and mitochondrial respiration [25, 26]. Our data showed that HepG2/ADR and MCF-7/ADR cells exhibited no significant difference in glucose consumption (Figure

5A) and lactate production (Figure 5B) rates compared to that of their corresponding parental cells. Further, the activity of pyruvate dehydrogenase (PDH), which converts pyruvate to acetyl-CoA (Ac-CoA) and then enters the TCA cycle [27], did not vary between chemoresistant and parental cells (Figure 5C). However, the extracellular ATP levels in chemoresistant cells were significantly greater than

that in the parental cells (Figure 5D). Seahorse analysis showed that HepG2/ADR cells displayed an increased basal and maximal oxygen consumption rate (OCR), an indicator of mitochondrial oxidative respiration (Figure 5E), but had comparable levels of the extracellular acidification rate (ECAR), which reflects the overall glycolytic flux (Figure S5A), than that of HepG2 cells. However, the mitochondrial mass between HepG2/ADR and HepG2 cells had no significant difference (Figure S5B). These data suggested that chemoresistant cells showed increased ATP production and OCR than that of parental cells.

We further investigated the potential roles of ERR $\gamma$  in metabolic programming of chemoresistant cancer cells. Our data showed that knockdown of ERR $\gamma$  had no significant effect on glucose consumption, lactate production, or mitochondrial mass in HepG2/ADR cells (Figure 5F). However, knockdown of ERR $\gamma$  decreased the ATP levels of HepG2/ADR cells (Figure 5G) as well as the basal and maximum OCRs (Figure 5H), but had no significant

effect on ECAR (Figure S5C) in HepG2/ADR cells. This was further confirmed by overexpression of ERR $\gamma$  resulting in increased basal and maximum OCRs without significantly altering ECAR in HepG2 cells (Figure S5D and S5E). Moreover, knockdown of ERR $\gamma$  more effectively decreased the basal (Figure 5I) and maximum (Figure 5J) OCRs of HepG2/ADR cells than that in HepG2 cells. Both overexpression of ERR $\gamma$  in HepG2 cells (Figure S5F) and knockdown of ERR $\gamma$  in HepG2/ADR cells (Figure S5G) had no significant effect on key gene expression involved in oxidative phosphorylation (OxPhos) such as *CS*, *NDUFA4*, *SDHB*, *COX5B*, or *ATP5B*, suggesting that ERR $\gamma$ -upregulated OCR and ATP production were not related to the OxPhos pathway. All the data indicated that ERR $\gamma$  dictates metabolic reprogramming in chemoresistant cancer cells without affecting glycolysis, mitochondrial mass, and PDH activities, but it does increase ATP generation and OCR.



**Figure 5. ERR $\gamma$  dictates the metabolic reprogramming in chemoresistant cancer cells.** (A–D) Relative glucose consumption (A), lactate production (B), PDH activity (C), and ATP levels (D) measured in HepG2/ADR or MCF-7/ADR cells following comparison with those measured in their corresponding parental cells; (E) OCR in HepG2/ADR and HepG2 cells measured by Seahorse XF24 analyzer; (F) Relative glucose consumption, lactate production, and mitochondrial mass in HepG2/ADR cells transfected with sh-Con or sh-ERR $\gamma$ ; (G) Relative ATP levels in HepG2/ADR cells transfected with sh-Con or sh-ERR $\gamma$ ; (H) OCR in HepG2/ADR cells transfected with sh-Con or sh-ERR $\gamma$  measured by Seahorse XF24 analyzer; (I–J) Basal (I) and maximal (J) OCR measured by Seahorse XF24 analyzer in HepG2/ADR or HepG2 cells transfected with sh-Con or sh-ERR $\gamma$ . Data were presented as means  $\pm$  SD from three independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$ .

## ERR $\gamma$ regulates FAO via Cpt1b in chemoresistant cancer cells

A recent study indicates that ERR $\gamma$  in kidney cells can regulate mitochondrial FAO functions via direct binding to FAO genes such as *CPT1B*, *CPT2*, *ACADM*, and *HADHA* [28]. We then investigated the potential effects of ERR $\gamma$  on mitochondrial FAO functions in cancer cells. We found the FA uptake (Figure 6A) and FAO rate (Figure 6B) in HepG2/ADR cells were significantly greater than that in HepG2 cells. Knockdown of ERR $\gamma$  significantly inhibited the FA uptake (Figure 6C) and FAO rate (Figure 6D) in both HepG2/ADR and MCF-7/ADR cells. Further, overexpression of ERR $\gamma$  decreased Dox sensitivity of HepG2 cells, while this effect could be blocked by the FAO inhibitor etomoxir (ETO) (Figure 6E), suggesting that upregulation of FAO was involved in ERR $\gamma$ -induced chemoresistance of cancer cells.

To investigate the mechanisms of ERR $\gamma$ -regulated FAO in cancer cells, qRT-PCR was performed to evaluate the expression of genes involved in cellular FAO (Table S1) [29]. Among all of the FAO-related genes examined, expression of *CPT1B* was significantly increased in HepG2/ADR cells as compared with that in HepG2 cells (Figure 6F). Knockdown performed with sh-ERR $\gamma$  significantly decreased *CPT1B* expression in HepG2/ADR cells (Figure 6G). Consistently, overexpression of ERR $\gamma$  increased the expression of *CPT1B* in HepG2 cells (Figure S6A), while both si-ERR $\gamma$ -1 and si-ERR $\gamma$ -2 significantly decreased the protein levels of Cpt1b in HepG2/ADR cells (Figure S6B). To investigate whether Cpt1b was involved in ERR $\gamma$ -regulated FAO and chemoresistance, we overexpressed Cpt1b in HepG2/ADR cells transfected with sh-Con or sh-ERR $\gamma$  (Figure S6C). Our data showed that overexpression of Cpt1b significantly attenuated sh-ERR $\gamma$ -increased sensitivity of Dox in HepG2/ADR cells (Figure 6H), and also sh-ERR $\gamma$ -downregulated ATP levels (Figure 6I) and FAO rate (Figure 6J), suggesting that Cpt1b is involved in ERR $\gamma$ -regulated FAO and chemoresistance of cancer cells.

Cpt1b has been indicated as a direct transcriptional target of ERR $\gamma$  [28], but the binding site(s) in the *CPT1B* promoter has not been well studied. Analysis of the region 1.0 kb upstream from the transcription start site in the *CPT1B* promoter revealed one putative ERRE, showing 78% homology (7/9) to the consensus ERRE (Figure 6K). ChIP assay confirmed that binding of ERR $\gamma$  to *CPT1B* in HepG2/ADR cells was greater than that in HepG2 cells (Figure 6L). We then cloned the promoter of *CPT1B* to generate pGL3-*CPT1B*-WT-Luc and

mutated the bind site (to GAAACCG) to generate pGL3-*CPT1B*-Mut-Luc (Figure 6K). The promoter activity of pGL3-*CPT1B*-WT-Luc in HepG2/ADR cells was significantly greater than that in HepG2 cells; however, pGL3-*CPT1B*-Mut-Luc attenuated this difference between HepG2/ADR and HepG2 cells (Figure 6M). In general, the promoter activity of pGL3-*CPT1B*-WT-Luc was greater than that of pGL3-*CPT1B*-Mut-Luc in both HepG2/ADR and HepG2 cells (Figure 6M).

We further investigated whether p65/ERR $\gamma$  complex was involved in the upregulation of Cpt1b in chemoresistant cells. BAY 11-7082, the inhibitor of p65/NF- $\kappa$ B, suppressed the mRNA (Figure S6D) and protein (Figure S6E) levels of Cpt1b in both HepG2/ADR and MCF-7/ADR cells and also significantly decreased ERR $\gamma$ -induced promoter activity of pGL3-*CPT1B*-WT-Luc, while it had less effect on the activity of pGL3-*CPT1B*-Mut-Luc in HepG2 cells (Figure 6N). Our data suggest that ERR $\gamma$  forms a complex with p65, binds *CPT1B* promoter to increase its expression, elevates FAO, and thus mediate chemoresistance of cancer cells (Figure 6O).

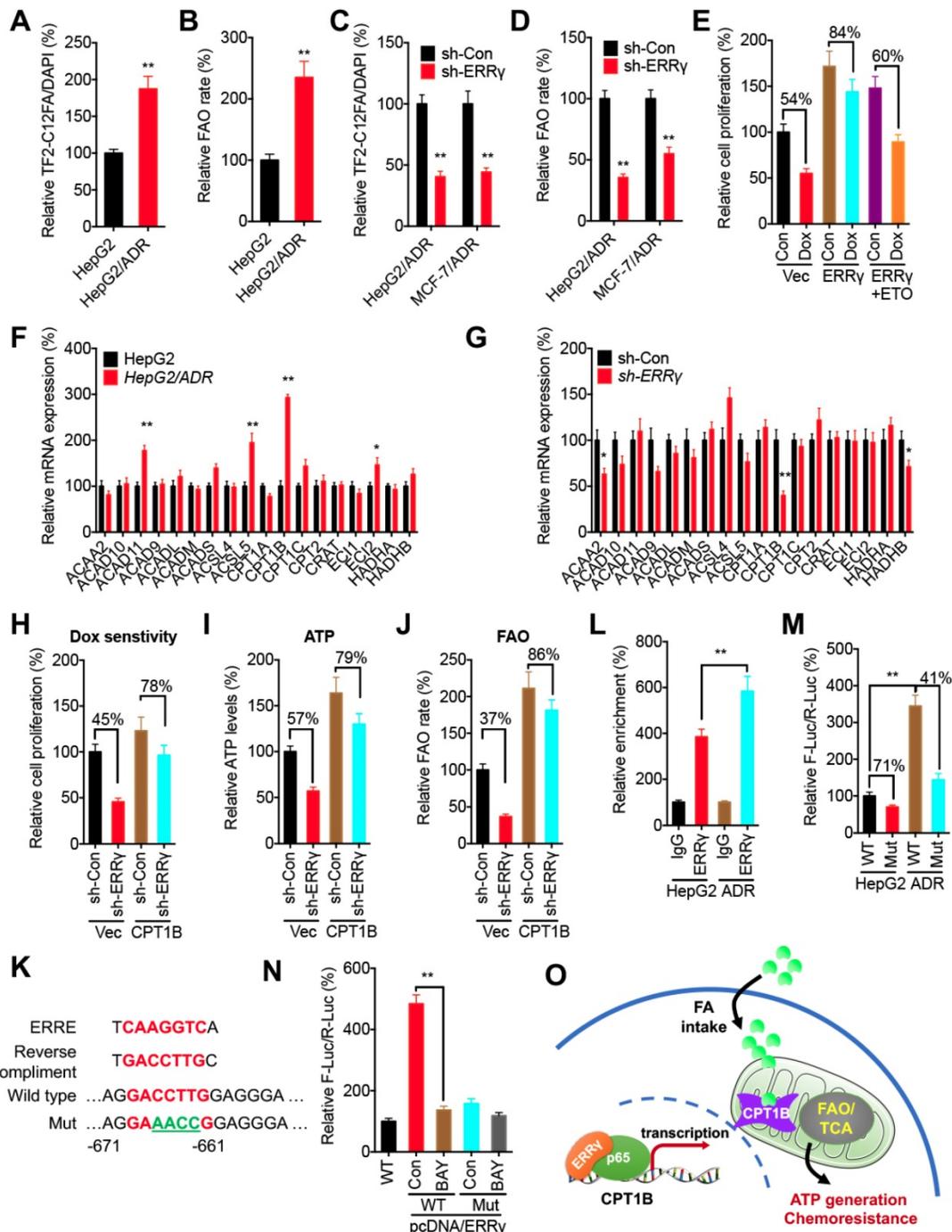
## The m<sup>6</sup>A-facilitated splicing increases the expression of ERR $\gamma$

The potential epigenetic mechanisms responsible for the upregulation of ERR $\gamma$  in chemoresistant cells were investigated. Firstly, treatment with 5-aza-dC (a DNA methyltransferase inhibitor) had no significant effect on ERR $\gamma$  expression in either HepG2 or HepG2/ADR cells (Figure S7 A), suggesting that DNA methylation might not be involved in ERR $\gamma$  expression. Further, broad-spectrum HDAC inhibitors including SAHA and NaB also had no obvious effect on the expression of ERR $\gamma$  in HepG2 or HepG2/ADR cells (Figure S7B), indicating histone acetylation might not be responsible for the upregulation of ERR $\gamma$  in chemoresistant cells.

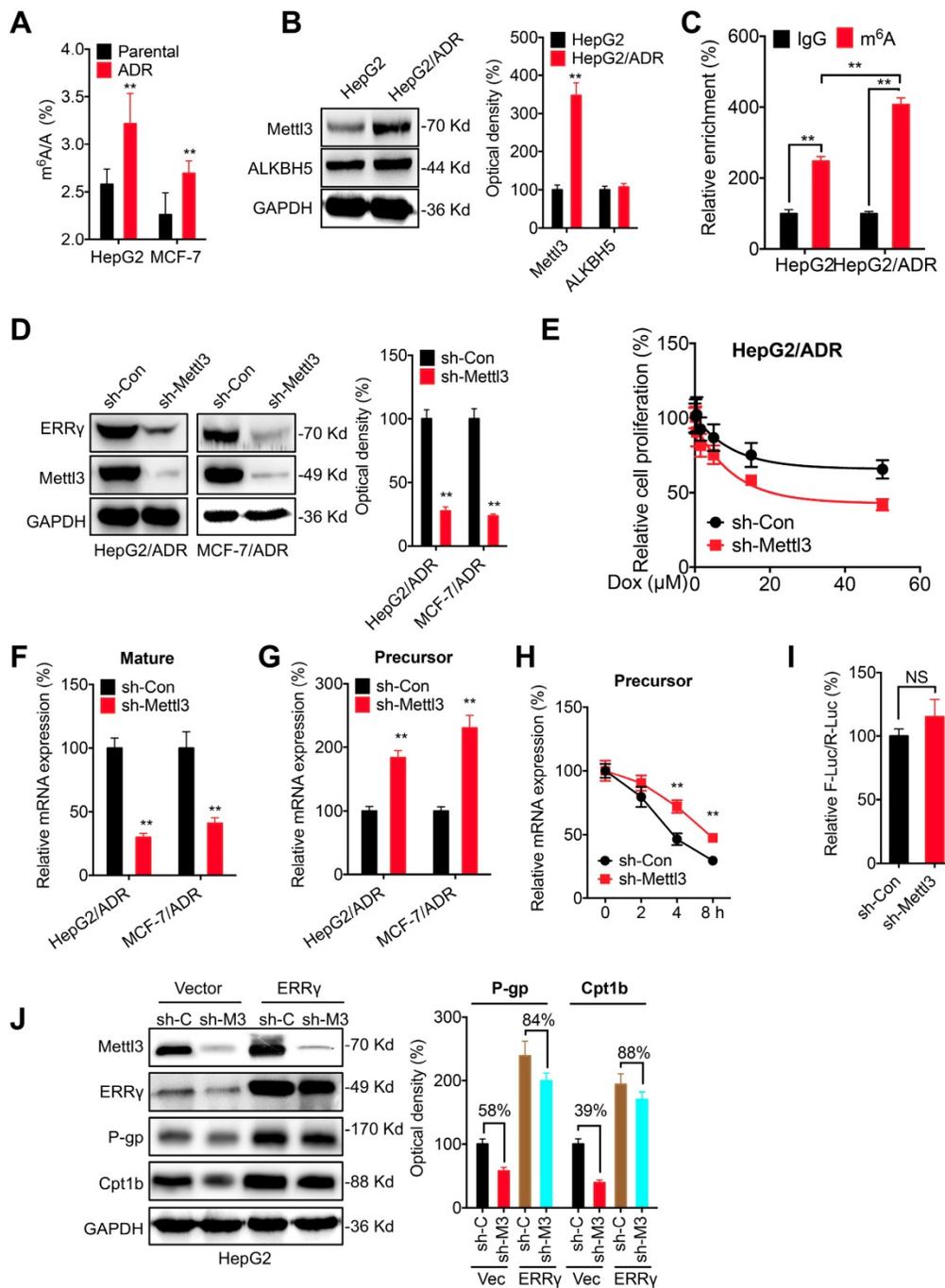
Recent investigations indicated that N<sup>6</sup>-methyladenosine (m<sup>6</sup>A) modification can regulate gene expression and be involved in chemoresistance of cancer cells [30, 31]. Intriguingly, we found that the m<sup>6</sup>A of mRNA was increased in HepG2/ADR and MCF/ADR cells as compared to their parental cells (Figure 7 A). Western blot analysis showed that the expression of m<sup>6</sup>A methyltransferase Mettl3 was upregulated in HepG2/ADR cells, while the expression of demethylase ALKBH5 had no variation (Figure 7 B). m<sup>6</sup>A-RIP-qPCR confirmed that a 2-fold m<sup>6</sup>A antibody enriched *ESRRG* mRNA in HepG2 cells, while this enrichment was significantly increased in HepG2/ADR cells (Figure 7 C). We found that knockdown of Mettl3 can decrease the expression of ERR $\gamma$  in both HepG2/ADR and

MCF-7/ADR cells (Figure 7 D). Consistently, over expression of *Mettl3* increased the expression of *ERRγ* in HepG2 cells (Figure S7 C). Further, knockdown of *Mettl3* can significantly increase the Dox sensitivity

of HepG2/ADR cells (Figure 7 E). It indicated that m<sup>6</sup>A can increase the expression of *ERRγ* in cancer cells.



**Figure 6.** *ERRγ* regulates the FAO via *Cpt1b* in chemoresistant cancer cells. (A–B) Relative FA uptake (A) and FA β oxidation rate (B) between HepG2 and HepG2/ADR cells; (C–D) Relative FA uptake (C) and FA β oxidation rate (D) in cells transfected with sh-Con or sh-*ERRγ*; (E) Cell proliferation measured by CCK-8 kit in HepG2 cells pre-transfected with vector control or pcDNA/*ERRγ* for 6 h and then treated with or without Dox (1 μM) combined with or without ETO for 24 h; (F) mRNA levels of FAO-related genes measured by qRT-PCR in HepG2 and HepG2/ADR cells; (G) mRNA levels of FAO-related genes measured by qRT-PCR in HepG2/ADR cells transfected with sh-Con or sh-*ERRγ*; (H) Cell proliferation measured by CCK-8 kit in HepG2/ADR cells pre-transfected with sh-Con or sh-*ERRγ* and then transfected with vector or a *Cpt1b* expression construct, followed by further treatment with Dox (10 μM) for 24 h; (I–J) ATP (I) and FA β oxidation rate (J) measured in HepG2/ADR cells transfected with sh-Con or sh-*ERRγ* with further transfection with vector or a *Cpt1b* expression construct for 24 h; (K) Nucleotide sequences of ERREs in *CPT1B* and the mutated (GACCTTG to AGAACCG) nucleotides in pGL3-*CPT1B*-Mut-Luc vector; (L) ChIP assay measuring *ERRγ* binding to *CPT1B* promoter in both HepG2 and HepG2/ADR cells; (M) Dual-luciferase reporter gene assay performed in HepG2 and HepG2/ADR cells transfected with pGL3-*CPT1B*-WT-Luc or pGL3-*CPT1B*-Mut-Luc; (N) Dual-luciferase reporter gene assay performed in HepG2 cells transfected with pGL-*ABC1*-WT or pGL3-*CPT1B*-Mut reporter with or without pcDNA/*ERRγ* for 12 h and then further treated with or without BAY 11-7082 for 12 h; (O) Model for *ERRγ*-regulated FAO via *Cpt1b* in chemoresistant cancer cells. Data were presented as means ± SD from three independent experiments. \*p < 0.05.



**Figure 7. The m<sup>6</sup>A-facilitated splicing is responsible for the upregulation of ERR $\gamma$ .** (A) The m<sup>6</sup>A/A ratio of total mRNA in HepG2/ADR and MCF-7/ADR cells were determined by LC-MS/MS and compared with that in their parental cells; (B) The expression of Mettl3 and ALKBH5 in HepG2/ADR and HepG2 cells was checked by western blot analysis and quantitatively analyzed; (C) m<sup>6</sup>A RIP-qPCR analysis of ERR $\gamma$  mRNA in HepG2/ADR and HepG2 cells; (D) The expression of ERR $\gamma$  in HepG2/ADR and MCF-7/ADR cells transfected with sh-Con or sh-Mettl3 was checked by western blot analysis and quantitatively analyzed; (E) HepG2/ADR cells transfected with sh-Con or sh-Mettl3 were further treated with increasing concentrations of Dox, the cell proliferation was tested by CCK-8 kit; (F-G) The mature (F) and precursor (G) mRNA of ERR $\gamma$  in HepG2/ADR cells transfected with sh-Con or sh-Mettl3 were checked by qRT-PCR; (H) HepG2/ADR cells transfected with sh-Con or sh-Mettl3 were pre-treated with Act-D for 90 min, then the precursor mRNA of ERR $\gamma$  was checked by qRT-PCR; (I) The promoter activity of pGL-ESRRG-Basic in HepG2/ADR cells transfected with sh-Con or sh-Mettl3 was checked by dual luciferase assay; (J) HepG2 cells were transfected with sh-Con, sh-Mettl3, vector control or ERR $\gamma$  construct alone or together for 24 h, the expression of targets was measured and quantitatively analyzed. Data were presented as means  $\pm$  SD from three independent experiments. \*\**p* < 0.01. NS, no significant.

We then investigated the potential mechanisms responsible for m<sup>6</sup>A regulated expression of ERR $\gamma$ . Firstly, knockdown of Mettl3 had no significant effect on the protein stability of ERR $\gamma$  in HepG2/ADR cells (Figure S7 D). However, knockdown of Mettl3 can significantly decrease mature mRNA of ERR $\gamma$  (Figure

7 F). Consistently, over expression of Mettl3 can increase the mRNA expression of ERR $\gamma$  in both HepG2 and MCF-7 cells (Figure S7 E). The Mettl3 induced upregulation might not be due to the nuclear turnover or mRNA degradation since knockdown of Mettl3 had no significant effect on either the

subcellular localization of mature mRNA (Figure S7 F) or the mRNA stability (Figure S7 G). Intriguingly, we found that knockdown of *Mettl3* can increase the precursor mRNA of *ERRγ* (Figure 7 G), which might be due to that knockdown of *Mettl3* can increase the half-life of precursor mRNA (Figure 7 H). It suggested that the decrease of m<sup>6</sup>A can delay the splicing of precursor mRNA of *ERRγ* to suppress its expression. This was confirmed by the promoter activity assay which showed that knockdown of *Mettl3* had no significant effect on the pGL-*ESRRG*-Basic (Figure 7 I), suggesting that m<sup>6</sup>A had no effect on the transcription of *ERRγ*. Further, we found that in HepG2 cells, knockdown of *Mettl3* resulted in the down regulation of P-gp and *Cpt1b*, however, over expression of *ERRγ* can significantly attenuate knockdown of *Mettl3* induced down regulation of P-gp and *Cpt1b* (Figure 7 J). Collectively, these data suggested that m<sup>6</sup>A can trigger the splicing of *ERRγ* precursor mRNA and then regulate the phenotype of chemoresistance.

### The m<sup>6</sup>A/*ERRγ* axis and *in vivo* cancer progression

To define the molecular basis of the *ERRγ* in cancer progression, we checked the expression of *Mettl3* and *ERRγ* in xenografts based on HepG2 and HepG2/ADR cells. Our data showed that when the tumor volume was comparable, the expression of *ERRγ* and *Mettl3* was increased in HepG2/ADR groups as compared to that in parental cells (Figure 8 A). We further checked the *in vivo* effects of sh-*ERRγ* on the expression of P-gp and *Cpt1b* in chemoresistance cells. Our data showed that knockdown of *ERRγ* can further decrease the expression of P-gp and *Cpt1b* in HepG2/ADR xenograft model (Figure 8 B). These data indicated that m<sup>6</sup>A/*ERRγ*/P-gp-*Cpt1b* axis was involved in the *in vivo* progression and chemoresistance of cancer cells. Further, we established mouse xenograft tumors by using sh-*ERRγ*-transfected HepG2/ADR cells. Then the xenograft tumors were then treated with Dox combined with elacridar (P-gp inhibitor) [32] or etomoxir (*Cpt1* inhibitor) [8], our data showed that the combination of elacridar or etomoxir can increase the *in vivo* Dox sensitivity of HepG2/Dox cells. Further, the combination of elacridar and etomoxir had synergistic effects to increase the sh-*ERRγ*-restored *in vivo* Dox sensitivity of HepG2/Dox cells (Figure 8C). It confirmed that *ERRγ* can regulate the *in vivo* chemoresistance via modulation of P-gp and *Cpt1b*.

We then analyzed the expression of m<sup>6</sup>A/*ERRγ* axis and their correlation with clinical characteristics of breast and liver cancers. Increased expression of *ERRγ* in liver cancer versus normal tissue has been

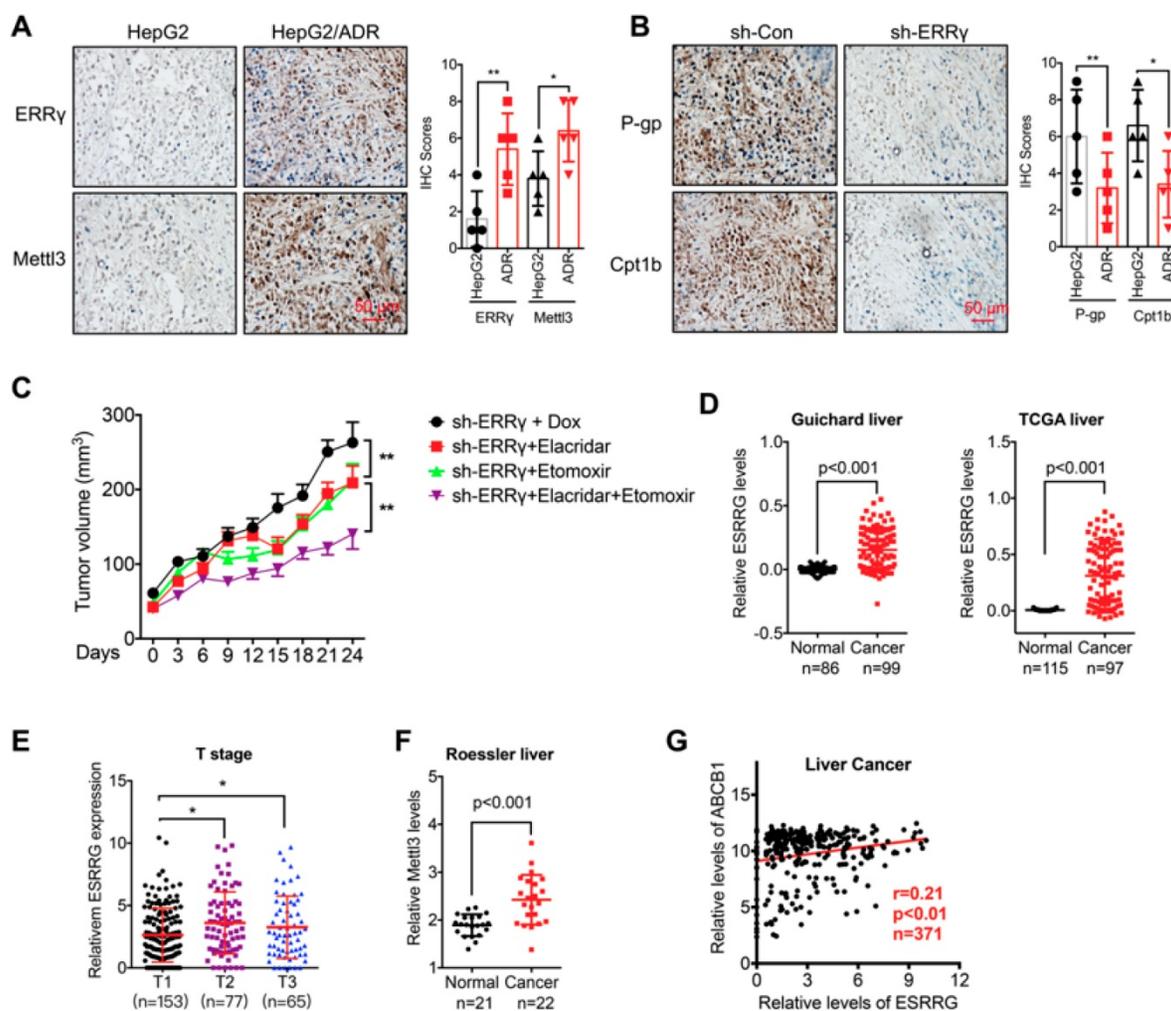
observed in Guichard and TCGA data from Oncomine database (Figure 8D). Consistently, increased expression of *ERRγ* was also observed in breast cancer versus normal tissues in Finak data from Oncomine database (Figure S8 A). Further, significant rising expression levels of *ESRRG* from T1 to T3 stage of liver cancer tissues were observed (Figure 8 E), implying an increasing tendency of *ESRRG* expression during malignant transformation.

In addition, increased expression of *Mettl3* has also be found in Roessler liver cancer (Figure 8 F). Further, we assessed the correlation between *ERRγ* and P-gp in cancer patients with data downloaded from LinkedOmics (<http://www.linkedomics.org>). Our data showed that the expression of *ERRγ* was positively correlated with the *ABCB1* in liver (Figure 8 G) and breast (Figure S8 B) cancer patients. All these data suggested that *ERRγ* regulated chemoresistance and metabolic reprogramming might be involved in the *in vivo* cancer progression.

### Discussion

Previous studies indicated that ERR signaling can regulate the progression of various cancers [19, 33, 34]. Our present study found that *ERRγ* was upregulated in chemoresistant cancer cells and targeted inhibition of *ERRγ* restored the chemosensitivity. Mechanistically, *ERRγ* can interact with p65 and bind to the promoter of *ABCB1*, which encodes a key transporter that pumps many foreign substances out of cells, to increase its transcription and expression. At the same time, *ERRγ* can facilitate FAO of chemoresistant cancer cells via upregulation of *Cpt1b*. Intriguingly, chemoresistant cells showed increased levels of m<sup>6</sup>A and expression of *Mettl3* than that of parental cells, which can trigger the splicing of precursor of *ESRRG* mRNA to increase its expression. Collectively, we found that m<sup>6</sup>A-induced *ERRγ* is essential for chemoresistance of cancer cells through upregulation of *ABCB1* and metabolic reprogramming.

Since *ERRγ* is upregulated in resistant cells, targeted inhibition of *ERRγ* can increase *in vitro* and *in vivo* sensitivity to chemotherapy. In hormone therapy of breast cancer cells, *ERRγ* is upregulated during the acquisition of TAM resistance in estrogen receptor-positive (ER+) breast cancer cells, thus overexpression of *ERRγ* is sufficient to induce TAM resistance [18] via cooperation with proline, glutamic acid and leucine rich protein 1 (PELP1) to inhibit TAM-mediated cell death [35]. *ERRγ* target genes are poor prognostic factors in TAM-treated breast cancer [36]. As to cancer progression, the role of *ERRγ* seems to be paradoxical. *ERRγ* acts as a tumor suppressor in gastric cancer by directly targeting the Wnt signaling



**Figure 8. The m<sup>6</sup>A/ ERR $\gamma$  axis and *in vivo* cancer progression.** (A) IHC (ERR $\gamma$  and Mettl3)-stained paraffin-embedded sections obtained from HepG2 and HepG2/ADR xenografts when the tumor volumes were about 100 mm<sup>3</sup> for each group; (B) The sh-control and sh-Mettl3 HepG2/ADR cells were subcutaneously inoculated in nude mice. IHC (P-gp and Cpt1b)-stained paraffin-embedded sections obtained at the end of the experiment; (C) Tumor volume measurement in mouse xenografts. HepG2/ADR cells stably transfected with sh-ERR $\gamma$  were subcutaneously inoculated in nude mice. We randomly divided the mice into sh-ERR $\gamma$ , sh-ERR $\gamma$  + Elacridar, sh-ERR $\gamma$  + Etomoxir, and sh-ERR $\gamma$  + Elacridar + Etomoxir and then treated with Dox as described in the Methods. Tumor growth curves were constructed based on the tumor volumes measured in the mice; (D) Expression of ESRRG in HCC tumor tissues and normal liver tissues from Oncomine database (Guichard and TCGA liver cancers); (E) ESRRG expression in liver cancers of T1 (n=153), T2 (n=77), and T3 (n=65) stages from TCGA database; (F) Expression of Mettl3 in HCC tumor tissues and normal liver tissues from Oncomine database (Roessler liver); (G) Correlation between ESRRG and ABCB1 in liver cancer patients (n=371) from TCGA database; Data were presented as means  $\pm$  SD from three independent experiments. \*p<0.05, \*\*p<0.01.

pathway [37]. Conversely, ERR $\gamma$  is upregulated in liver cancer and exerts oncogenic potential by suppressing *p21* and *p27* [38]. The diverse roles of ERR $\gamma$  is likely dependent on cell context and its functional interactions with cell-specific transcription factors and co-regulators. Our present study revealed that essential roles of ERR $\gamma$  in chemoresistance of cancer cells. Nowadays, GSK5182, the inverse agonist of ERR $\gamma$ , has been reported to inhibit the biological functions of ERR $\gamma$  in cardiac hypertrophy [39], iron homeostasis [40], and cancer cell proliferation [41]. The therapeutic potential of GSK5182 on cancer chemoresistance needs further investigations.

Continuing our finding that ERR $\gamma$  interacts with p65 to trigger the transcription and expression of ABCB1, we identified two ERREs in the promoter region of ABCB1 and proved that both ERREs are

involved in ERR $\gamma$ -regulated expression of P-gp. NF- $\kappa$ B appears to play a dual role in the regulation of ABCB1 [42]. It can bind to -167 and -158 of the ABCB1 promoter to activate its transcription in liver cancer cells [43]. In our study, the inhibitor (BAY) of NF- $\kappa$ B can attenuate ERR $\gamma$ -induced transcription of ABCB1, potentially attributed to BAY-abrogated NF- $\kappa$ B binding to DNA [44] and thus reducing ERR $\gamma$ /p65 association with the ABCB1 promoter. Our results also revealed an essential role of NF- $\kappa$ B in ERR $\gamma$ -induced expression of ABCB1 and chemoresistance, which is further supported by clinical data confirming that expression of ABCB1 and ERR $\gamma$  was positively correlated in liver cancer tissues.

Metabolic reprogramming is one of the hallmarks for cancer cell growth and progression, as well as resistance to chemotherapy [45]. We found

that chemoresistant cells reprogram metabolic pathways without affecting glycolysis, mitochondrial mass, and PDH activities but increasing ATP generation and OCR through acceleration of FAO. Recent evidence underscores the idea that FAO, also called  $\beta$ -oxidation, is an important energy resource required for cancer cell growth, survival, and metastasis [13, 46]. Inhibition of FAO is identified as a new therapeutic approach for MYC-overexpressing triple-negative breast cancer (TNBC) [8]. Some studies indicate that FAO is able to support breast cancer stem cell self-renewal and is a characteristic of chemoresistant cancer cells [10]. The upregulation of FAO may thus confer the chemoresistance through maximizing ATP production, decrease intracellular ROS, and eventually protect cancer cells from death [13, 47]. Our data confirmed that targeted FAO might be helpful in overcoming chemoresistance of cancer cells.

According to our data, ERR $\gamma$ -regulated Cpt1b was responsible for facilitated FAO in chemoresistant cancer cells. ERR $\gamma$  plays an important role in metabolism to promote energy-generating mitochondrial functions in several energy-demanding cell types [17]. Genomic studies revealed that ERR $\gamma$  binds directly to and activates the transcription of hundreds of genes involved in mitochondrial OxPhos/FAO functions, including *CPT1B*, *CPT2*, *ACADM*, and *HADHA* [28, 48]. Consistently, we identified the ERRE located at -671 to -661 of the *CPT1B* promoter is involved in ERR $\gamma$ -regulated transcription and expression. Cpt1b, which is responsible for fatty acid transport into mitochondria for  $\beta$ -oxidation, localizes at the outer mitochondrial membrane and acts as the rate-limiting FAO enzyme [49]. We found that overexpression of Cpt1b can reverse sh-ERR $\gamma$ -sensitized Dox treatment and downregulation of ATP and FAO, while elevated Cpt1b expression correlates with poorer response to chemotherapy [10]. Considering ERR $\gamma$  cisomes may exhibit cell-type-specific features to match the metabolic profiles of individual cell types [17], the roles of ERR $\gamma$ /Cpt1b axis-regulated metabolic reprogramming in chemoresistance of other cancers will need more studies.

Intriguingly, we found that m<sup>6</sup>A was upregulated in chemoresistant cells and facilitated the splicing of precursor of *ESRRG* mRNA to elevate its expression. The roles of mRNA modification in controlling the cancer progression have just begun to be studied. Our recent study indicated that m<sup>6</sup>A can trigger the epithelial to mesenchymal transition of cancer cells via triggering the translation of Snail [50]. In the present study, we showed that knockdown of *Mettl3* can restore the chemosensitivity of

HepG2/ADR cells, which is consistent with recent study that *Mettl3* can promote the chemo- and radioresistance of pancreatic cancer cells [51]. m<sup>6</sup>A can regulate the all stages in the life cycle of RNA including RNA processing, nuclear export and translation modulation [31, 52, 53]. It has been reported that splicing regulators and m<sup>6</sup>A “reader” HNRNPC can regulate the splicing of target mRNAs in a m<sup>6</sup>A switch regulated manner [54]. Whether HNRNPC is involved in *Mettl3* and chemoresistance triggered splicing of *ESRRG* precursor mRNA need further studies.

In conclusion, we identified a key role of ERR $\gamma$  in chemoresistance of cancer cells via upregulation of *ABCB1* and facilitation of FAO. We further uncovered novel mechanisms for ERR $\gamma$  regulated transcription of *ABCB1*, revealed that Cpt1b mediated FAO is essential for chemoresistance, and found that m<sup>6</sup>A can trigger the cleave of precursor mRNA of *ESRRG* to decrease chemosensitivity. Our results provided a potent target that may serve as a predictive marker of chemotherapy and as an effective target for overcome chemoresistance.

## Abbreviations

ABC, ATP-binding cassette; *ABCB1*, ATP binding cassette subfamily B member 1; Dox, doxorubicin; ECAR, extracellular acidification rate; ER, estrogen receptor; ERR $\gamma$ , estrogen related receptor  $\gamma$ ; ERRE, ERR response elements; ETX, etomoxir; FA, fatty acid; FAO, fatty acid oxidation; LBD, ligand-binding domain; m<sup>6</sup>A, N<sup>6</sup>-methyladenosine; MDR, multidrug resistance; TAM, tamoxifen; TCA, tricarboxylic acid cycle; TNBC, triple-negative breast cancer; OCR, oxygen consumption rate; OxPhos, oxidative phosphorylation; P-gp, P-glycoprotein; PDH, pyruvate dehydrogenase; PELP1, proline, glutamic acid and leucine rich protein 1; Tax, taxol; WT, wide-type.

## Supplementary Material

Supplementary figures, tables, materials and methods. <http://www.thno.org/v10p3382s1.pdf>

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## Consent for publication

Written informed consent for publication was obtained from all participants.

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Writing, review, and/or revision of the manuscript: Hongsheng Wang, Wei Ling He, Zhuojia Chen, Cheng-Ming Chiang.

## Competing Interests

The authors have declared that no competing interest exists.

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