



ORIGINAL RESEARCH

RNA Structural Dynamics Modulate EGFR-TKI Resistance Through Controlling *YRDC* Translation in NSCLC Cells



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Abstract Epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKIs) positively affect the initial control of **non-small cell lung cancer** (NSCLC). Rapidly acquired resistance to EGFR-TKIs is a major hurdle in successful treatment. However, the mechanisms that control the resistance of EGFR-TKIs remain largely unknown. **RNA structures** have widespread and crucial functions in many biological regulations; however, the functions of RNA structures in regulating cancer drug resistance remain unclear. Here, the psoralen analysis of RNA interactions and structures (PARIS) method is used to establish the higher-order RNA structure maps of EGFR-TKIs-resistant and -sensitive cells of NSCLC. Our results show that RNA structural regions are enriched in untranslated regions (UTRs) and correlate with translation efficiency (TE). Moreover, *yrDC* *N*⁶-*threonylcarbamoyltransferase domain containing (YRDC)* promotes resistance to EGFR-TKIs. RNA structure formation in *YRDC* 3' UTR suppresses embryonic lethal abnormal vision-like 1 (**ELAVL1**) binding, leading to EGFR-TKI sensitivity by impairing *YRDC* translation. A potential therapeutic strategy for cancer treatment is provided using antisense oligonucleotide

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(ASO) to perturb the interaction between RNA and protein. Our study reveals an unprecedented mechanism through which the RNA structure switch modulates **EGFR-TKI resistance** by controlling *YRDC* mRNA translation in an ELAVL1-dependent manner.

Introduction

Non-small cell lung cancer (NSCLC) is a primary lung cancer (LC) histological subtype, constituting 85% of all LC cases [1]. Although conventional cytotoxic chemotherapy plays a critical role in treating advanced NSCLC, the latest advancements in individualized medicine have enhanced the responsiveness of oncogenic mutation-harboring NSCLC patients to targeted treatment without inducing severe side effects. Particularly, epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKIs) have been widely studied [2], and *EGFR* genetic mutations are usually present among NSCLC cases (incidence of > 50%) [3]. NSCLC patients containing *EGFR* mutations are sensitive to the first-generation and the second-generation EGFR-TKIs [4,5]. Although disease control and clinical responsiveness rates attained with EGFR-TKIs were excellent, resistance soon developed in such cases (mean, 1 year) [6]. Among the various resistance mechanisms of EGFR-TKIs, EGFR gatekeeper threonine 790 (T790M) point mutation near the catalytic site has the highest prevalence. EGFR T790M mutation elevates adenosine-triphosphate (ATP) affinity in receptor tyrosine kinase and shows steric hindrance of EGFR-TKI binding, resulting in TKI treatment failure [7]. Although third-generation EGFR-TKIs could overcome EGFR T790M resistance, and has been used as the new first-line standard, acquired resistance is inevitable [8], indicating that genetic events are insufficient to explain TKI resistance. Consequently, it is crucial and urgent to identify novel therapeutics or treatments to treat LC with EGFR-TKI resistance.

RNA molecules can fold into complicated structures necessary for the diverse roles and regulation, such as transcription, splicing, polyadenylation, degradation, translation, and localization in cells [9–12]. Recently, combinations of chemical probing with high-throughput sequencing methods have been used to study whole-transcriptome secondary structures. Whole-genome RNA secondary structural probing with dimethyl sulfate sequencing (DMS-seq) [13,14], and selective 2'-hydroxyl acylation analyzed by primer extension sequencing (SHAPE-sequencing) [15,16] are conducted in living cells, which reveal the active mRNA structural unfolding, indicating the contribution of RNA structures to its overall processing. The aforementioned approaches indicated considerable progress and offered specific data on the single- or double-stranded RNA regions, but did not detect the direct pairing information between RNA sequences. Using psoralen to cross-link the double-stranded RNAs under 365-nm ultraviolet (UV) [17–19], psoralen analysis of RNA interactions and structures (PARIS) could identify the detailed base-pairing regions *in vivo* [20].

Over the past few years, many studies revealed that the widespread regulation of RNA processing, including post-transcriptional regulation in cancer, impacts multiple facets of tumorigenesis and drug resistance [21–23]. Meanwhile, RNA structures are crucial in physiological processes, including embryogenesis [24,25], cardiac specification [26], neurogenesis [27,28], and viral infection [29–33]. Consequently,

regulation based on RNA structures is expected to affect tumorigenesis and drug resistance critically. Our study illustrated the *in vivo* RNA structural landscapes in cells with EGFR-TKI resistance and sensitivity using PARIS, and found that the RNA structure switch modulates EGFR-TKI resistance by regulating *yrdc* *N*⁶-*threonylcarbamoyltransferase domain containing* (*YRDC*) mRNA translation in an embryonic lethal abnormal vision-like 1 (ELAVL1)-dependent manner. Collectively, we reveal a role of RNA structure-dependent regulation of EGFR-TKI resistance.

Results

Higher-order RNA structure maps of cells with EGFR-TKI resistance and sensitivity

The AZD9291-resistant (AZD9291-R) (Figure 1A) and gefitinib-resistant (gefitinib-R) (Figure 1B) cells were established using *EGFR*-mutant NSCLC cell line (PC9) to explore the mechanisms of RNA structures in LC-acquired EGFR-TKI resistance. PARIS assay [20], a high-throughput sequencing technology that allows the potent measurement of RNA duplexes in PC9, PC9 (gefitinib-R), or PC9 (AZD9291-R) cells, was performed (Figure 1C). We detected the RNA structures using the PARIS method [20] and acquired RNA structural maps (Figure S1A–D; Table S1). Overall, about 150,000 RNA duplexes from more than 15,000 transcripts were obtained from each of the cell lines (Figure 1D), including intramolecular (Figure S1E) and intermolecular RNA duplexes (Figure S1F). Our study reveals that RNA structures exist in most functional classes of RNA, including messenger RNA (mRNA), microRNA (miRNA), long non-coding RNA (lncRNA), small nucleolar RNA (snoRNA), and small nuclear RNA (snRNA) (Figure 1E, Figure S1G and H). Overall, our study constructed RNA structure maps of cells with EGFR-TKI resistance and sensitivity at the transcriptome level.

Having established the RNA structure maps of cells with EGFR-TKI resistance and sensitivity, we performed bioinformatic analysis to investigate the global characteristics of RNA structures. Unlike the previous study which can only identify local structures mainly within the window of < 200 nt, our study identified numerous RNA duplexes (more than 50%) spanning > 200 nt, with more than 30% of them spanning over 1000 nt in cells with EGFR-TKI resistance and sensitivity (Figure 2A). To study the RNA structural distribution of mRNA, the two-dimensional heatmaps of enriched RNA structural sites down the mRNA length that aligned transcripts in line with the sites of translation initiation and termination codons were plotted (Figure 2B, Figure S2A and B). Intriguingly, we found that RNA structures were also enriched in untranslated regions (UTRs), translation initiation sites, and stop codons. Except for the local RNA structures, there were many long-range RNA structures across different regions, especially in the UTRs. UTRs are crucial for RNA processing

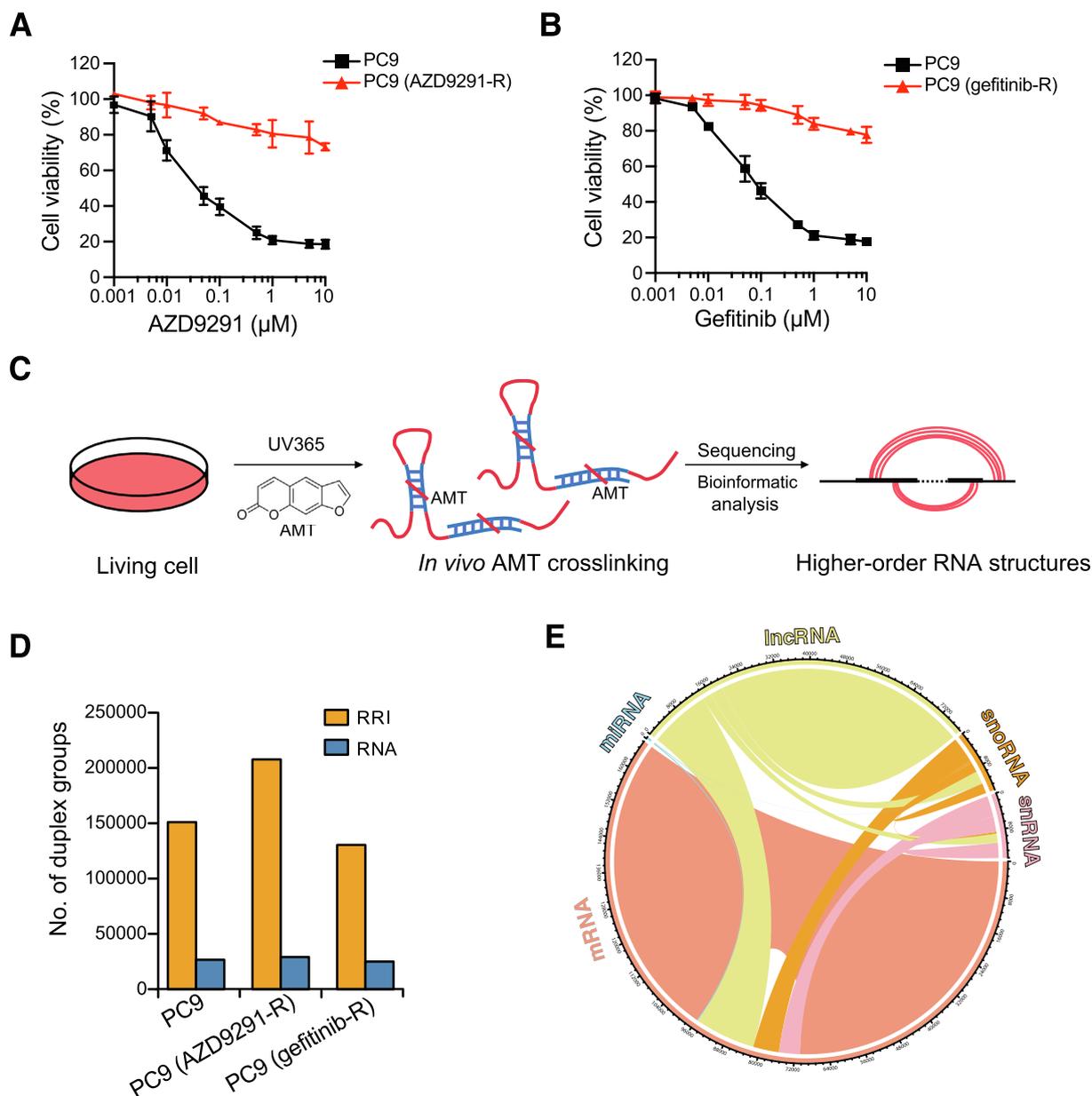


Figure 1 Comprehensive analysis of RNA structures in EGFR-TKI-resistant and -sensitive cells

A. CCK-8 assays in PC9 and PC9 (AZD9291-R) cells treated with AZD9291. **B.** CCK-8 assays in PC9 and PC9 (gefitinib-R) cells treated with gefitinib. **C.** Schematic view of *in vivo* RNA structure maps in EGFR-TKI-resistant and -sensitive cells using PARIS. **D.** The number of RNA–RNA duplexes and transcripts in EGFR-TKI-resistant and -sensitive cells. **E.** Circos plot showing the landscape of RNA–RNA duplexes detected by PARIS in PC9 cells. EGFR-TKI, epidermal growth factor receptor-tyrosine kinase inhibitor; PARIS, psoralen analysis of RNA interactions and structures; CCK-8, cell counting kit-8; UV, ultraviolet; AMT, 4'-aminomethyltrioxsalen; RRI, RNA–RNA interaction; mRNA, messenger RNA; miRNA, microRNA; lncRNA, long non-coding RNA; snoRNA, small nucleolar RNA; snRNA, small nuclear RNA.

regulation. Thus, RNA structures are the possible regulators for RNA processing modulation in cells with EGFR-TKI resistance and sensitivity.

To explore whether RNA structures are involved in RNA translation, we next performed ribosome profiling (Ribo-seq) and RNA sequencing (RNA-seq) in PC9, PC9 (AZD9291-R), and PC9 (gefitinib-R) cells, respectively (Figure S3A–C). Intriguingly, for structural mRNAs, their translation efficiency (TE) experienced notable impairment

compared to non-structural counterparts in these three cell lines (Figure S3D). Furthermore, we tested the TE of mRNAs with or without RNA structures, and found that RNA structures impaired TE of mRNAs (Figure 2C–E). These results suggest conserved regulation of RNA structures on translation in cells with EGFR-TKI resistance and sensitivity. To further evaluate RNA structure changes in the transition of EGFR-TKI resistance, the RNA duplexes were compared between cells with EGFR-TKI resistance

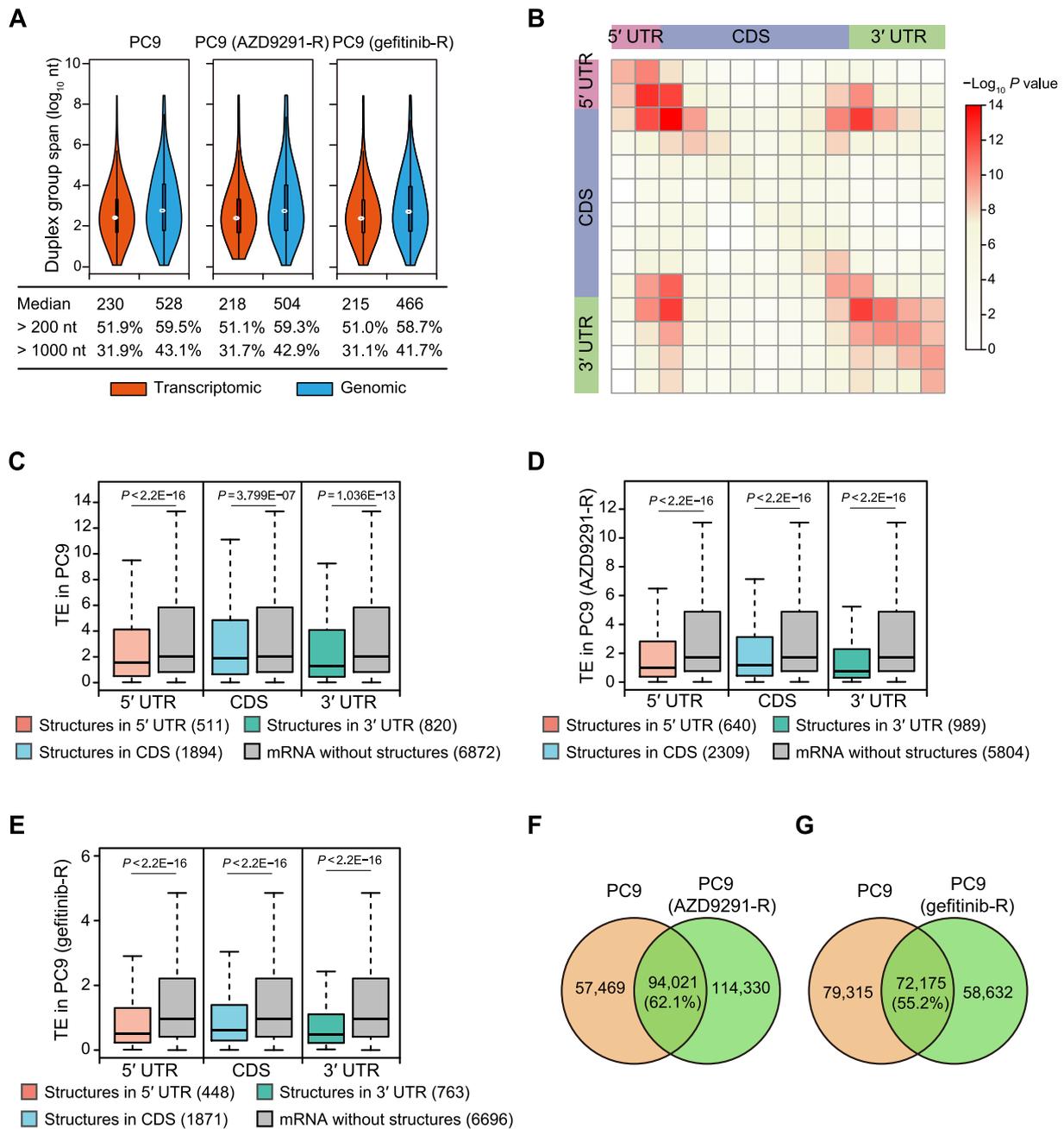


Figure 2 Genome-wide analysis of features in RNA structures in EGFR-TKI-resistant and -sensitive cells

A. Size distribution of RNA duplexes in transcriptome and genome. Genomic span is the distance between the ends of gapped reads in the genome, while the transcriptomic span excludes introns. **B.** Two-dimensional heatmap showing the enrichment of mRNA duplexes based on the location of chimera ends in PC9 cells. **C.** Box plots showing decreased TE of mRNAs displaying RNA structures within UTR or CDS compared to mRNAs without RNA structures in PC9 cells. P values were calculated by the Wilcox test. **D.** Box plots showing decreased TE of mRNAs displaying RNA structures within UTR or CDS compared to mRNAs without RNA structures in PC9 (AZD9291-R) cells. P values were calculated by the Wilcox test. **E.** Box plots showing decreased TE of mRNAs displaying RNA structures within UTR or CDS compared to mRNAs without RNA structures in PC9 (gefitinib-R) cells. P values were calculated by the Wilcox test. **F.** Overlay of RNA duplex groups between PC9 and PC9 (AZD9291-R) cells. **G.** Overlay of RNA duplex groups between PC9 and PC9 (gefitinib-R) cells. TE, translation efficiency; UTR, untranslated region; CDS, coding sequence.

and sensitivity. Almost 60% of RNA duplexes were conserved between cells with EGFR-TKI resistance and sensitivity (Figure 2F and G), indicating the dynamic RNA structures in the transition of EGFR-TKI resistance. In summary, the RNA structural changes between cells with

EGFR-TKI resistance and sensitivity are not found at the whole-transcriptome level. Considering that drug resistance turns up from the evolutionary pressure [34,35], we speculate that the changes in RNA structural regulation mainly occur at the transcript level.

YRDC facilitates the resistance to EGFR-TKIs in NSCLC cells

To determine whether the translation regulation is a crucial regulator for EGFR-TKI resistance, the TE of cells with EGFR-TKI resistance was compared to that with EGFR-TKI sensitivity. As a result, EGFR-TKI resistance causes translation changes in both PC9 (gefitinib-R) and PC9 (AZD9291-R) cells compared to PC9 cells (Figure 3A). Intriguingly, an 80.1% [1766 / (1766 + 437)] overlap in PC9 (gefitinib-R) and PC9 (AZD9291-R) cells was observed between the up-regulated genes in these two EGFR-TKI-resistant cells, and a 93.7% [6524 / (6524 + 437)] overlap in PC9 (gefitinib-R) and PC9 (AZD9291-R) cells was observed between the down-regulated genes (Figure 3A). Hence, the translation regulation between EGFR-TKI-resistant and -sensitive cells exhibits the similar tendency on TE of these changed genes. Based on the aforementioned findings, translation control is speculated to regulate EGFR-TKI resistance. Gene Ontology (GO) analysis revealed that common up-regulated genes in EGFR-TKI-resistant cells are enriched in the processes of intracellular signal transduction, MAPK cascade, Ras protein signal transduction, regulation of Wnt signaling pathway, and small GTPase mediated signal transduction (Figure 3B; Table S2). Common down-regulated genes in EGFR-TKI-resistant cells are enriched in the processes of DNA repair, cell cycle, cellular response to DNA damage stimulus, and response to drug (Figure 3C; Table S2). These results suggest that the translation changes play a crucial role in EGFR-TKI resistance.

Previous studies have reported that *YRDC* regulates RNA translation via involvement in tRNA's *N*⁶-threonylcarbamoyl adenosine (t⁶A) synthesis [36–38], and it has been reported that *YRDC* can regulate hepatocellular carcinoma (HCC) cell resistance to lenvatinib by regulating KRAS translation [39]. In our study, we found that *YRDC* has a higher TE in EGFR-TKI-resistant cells than in EGFR-TKI-sensitive cells (Figure 3A). *YRDC* protein expression was verified through Western blot (Figure 3D). We then investigated the expression of *YRDC* mRNA in The Cancer Genome Atlas (TCGA) and observed increased *YRDC* expression in NSCLC specimens compared to normal tissue (Figure S4A), indicating its role in tumorigenesis. Thus, we speculate that *YRDC* might be associated with the regulation of EGFR-TKI resistance in NSCLC cells. The coding sequence (CDS) of *YRDC* was overexpressed in the EGFR-TKI-sensitive cells by plasmid transfection (Figure 3E) to test this hypothesis. Further, a cell counting kit-8 (CCK-8) assay was conducted to detect the cell viability under the treatments of AZD9291 and gefitinib, respectively. *YRDC* overexpression induced a higher EGFR-TKI resistance than the control group in PC9 cells (Figure 3F and G). *YRDC* knockdown in the EGFR-TKI-resistant cells was also performed by small interfering RNA (siRNA) transfection (Figure 3H). The resistance to EGFR-TKIs was impaired under *YRDC* knockdown (Figure 3I and J), indicating that *YRDC* facilitates the resistance to EGFR-TKIs in NSCLC cells.

Intriguingly, by analyzing PARIS data, we found that 3' UTR of *YRDC* mRNA forms a double-stranded structure only in EGFR-TKI-sensitive cells (Figure S4B), which unwound in both EGFR-TKI-resistant cell lines. Our results have suggested that the RNA structures impair the TE in cells with EGFR-TKI resistance and sensitivity; thus, we speculate

that the structural changes may influence the translation of *YRDC* mRNA.

RNA structure in *YRDC* 3' UTR is necessary for EGFR-TKI resistance

For investigating RNA structure's effect on *YRDC* 3' UTR for translation control and EGFR-TKI resistance, four mutant plasmids containing endogenous CDS and 3' UTR of *YRDC* with point mutations (Mut-1: U1577C + G1582C, Mut-2: G1585C + G1590U, Mut-3: U1631G + A1636C, Mut-4: C1625A + U1628G) that disrupt base pairing (Figure 4A; Figure S5) were constructed and transfected into PC9 cells, with the wild-type (WT) *YRDC* as a control. Only the Mut-1 group showed increased protein expression level (Figure 4B) in the Western blot assay; however, mRNA expression was not changed considerably between WT and/or mutants (Figure 4C). As a result, RNA structure in *YRDC* 3' UTR impairs the *YRDC* protein translation instead of the mRNA abundance. Meanwhile, only Mut-1 abolishes this RNA structure's translation inhibition, indicating that the stem formed by 1575–1584 nt and 1629–1638 nt of *YRDC* mRNA was essential for translation control. Furthermore, Mut-1 and Mut-3 can disrupt the base pairing of 1575–1584 nt and 1629–1638 nt of *YRDC* mRNA. However, only Mut-1 (mutant position at 1575–1584 nt of *YRDC* mRNA) abolished the translation inhibition, but not Mut-3 (mutant position at 1629–1637 nt of *YRDC* mRNA), indicating that the region of 1629–1637 nt is a functional region that modulates translation of *YRDC* mRNA. Instead, the region of 1575–1584 nt may play the role of RNA structural regulator as a flanking sequence. The results of the CCK8 assay also showed that only Mut-1 overexpression induces a higher resistance to EGFR-TKIs (Figure 4D and E), supporting that the RNA structure in *YRDC* mRNA 3' UTR possibly has an essential effect on translation control and EGFR-TKI resistance. Antisense oligonucleotide (ASO) was also used to modulate the RNA structure in *YRDC* 3' UTR in EGFR-TKI-resistant cells, to test whether this RNA structure can influence EGFR-TKI resistance. ASO was prepared with the phosphorothioate backbone and 2'-*O*-methoxyethyl (2'-MOE) modifications to reduce cell toxicity and enhance nuclease resistance. The results showed that ASO-*YRDC* (antisense of 1624–1643 nt in *YRDC* mRNA) transfection groups had a lower *YRDC* protein expression level than non-binding ASO (ASO-NC) groups in EGFR-TKI-resistant cells (Figure 4F and G). Besides, the mRNA expression level was not influenced by ASO transfection (Figure 4H and I). CCK8 assay showed that ASO-*YRDC* transfection decreased the resistance to EGFR-TKIs (Figure 4J and K), suggesting that the RNA structure modulation in EGFR-TKI resistance cells by ASO transfection can restore the sensitivity to EGFR-TKIs.

ELAVL1 shows a higher affinity to the single-stranded RNA within 3' UTR of *YRDC* *in vitro* and *in vivo*

According to previous studies, RNA structures can regulate RNA-binding proteins (RBPs) binding to RNA by structural switching [15,24]. Therefore, RNA structure in *YRDC* 3' UTR might regulate translation by modulating RBP binding. By analyzing the sequence of 1629–1637 nt of *YRDC* mRNA, we found that this region contains the sequence of UUUUAUA, an AU-rich

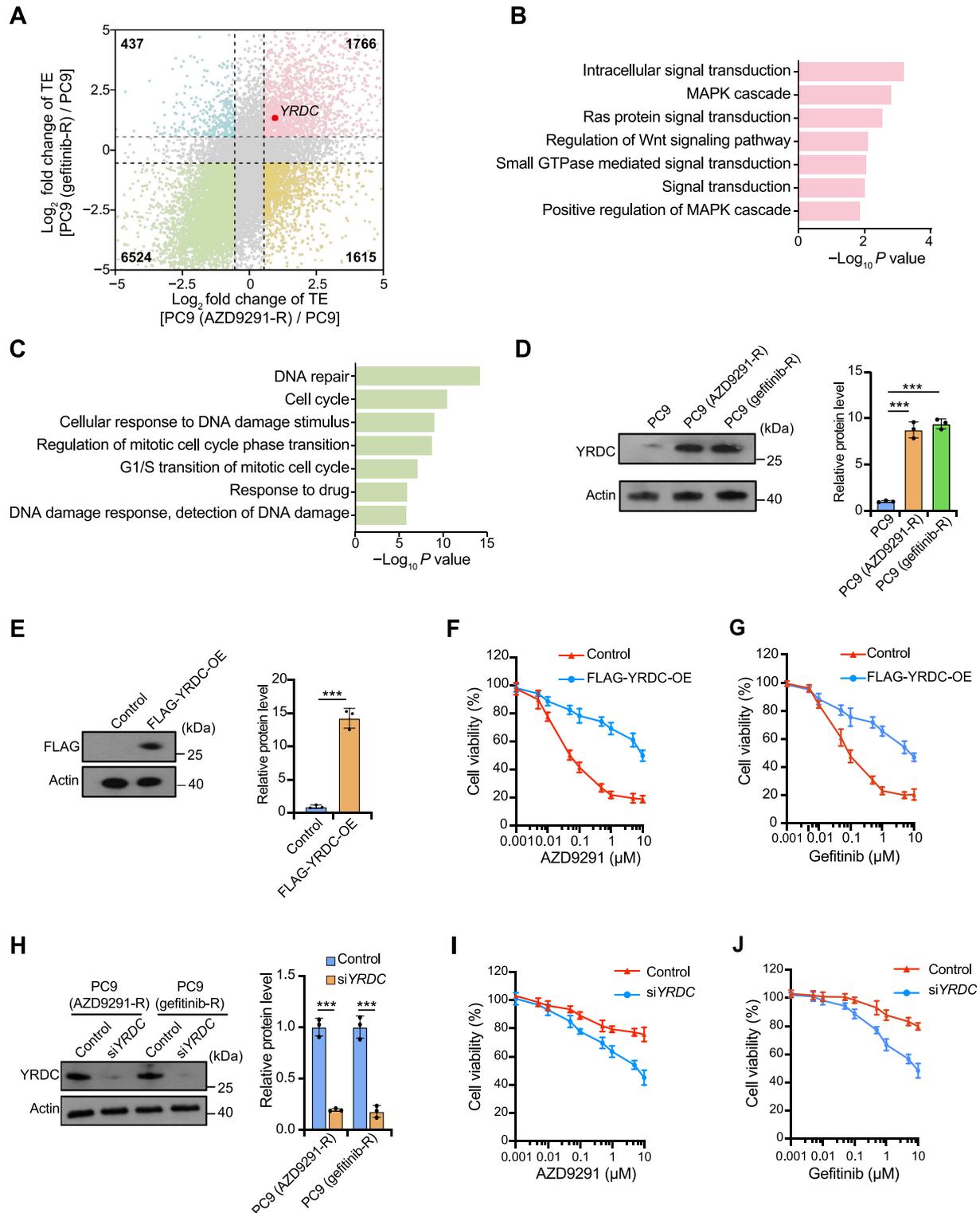
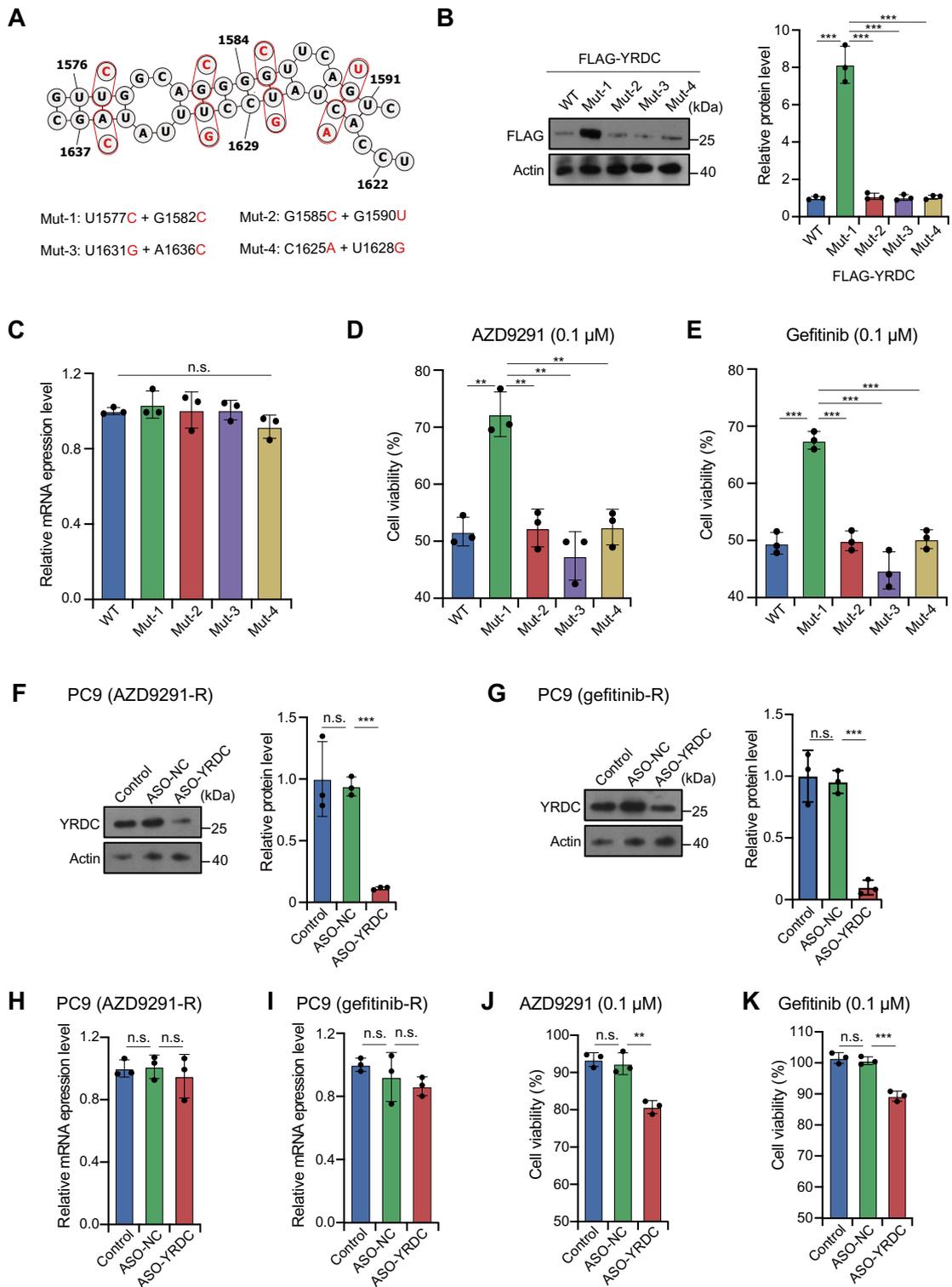


Figure 3 YRDC is required for EGFR-TKI resistance

A. Volcano plot showing the log₂ fold change of TE in PC9 (AZD9291-R) or PC9 (gefitinib-R) cells versus PC9 cells. **B.** GO enrichment analysis of common up-regulated genes in PC9 (AZD9291-R) and PC9 (gefitinib-R) cells compared to PC9 cells. **C.** GO enrichment analysis of common down-regulated genes in PC9 (AZD9291-R) and PC9 (gefitinib-R) cells compared to PC9 cells. **D.** Western blot (left) and statistical analysis (right) show the protein level of YRDC in PC9, PC9 (AZD9291-R), and PC9 (gefitinib-R) cells. **E.** Western blot (left) and statistical analysis (right) show the protein level of control and overexpressed FLAG-YRDC in PC9 cells. **F.** CCK-8 assays for PC9 cells transfected with FLAG-YRDC or control for 24 h followed by AZD9291 treatment for another 48 h. **G.** CCK-8 assays for PC9 cells transfected with FLAG-YRDC or control for 24 h followed by gefitinib treatment for another 48 h. **H.** Western blot (left) and statistical analysis (right) show the protein level of YRDC in PC9 (AZD9291-R) and PC9 (gefitinib-R) cells with or without YRDC knockdown. **I.** CCK-8 assays for PC9 (AZD9291-R) cells with or without YRDC knockdown for 24 h followed by AZD9291 treatment for another 48 h. **J.** CCK-8 assays for PC9 (gefitinib-R) cells with or without YRDC knockdown for 24 h followed by gefitinib treatment for another 48 h. Data were represented by mean ± SD. Statistical significance was determined by Student's *t*-test (***, *P* < 0.001). YRDC, *YrdC* N⁶-threonylcarbamoyltransferase domain containing; GO, Gene Ontology; SD, standard deviation.



element (ARE). ARE is an important *cis*-element for RNA processing, and a previous study has reported that ARE could regulate ELAVL1 binding by RNA structure switch [24]. ELAVL1 is well-known for regulating RNA stability and translation [40]. Thus, we speculated that RNA structure in *YRDC* 3' UTR regulates *YRDC* mRNA translation by controlling ELAVL1 protein binding. FLAG-ELAVL1 RNA-binding protein immunoprecipitation-quantitative real-time polymerase chain reaction (RIP-qPCR) was performed to test the ELAVL1 binding on *YRDC*. The results showed that ELAVL1 could bind *YRDC* in PC9 (AZD9291-R) and PC9 (gefitinib-R) cells, but not in PC9 cells (Figure 5A, Figure S6A). To further examine the binding ability of ELAVL1, biotin-labeled RNA pull-down assays were conducted using an RNA probe of 1621–1638 nt of *YRDC* mRNA. The results showed that the ELAVL1 can be pulled down by the *YRDC* RNA probe (Figure 5B and C). Similarly, electrophoretic mobility shift assay (EMSA) (Figure S6B) also illustrated that ELAVL1 could bind to the region of 1621–1638 nt of *YRDC* mRNA. Considering the RNA structure switch between cells with EGFR-TKI resistance and those with EGFR-TKI sensitivity, these results suggest that RNA structure in *YRDC* 3' UTR regulates ELAVL1 binding in a structure-dependent manner. To test the role of RNA structure in ELAVL1's binding on 3' UTR of *YRDC* mRNA, RNA probes were synthesized to reconstruct WT RNA structure formed by 1575–1592 nt and 1621–1638 nt of *YRDC* mRNA, rescue and mutant mRNAs with restored and disrupted base pairing within the ARE region, respectively (Figure 5D). These assays were repeated by adopting diverse biotin-labeled probe structures. According to RNA pull-down (Figure 5E and F) and EMSA (Figure 5G) results, ELAVL1 preferentially binds to 3' UTR of *YRDC* mRNA with single strand than double strand. Overall, the results indicate that the 3' UTR of *YRDC* mRNA could efficiently affect ELAVL1 protein binding by switching the RNA structure between EGFR-TKI-resistant and -sensitive cells.

RNA structure switch modulates EGFR-TKI resistance by regulating *YRDC* mRNA translation in an ELAVL1-dependent manner

To test the sufficiency of RNA structure in ELAVL1-regulated *YRDC* mRNA translation and EGFR-TKI resistance, *YRDC* reporter mRNAs based on CDS and 3' UTR

of *YRDC* with diverse structures (Figure 5D), including the WT where the ARE region was base-paired, the mutant disrupting the ARE region's base pairing, as well as the rescue restoring base pairing, were constructed. PC9 cells were transfected with WT, mutant, and rescue *YRDC* reporter plasmids under control and *ELAVL1* knockdown condition. Western blot results showed that the protein level of the mutant *YRDC* reporter with a single-stranded flanking sequence was higher than those of the WT and rescue *YRDC* reporters with the double-stranded flanking sequence. In contrast, no difference was observed after *ELAVL1* knockdown (Figure 6A, Figure S6C). The mRNA expression of *YRDC* reporter genes was not influenced under *ELAVL1* knockdown (Figure 6B), indicating that ELAVL1 was not related to *YRDC* mRNA stability. The CCK8 assay also showed that the only mutant *YRDC* reporter gene overexpression induced a higher resistance to EGFR-TKIs, and these differences were abolished under *ELAVL1* knockdown (Figure 6C and D). Thus, RNA structure modulates *YRDC* mRNA translation and EGFR-TKI resistance via affecting ELAVL1's accessibility to the ARE region.

Moreover, ASO transfection was performed to test this mechanism. The results showed that ASO-*YRDC* transfection can impair the expression of *YRDC* protein, and *ELAVL1* knockdown abolished the difference in *YRDC* protein levels under ASO-NC or ASO-*YRDC* transfection (Figure 6E and F, Figure S6D and E). The mRNA expression level of *YRDC* in ASO-NC or ASO-*YRDC* transfection groups was not influenced by *ELAVL1* knockdown (Figure 6G and H). ASO-*YRDC* transfection also can impair the EGFR-TKI resistance, and *ELAVL1* knockdown also abolished the difference of EGFR-TKI resistance under ASO-NC or ASO-*YRDC* transfection (Figure 6I and J), consistent with the *YRDC* protein level. Combined with our previous findings that RNA structure switch modulates ELAVL1's accessibility to the ARE region, these results suggest that ASO can be used to perturb the interaction between RNAs and RBPs, and modulate the EGFR-TKI resistance in NSCLC cells.

In summary, our study put forward one model that depicted the functions of RNA structural alterations in regulating *YRDC* translation and EGFR-TKI resistance by affecting ELAVL1 binding ability to *YRDC* (Figure 7).

Figure 4 RNA structure in *YRDC* 3' UTR contributes to EGFR-TKI resistance via controlling *YRDC* mRNA translation

A. Predicted secondary structure model of the RNA structure in the 3' UTR of *YRDC* mRNA, annotated with genomic coordinates. Red circles represent designs of mutations in the transfection study. B. Western blot (left) and statistical analysis (right) show the protein level of WT and mutant *FLAG-YRDC* overexpressed in PC9 cells. C. Relative mRNA level of the WT and mutant *FLAG-YRDC* reporter genes in PC9 cells. D. CCK-8 assays for PC9 cells transfected with WT and mutant *FLAG-YRDC* vectors for 24 h followed by AZD9291 (0.1 μ M) treatment for another 48 h. E. CCK-8 assays for PC9 cells transfected with WT and mutant *FLAG-YRDC* vectors for 24 h followed by gefitinib (0.1 μ M) treatment for another 48 h. F. Western blot (left) and statistical analysis (right) show the protein level of *YRDC* in PC9 (AZD9291-R) cells transfected with control, ASO-NC (non-binding ASO), or ASO-*YRDC*. G. Western blot (left) and statistical analysis (right) show the protein level of *YRDC* in PC9 (gefitinib-R) cells transfected with control, ASO-NC (non-binding ASO), or ASO-*YRDC*. H. Relative mRNA level of *YRDC* in PC9 (AZD9291-R) cells transfected with control, ASO-NC (non-binding ASO), or ASO-*YRDC*. I. Relative mRNA level of *YRDC* in PC9 (gefitinib-R) cells transfected with control, ASO-NC (non-binding ASO), or ASO-*YRDC*. J. CCK-8 assays for PC9 (AZD9291-R) cells transfected with control, ASO-NC (non-binding ASO), or ASO-*YRDC* for 24 h followed by AZD9291 (0.1 μ M) treatment for another 48 h. K. CCK-8 assays for PC9 (gefitinib-R) cells transfected with control, ASO-NC (non-binding ASO), or ASO-*YRDC* for 24 h followed by gefitinib (0.1 μ M) treatment for another 48 h. Data were represented as mean \pm SD. Statistical significance was determined by Student's *t*-test (**, $P < 0.01$; ***, $P < 0.001$; n.s., not significant). WT, wild-type; ASO, antisense oligonucleotide.

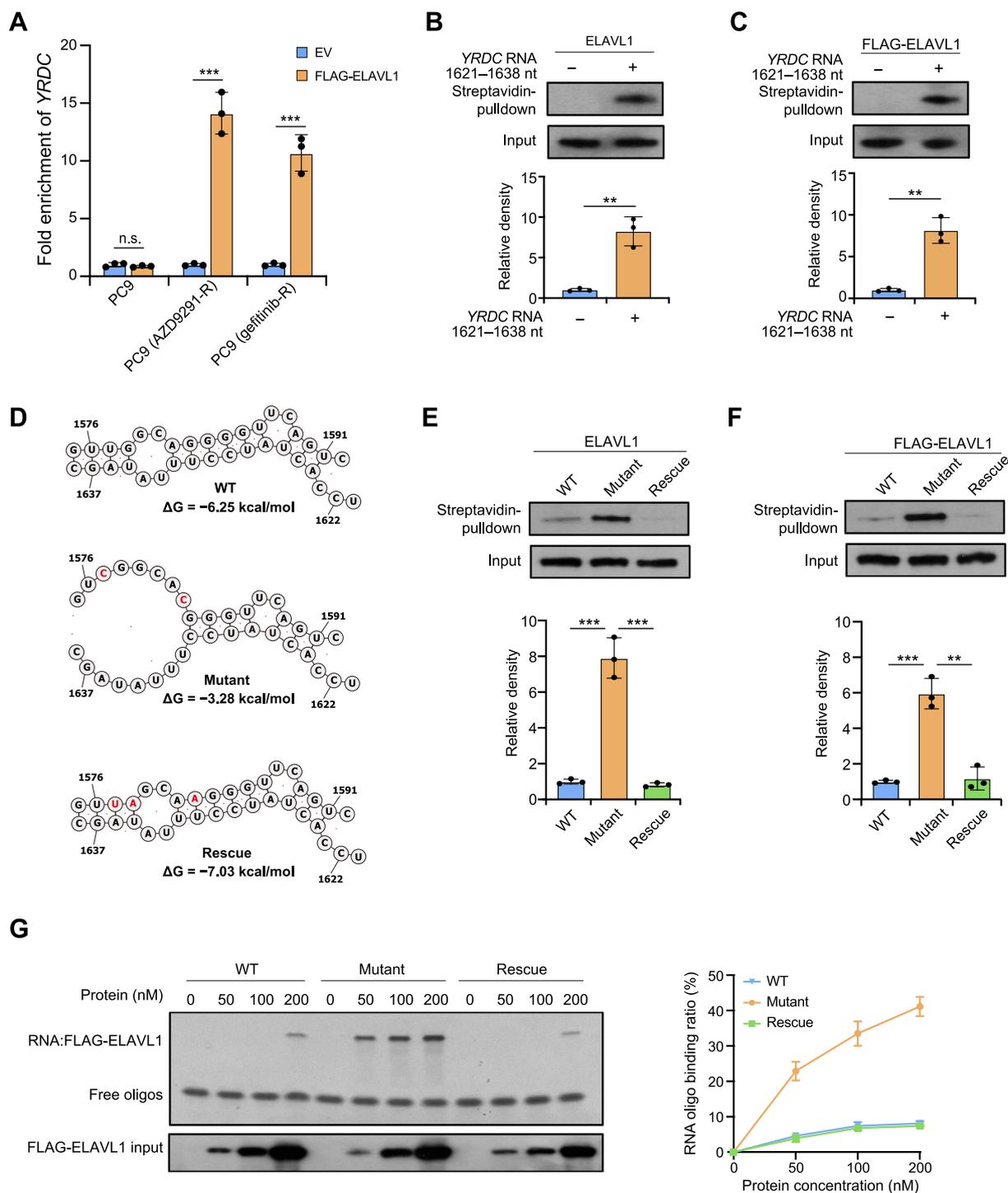


Figure 5 ELAVL1 prefers to bind single-stranded RNA in 3' UTR of *YRDC*

A. RIP-qPCR shows the fold enrichment of ELAVL1 binding sites in 3' UTR of *YRDC* mRNA upon FLAG pull down in PC9 cells, PC9 (AZD9291-R) cells, and PC9 (gefitinib-R) cells. **B.** Demonstration of endogenous ELAVL1 protein pulled down by an RNA probe of 1621–1638 nt of *YRDC* mRNA. **C.** Demonstration of purified FLAG-ELAVL1 protein pulled down by an RNA probe of 1621–1638 nt of *YRDC* mRNA. **D.** The RNA probe structure prediction models of WT, mutant, and rescue. **E.** Demonstration of ELAVL1 protein pulled down by WT, mutant, and rescue RNA probes of *YRDC*. **F.** Demonstration of FLAG-ELAVL1 protein pulled down by WT, mutant, and rescue RNA probes of *YRDC*. **G.** EMSA showing the binding ability of FLAG-ELAVL1 with WT, mutant, and rescue RNA probes of *YRDC*. Data were represented by mean \pm SD. Statistical significance was determined by Student's *t*-test (**, $P < 0.01$; ***, $P < 0.001$). RIP-qPCR, RNA-binding protein immunoprecipitation-quantitative real-time polymerase chain reaction; EMSA, electrophoretic mobility shift assay; ELAVL1, embryonic lethal abnormal vision-like 1.

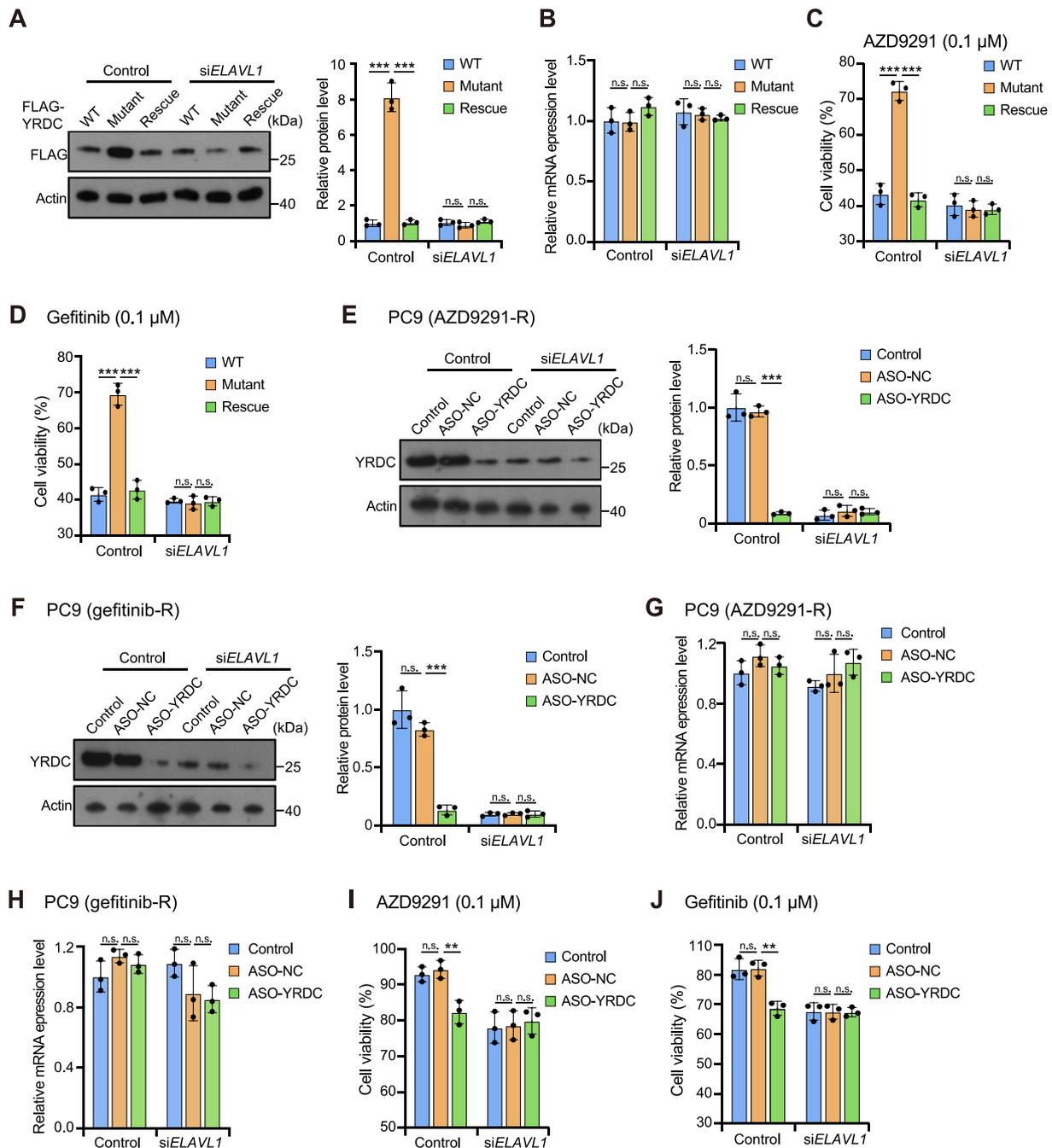


Figure 6 RNA structure modulates *YRDC* mRNA translation and EGFR-TKI resistance

A. Western blot (left) and statistical analysis (right) show the protein levels of the WT, mutant, and rescue FLAG-YRDC reporters upon control and *ELAVL1* knockdown in PC9 cells. **B.** Relative mRNA levels of the WT, mutant, and rescue *FLAG-YRDC* reporter genes upon control and *ELAVL1* knockdown in PC9 cells. **C.** CCK-8 assays for PC9 cells transfected with the WT, mutant, and rescue *FLAG-YRDC* reporter genes upon control and *ELAVL1* knockdown for 24 h followed by AZD9291 (0.1 μ M) treatment for another 48 h. **D.** CCK-8 assays for PC9 cells transfected with the WT, mutant, and rescue *FLAG-YRDC* reporter genes upon control and *ELAVL1* knockdown for 24 h followed by gefitinib (0.1 μ M) treatment for another 48 h. **E.** Western blot (left) and statistical analysis (right) show the protein levels of YRDC in PC9 (AZD9291-R) cells transfected with control, ASO-NC (non-binding ASO), or ASO-YRDC upon control and *ELAVL1* knockdown. **F.** Western blot (left) and statistical analysis (right) show the protein levels of YRDC in PC9 (gefitinib-R) cells transfected with control, ASO-NC (non-binding ASO), or ASO-YRDC upon control and *ELAVL1* knockdown. **G.** Relative mRNA level of *YRDC* in PC9 (AZD9291-R) cells transfected with control, ASO-NC (non-binding ASO), or ASO-YRDC upon control and *ELAVL1* knockdown. **H.** Relative mRNA level of *YRDC* in PC9 (gefitinib-R) cells transfected with control, ASO-NC (non-binding ASO), or ASO-YRDC upon control and *ELAVL1* knockdown. **I.** CCK-8 assays for PC9 (AZD9291-R) cells transfected with control, ASO-NC (non-binding ASO), or ASO-YRDC upon control and *ELAVL1* knockdown for 24 h followed by AZD9291 (0.1 μ M) treatment for another 48 h. **J.** CCK-8 assays for PC9 (gefitinib-R) cells transfected with control, ASO-NC (non-binding ASO), or ASO-YRDC upon control and *ELAVL1* knockdown for 24 h followed by gefitinib (0.1 μ M) treatment for another 48 h. Data were represented as mean \pm SD. Statistical significance was determined by Student's *t*-test (**, $P < 0.01$; ***, $P < 0.001$; n.s., not significant).

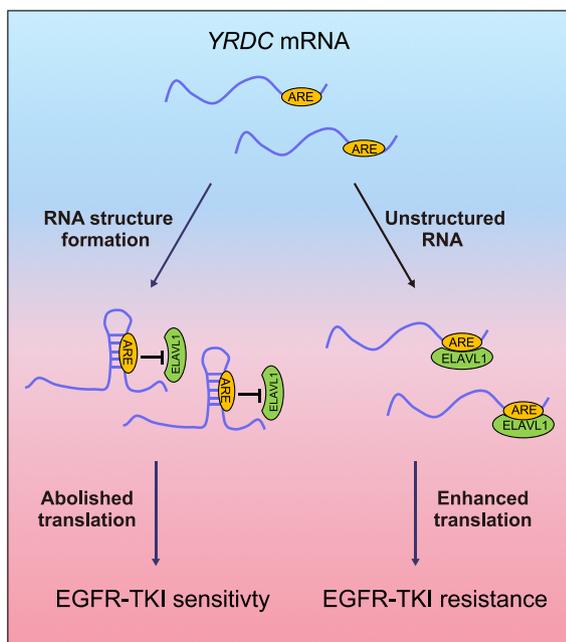


Figure 7 ELAVL1 regulates *YRDC* mRNA translation in a RNA structure-dependent manner to modulate EGFR-TKI resistance. ARE, AU-rich element.

Discussion

In this study, transcriptome-scale RNA structures were profiled using an acquired resistant model of NSCLC cells that revealed an RNA structure switching-dependent mechanism in regulating EGFR-TKI resistance. Intriguingly, we show that (1) RNA structural regions are enriched in UTR, translation start site, and stop codon, indicating its role of post-transcriptional regulation and translation control, (2) RNA TE is dynamic during the period of drug resistance, and impaired by the RNA structures, and (3) the RNA structure within *YRDC* mRNA's 3' UTR could modulate NSCLC cell translation and EGFR-TKI resistance by regulating ELAVL1 binding. Overall, our study demonstrates RNA structure-based regulation of translation control, which is critical for the drug sensitivity to resistance in NSCLC cells. Furthermore, RNA structures serve as molecular switches for controlling translation in cancer drug resistance, thereby allowing us to overcome drug resistance by modulating RNA structure.

LC is an important factor in cancer-associated death worldwide, while NSCLC is a frequent subtype [1]. Nearly 2/3 of NSCLC cases harbor the carcinogenic driver mutation [41], and the TKIs for sensitizing EGFR mutations are investigated widely. Nevertheless, many cases acquired EGFR-TKI resistance in a short time. The mechanisms of resistance are divided into “on-target” and “off-target” [42]. The former occurs in the case of a changed drug's primary target, which limits the target inhibition capacity of the drug, among which the T790M point mutation of EGFR is the most prevalent for gefitinib, erlotinib, or afatinib resistance. Third-generation EGFR-TKI (AZD9291) solved this problem; however, accompanied by inevitable acquired resistance [8]. The accumulating evidence indicated that the on-target resistance was insufficient

to explain the resistance of EGFR-TKIs. By contrast, off-target resistance occurs by activating collateral signaling in the downstream signaling or paralleling to the signaling via a driver oncoprotein. Therefore, exploring the new mechanism for overcoming the resistance to EGFR-TKIs in LC is crucial for therapy. Our work profiled RNA structure atlas during EGFR-TKI resistance in NSCLC cells, which provided a new perspective that RNA structural switch-mediated translation control modulates drug resistance. Meanwhile, this study used gefitinib and AZD9291 (the first- and third-generation EGFR-TKIs, respectively) for drug resistant-cell construction to obtain the comprehensive RNA structure atlases and general regulation mechanism of EGFR-TKI resistance.

RNA structures are involved in almost all RNA processing, such as polyadenylation, splicing, localization, degradation, and translation [9]. Moreover, recent studies proved that RNA structures are crucial in physiological processes, including embryogenesis [24,25,43], cardiac specification [26], neurogenesis [27], and viral infection [29–33]. Although RNA structures are related to RNA processing and essential for RNA functions, gene regulation based on RNA structure in cancer drug resistance remains unknown. RNA structure is highly dynamic *in vivo*, and is influenced by many factors such as cellular energy state, temperature, RNA helicases, chaperone proteins, and other RBPs [11]. We think there are other regulatory mechanisms promote RNA structural switch in drug resistance, especially for the protein-directed RNA switch. This work constructed an RNA structure atlas in NSCLC cells, associating RNA structure with post-transcriptional regulation. Firstly, RNA structures were found enriched in UTRs. Previous studies have reported that 35% of conserved structured elements was in UTRs [12], indicating that the RNA structures in UTRs are crucial for RNA fate determination and function. Secondly, RNA structures were found to impair TE in NSCLC cells. RNAs that had more structure within 5' UTR decreased the binding affinity of ribosomes, lowering TE [12]. Meanwhile, ribosomes have an essential effect on unlocking secondary structures in CDS [12,24,25]. Additionally, RNAs double-stranded structures in 3' UTR impaired translation, possibly due to their higher accessibility to RNA decomposition and translation inhibition mechanisms [9].

YRDC is involved primarily in adenosine N^6 -threonyl-carbamoylation in ANN-type tRNA synthesis for recognizing ANN codons [36]. t^6A represents the widely distributed modification necessary to maintain the accurate and efficient translation [37,38], and *YRDC* regulates HCC cell resistance to lenvatinib by regulating *KRAS* translation [39]. The present work found that *YRDC* facilitates EGFR-TKI resistance in NSCLC cells, and the RNA structure in 3' UTR of *YRDC* mRNA can modulate TE, which only exists in PC9 cells that is sensitive to EGFR-TKIs. Further studies found that RNA structure reduces the binding affinity of ELAVL1 and impairs the *YRDC* translation. ELAVL1 is crucial for mRNA stability and translation control [40] by binding the AU-rich motifs [44]. Many studies revealed the involvement of ELAVL1 in post-transcriptional regulation of several specific cancers [45,46]. Moreover, the RNA structure greatly affects the binding affinity of its zebrafish ortholog *Elavl1a* to the AU-rich motif and is essential for zebrafish embryogenesis [24]. Our study performed RNA pull-down assays *in vivo* and *in vitro* using structural mutant and rescue form of 3' UTR of *YRDC*.

The results showed that ELAVL1 protein preferentially binds to single-strand RNA of 3' UTR of *YRDC*, concordant with the result of the EMSA assay. Moreover, we found that the RNA structural switch in 3' UTR of *YRDC* modulates ELAVL1 binding, essential for controlling *YRDC* translation.

Previous studies reported several mechanisms that regulate translational modulation for shaping tumor development and treatment resistance [47]. The translation modulation by RNA structure reconstruction can be exploited in cancer treatment. Due to the progression of medicinal chemistry, targeted delivery, and molecular mechanisms, ASOs have substantially improved efficacy and properties [48]. Besides inducing target RNA degradation, ASO can also modulate splicing, translation, polyadenylation, etc. [48,49]. A recent study reported that ASO could disturb the RBP-RNA target interactions by reconstructing RNA structure [29]. In our study, we found the ASO transfection could decrease the *YRDC* translation and resistance to EGFR-TKIs, indicating that the ASO-mediated RNA structural switch serves as a possible way for overcoming drug resistance in cancer.

Our study establishes RNA structural atlas in EGFR-TKI resistance model, and uses this information to explore the potential RNA structure-dependent mechanism in drug resistance. There are some limitations in this study. First, the RNA structural atlas was obtained from the cellular models with EGFR-TKI resistance and sensitivity that were constructed using a representative NSCLC cell line, PC9. The LC was highly heterogeneous between different people, although we used two EGFR-TKI-resistant cell lines to profile RNA structures, so it was still difficult to represent the RNA structures in all LC cells. Second, although we have demonstrated the RNA structure-dependent mechanism in drug resistance and potential ASO treatment in different cells, the mechanisms and the action of ASO should be studied further with animal models.

Collectively, our study profiles the RNA structure landscape in cells with EGFR-TKI resistance and sensitivity, and reveals the novel RNA structure switching-dependent mechanism in regulating EGFR-TKI resistance. Notably, the usage of ASO in perturbing the interaction between RNA and protein provide a potential strategy for cancer therapy. Thus, we reasonably speculate that the RNA structure-dependent mechanism and RNA structure-targeted drugs could be used for treatment of NSCLC.

Materials and methods

Cell lines and culture conditions

PC9 cells was newly purchased from American Type Culture Collection (ATCC). PC9 (gefitinib-R) and PC9 (AZD9291-R) cells were passaged with low concentration (0.1 μ M) of gefitinib (Catalog No. ZD1839, Selleck, Shanghai, China) or AZD9291 (Catalog No. S7297, Selleck) and sequentially cultured in increasing concentrations of these TKIs. Cell lines were grown in Roswell Park Memorial Institute (RPMI)-1640 (Catalog No. 11875093, Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (Catalog No. 16140078, Gibco) and Antibiotic-Antimycotic (Catalog No. 15240062, Gibco) at 37 °C under 5% CO₂.

PARIS assay

PARIS experiments were performed as previously reported [20]. PC9, PC9 (gefitinib-R), and PC9 (AZD9291-R) cells were treated with AMT (Catalog No. A4330, Sigma-Aldrich, Saint Louis, MO) and crosslinked by 365-nm UV. The lysates were treated by S1 nuclease (Catalog No. EN0321, ThermoFisher Scientific, Carlsbad, MA) and purified by TRIzol (Catalog No. 15596026, Invitrogen, Carlsbad, MA). Purified RNA was treated with ShortCut RNase III (Catalog No. M0245L, New England Biolabs, Ipswich, MA). RNA was separated by 12% native polyacrylamide gel and then electrophoresed in a two-dimension 20% urea-denatured gel. Crosslinked RNA proximity ligated with T4 RNA ligase I (Catalog No. M0204L, New England Biolabs) and reversed crosslinking with 254-nm UV. The RNA molecules were used for library construction by the smRNA-Seq kit (Catalog No. 635031, Clontech, San Jose, CA).

Western blot

Western blot was performed as previously reported [50] and using these antibodies: monoclonal anti-HuR antibody (Catalog No. 390600, Invitrogen), anti-YRDC antibody (Catalog No. sc-390477, SANTA CRUZ, Dallas, TX), anti- β -Actin antibody (Catalog No. 4967, Cell Signaling Technology, Wuhan, China), and anti-FLAG antibody (Catalog No. F7425, Sigma-Aldrich).

Protein purification

HEK293 were transfected by FLAG-tagged *ELAVL1* plasmids using the Polyethylenimine (PEI) (Catalog No. BMS1003, Invitrogen). Cells were lysed with lysis buffer after 48 h and sonicated for 1 min. The cell debris was removed by centrifugation at 13,300 r/min for 10 min, and the lysates were incubated with the anti-FLAG M2 Affinity Gel (Catalog No. A2220, Sigma-Aldrich) at 4 °C for 4 h. Afterward the samples were washed five times with lysis buffer and twice with Tris-buffered saline (TBS) (Catalog No. T5912-1L, Sigma-Aldrich) buffer. The proteins were eluted by 3 \times FLAG peptide at 4 °C and concentrated by Vivaspin (Catalog No. VS0291, Vivaproducts, Littleton, MA).

RNA-seq

Total RNA was isolated from cells by TRIzol reagent. RNA HyperPrep kit (Catalog No. KK8540, KAPA, Saint Louis, MO) was used for RNA-seq library construction.

Ribo-seq

Ribo-seq was performed as previously reported [51]. Cells were incubated in 0.1 mg/ml medium for 5 min at 37 °C and then transferred into 200- μ l lysis buffer. After triturating ten times by a 26-G needle, the lysate was centrifugated for 15 min at 20,000 g at 4 °C. Then, 1 μ l of RNase I was added to the lysate and incubated at room temperature for 45 min. The lysate was loaded on the MicroSpin S-400 columns (Catalog No. 27-5140-01, GE, Marlborough, MA) and eluted by centrifugation. RNA was extracted from the flow-through using Trizol.

Purified RNAs were separated by 15% urea polyacrylamide gel electrophoresis (PAGE) gel. RNA fragments (27 nt to 30 nt) were recovered and used for library preparation by SMARTer smRNA-Seq kit (Catalog No. 635031, Clontech, San Jose, CA).

Real-time quantitative polymerase chain reaction

Real-time quantitative polymerase chain reaction (RT-qPCR) was performed with the SYBR Premix Ex Taq (Catalog No. DRR420A, TAKARA, Shiga, Japan) using CFX96 Real-Time PCR System (Catalog No. 1845096, BIO-RAD, Hercules, CA). The primers used are as follows: *YRDC*-F, 5'-GC CTCTTGTTAGGCATTCGGA-3'; and *YRDC*-R, 5'-AGTAC TTTCCAGGGCACAGC-3'.

RNA pull-down assay

In vivo or *in vitro* RNA pull-down assays were performed by cell extracts or purified proteins as previously reported [24]. In brief, the annealing biotin-labeled RNAs were incubated with cell extracts or purified ELAVL1 proteins for 2 h at 4 °C together with streptavidin magnetic beads (Catalog No. S1420S, New England Biolabs). The beads-bound proteins were eluted in NuPAGE LDS buffer (Catalog No. NP0008, Invitrogen) and detected by Western blot.

EMSA

EMSA was performed as previously reported [24]. Purified ELAVL1 proteins were diluted to different concentrations in binding buffer. RNA probes and purified proteins of different concentrations were mixed, and incubated for 30 min at room temperature. The RNA–protein mixture was added with Tris-Borate-EDTA buffer (TBE) sample buffer (Catalog No. LC6678, Invitrogen) and separated on 10% TBE gel (Catalog No. EC62752BOX, Invitrogen). The TBE gel was detected by Typhoon 9400 (Catalog No. 29187191, GE Healthcare, Boston, MA). ImageJ was used for quantification. Probe binding ratio was calculated by [RNA–protein / (free RNA + RNA–protein)].

siRNA and ASO transfection

ASO-YRDC (5'-i2MOErC*/i2MOErC*/i2MOErC*/i2MOErT//i2MOErG//i2MOErG//i2MOErC//i2MOErT//i2MOErA// i2MOErT//i2MOErA//i2MOErA//i2MOErA//i2MOErG//i2MOErG//i2MOErA//i2MOErT*/i2MOErA/-3') and ASO-NC (5'-i2MOErA*/i2MOErT*/i2MOErG*/i2MOErT// i2MOErG//i2MOErT//i2MOErC//i2MOErC//i2MOErT//i2MOErG//i2MOErT//i2MOErT//i2MOErA//i2MOErA//i2MOErC//i2MOErT//i2MOErC//i2MOErA*/i2MOErT*/i2MOErC*/i2MOErA/-3') were synthesized by Genscript (Nanjing, China). siRNAs targeting *ELAVL1* (5'-AAGAGGCAAUUACCAGUUUCA-3') and *YRDC* (5'-CAUUCGGAUCCUGAUCAU-3') were synthesized by Genscript. siRNAs and ASOs were transfected using Lipofectamine RNAiMAX (Catalog No. 13778030, Life Technologies, Carlsbad, MA) and used for drug treatment after 24 h.

CCK8 assay

Cell viability was detected by a CCK-8 Cell Counting Kit (Catalog No. A311-02, Vazyme, Nanjing, China) and calculated by GraphPad Prism.

ELAVL1 RIP-qPCR

PC9, PC9 (gefitinib-R), and PC9 (AZD9291-R) cells were transfected with FLAG-tagged *ELAVL1* plasmids using the Lipofectamine 3000 (Catalog No. L3000015, Life Technologies). Then, cells were harvested after 48 h and lysed in lysis buffer. Lysates were incubated with Anti-FLAG M2 Magnetic Beads (Catalog No. M8823, Merck, Saint Louis, MO) at 4 °C for 4 h. After washing three times, lysates were incubated by proteinase K at 37 °C for 20 min. RNA was purified by Trizol and used for RT-qPCR detection.

RNA-seq data analysis

The raw data were treated using FastQC. Low-quality reads and shorter reads were trimmed by cutadapt (v3.0) and Trimmomatic (v0.39) [52]. Filtered reads were mapped to the human genome (GRCh38) using Hisat2 (v2.2.1) with “-p -N -dta”. The HTSeq software was used to count reads mapped to each Ensembl gene. The fold change was calculated using the DESeq2 (v1.26.0) package.

Ribo-seq data analysis

The raw data were evaluated using FastQC. Low-quality reads were screened by cutadapt (v3.0) [53] and Trimmomatic (v0.39). Screened reads were mapped to the human rRNA transcriptome using Bowtie (v1.3.0) [54] and unmapped reads were retained for further analysis. The retained reads were mapped to human genome (GRCh38) using Bowtie (v1.3.0). The mRNA expression levels were calculated by Cufflinks (v2.2.1). TE was calculated by [fragments per kilobase per million (FPKM) of Ribo-seq] / (FPKM of RNA-seq). For replicated sample, we used the mean expression value.

PARIS data processing and analysis

The raw data were evaluated using FastQC. The low-quality reads and shorter reads were screened by cutadapt (v3.0) and Trimmomatic (v0.39). Other reads were mapped to human genome (GRCh38) from the Ensembl annotation using STAR (v2.5.3) with “--outReadsUnmapped Fastx --outFilterMultimapNmax 10 --outSAMattributes All --alignIntronMin 1 --outSAMmultNmax 2 --chimOutType WithinBAM SoftClip --outSAMtype BAM Unsorted --scoreGapNoncan -4 --scoreGapATAC -4 --chimSegmentMin 15 --limitOutSJcollapsed 9000000 --limitIObufferSize 95000000 --chimJunctionOverhangMin 15 --runThreadN 32”. The sam file was converted to bam file using SAMtools (v1.11). The gap regions and duplex group were classified by icSHAPE-pipe.

Statistical analysis

Statistical analysis was performed with GraphPad Prism 6.0, and the statistics were analyzed by unpaired Student's *t*-test. *P* values were provided as *, *P* < 0.05, **, *P* < 0.01, and ***, *P* < 0.001.

Data availability

The PARIS, ribo-seq, and RNA-seq raw data have been deposited in the Genome Sequence Archive for Human [55] at the National Genomics Data Center, Beijing Institute of Genomics, Chinese Academy of Sciences / China National Center for Bioinformatics (GSA-Human: HRA002235), and are publicly accessible at <https://ngdc.cncb.ac.cn/gsa-human>.

Competing interests

The authors have declared no competing interests.

CRediT authorship contribution statement

Boyang Shi: Conceptualization, Methodology, Formal analysis, Data curation, Validation, Investigation, Writing – original draft, Writing – review & editing. **Ke An:** Formal analysis, Data curation, Writing – original draft, Writing – review & editing. **Yueqin Wang:** Methodology, Resources. **Yuhan Fei:** Formal analysis. **Caixia Guo:** Methodology, Writing – review & editing. **Qiangfeng Cliff Zhang:** Formal analysis. **Yun-Gui Yang:** Conceptualization, Supervision, Funding acquisition, Project administration, Writing – review & editing. **Xin Tian:** Conceptualization, Supervision, Funding acquisition, Project administration, Writing – review & editing. **Quancheng Kan:** Conceptualization, Supervision, Funding acquisition, Project administration, Writing – review & editing. All authors have read and approved the final manuscript.

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Supplementary material

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References

- [1] Siegel RL, Miller KD, Fuchs HE, Jemal A. Cancer statistics 2021. *CA Cancer J Clin* 2021;71:7–33.
- [2] Hirsch FR, Scagliotti GV, Mulshine JL, Kwon R, Curran Jr WJ, Wu YL, et al. Lung cancer: current therapies and new targeted treatments. *Lancet* 2017;389:299–311.
- [3] Shi Y, Au JSK, Thongprasert S, Srinivasan S, Tsai CM, Kho MT, et al. A prospective, molecular epidemiology study of EGFR mutations in asian patients with advanced non-small-cell lung cancer of adenocarcinoma histology (PIONEER). *J Thorac Oncol* 2014;9:154–62.
- [4] Wu S, Fu L. Tyrosine kinase inhibitors enhanced the efficacy of conventional chemotherapeutic agent in multidrug resistant cancer cells. *Mol Cancer* 2018;17:25.
- [5] Piotrowska Z, Sequist LV. Treatment of EGFR-mutant lung cancers after progression in patients receiving first-line EGFR tyrosine kinase inhibitors : a review. *JAMA Oncol* 2016;2:948–54.
- [6] Yu HA, Arcila ME, Rekhtman N, Sima CS, Zakowski MF, Pao W, et al. Analysis of tumor specimens at the time of acquired resistance to EGFR-TKI therapy in 155 patients with EGFR-mutant lung cancers. *Clin Cancer Res* 2013;19:2240–7.
- [7] Recondo G, Facchinetti F, Olaussen KA, Besse B, Friboulet L. Making the first move in EGFR-driven or ALK-driven NSCLC: first-generation or next-generation TKI? *Nat Rev Clin Oncol* 2018;15:694–708.
- [8] Sullivan I, Planchard D. Osimertinib in the treatment of patients with epidermal growth factor receptor T790M mutation-positive metastatic non-small cell lung cancer: clinical trial evidence and experience. *Ther Adv Respir Dis* 2016;10:549–65.
- [9] Wang XW, Liu CX, Chen LL, Zhang QC. RNA structure probing uncovers RNA structure-dependent biological functions. *Nat Chem Biol* 2021;17:755–66.
- [10] Ganser LR, Kelly ML, Herschlag D, Al-Hashimi HM. The roles of structural dynamics in the cellular functions of RNAs. *Nat Rev Mol Cell Biol* 2019;20:474–89.
- [11] Lewis CJT, Pan T, Kalsotra A. RNA modifications and structures cooperate to guide RNA–protein interactions. *Nat Rev Mol Cell Biol* 2017;18:202–10.
- [12] Mustoe AM, Busan S, Rice GM, Hajdin CE, Peterson BK, Ruda VM, et al. Pervasive regulatory functions of mRNA structure revealed by high-resolution SHAPE probing. *Cell* 2018;173:181–95.
- [13] Ding Y, Tang Y, Kwok CK, Zhang Y, Bevilacqua PC, Assmann SM. *In vivo* genome-wide profiling of RNA secondary structure reveals novel regulatory features. *Nature* 2014;505:696–700.

- [14] Rouskin S, Zubradt M, Washietl S, Kellis M, Weissman JS. Genome-wide probing of RNA structure reveals active unfolding of mRNA structures *in vivo*. *Nature* 2014;505:701–5.
- [15] Spitale RC, Flynn RA, Zhang QC, Crisalli P, Lee B, Jung JW, et al. Structural imprints *in vivo* decode RNA regulatory mechanisms. *Nature* 2015;519:486–90.
- [16] Smola MJ, Rice GM, Busan S, Siegfried NA, Weeks KM. Selective 2'-hydroxyl acylation analyzed by primer extension and mutational profiling (SHAPE-MaP) for direct, versatile and accurate RNA structure analysis. *Nat Protoc* 2015;10:1643–69.
- [17] Graveley BR. RNA matchmaking: finding cellular pairing partners. *Mol Cell* 2016;63:186–9.
- [18] Aw JGA, Shen Y, Wilm A, Sun M, Lim XN, Boon KL, et al. *In vivo* mapping of eukaryotic RNA interactomes reveals principles of higher-order organization and regulation. *Mol Cell* 2016;62:603–17.
- [19] Sharma E, Sterne-Weiler T, O'Hanlon D, Blencowe BJ. Global mapping of human RNA–RNA interactions. *Mol Cell* 2016;62:618–26.
- [20] Lu Z, Zhang QC, Lee B, Flynn RA, Smith MA, Robinson JT, et al. RNA duplex map in living cells reveals higher-order transcriptome structure. *Cell* 2016;165:1267–79.
- [21] Goodall GJ, Wickramasinghe VO. RNA in cancer. *Nat Rev Cancer* 2021;21:22–36.
- [22] Delaunay S, Frye M. RNA modifications regulating cell fate in cancer. *Nat Cell Biol* 2019;21:552–9.
- [23] Yan F, Al-Kali A, Zhang Z, Liu J, Pang J, Zhao N, et al. A dynamic N⁶-methyladenosine methylome regulates intrinsic and acquired resistance to tyrosine kinase inhibitors. *Cell Res* 2018;28:1062–76.
- [24] Shi B, Zhang J, Heng J, Gong J, Zhang T, Li P, et al. RNA structural dynamics regulate early embryogenesis through controlling transcriptome fate and function. *Genome Biol* 2020;21:120.
- [25] Beaudoin JD, Novoa EM, Vejnar CE, Yartseva V, Takacs CM, Kellis M, et al. Analyses of mRNA structure dynamics identify embryonic gene regulatory programs. *Nat Struct Mol Biol* 2018;25:677–86.
- [26] Xue Z, Hennelly S, Doyle B, Gulati AA, Novikova IV, Sanbonmatsu KY, et al. A G-rich motif in the lncrna braveheart interacts with a zinc-finger transcription factor to specify the cardiovascular lineage. *Mol Cell* 2016;64:37–50.
- [27] Wang J, Zhang T, Yu Z, Tan WT, Wen M, Shen Y, et al. Genome-wide RNA structure changes during human neurogenesis modulate gene regulatory networks. *Mol Cell* 2021;81:4942–53.
- [28] Bernat V, Disney MD. RNA structures as mediators of neurological diseases and as drug targets. *Neuron* 2015;87:28–46.
- [29] Sun L, Li P, Ju X, Rao J, Huang W, Ren L, et al. *In vivo* structural characterization of the SARS-CoV-2 RNA genome identifies host proteins vulnerable to repurposed drugs. *Cell* 2021;184:1865–83.
- [30] Mizrahi O, Nachshon A, Shitrit A, Gelbart IA, Dobesova M, Brenner S, et al. Virus-induced changes in mRNA secondary structure uncover *cis*-regulatory elements that directly control gene expression. *Mol Cell* 2018;72:862–74.
- [31] Huston NC, Wan H, Strine MS, de Cesaris Araujo Tavares R, Wilen CB, Pyle AM. Comprehensive *in vivo* secondary structure of the SARS-CoV-2 genome reveals novel regulatory motifs and mechanisms. *Mol Cell* 2021;81:584–98.
- [32] Cao C, Cai Z, Xiao X, Rao J, Chen J, Hu N, et al. The architecture of the SARS-CoV-2 RNA genome inside virion. *Nat Commun* 2021;12:3917.
- [33] Ziv O, Price J, Shalamova L, Kamenova T, Goodfellow I, Weber F, et al. The short- and long-range RNA–RNA interactome of SARS-CoV-2. *Mol Cell* 2020;80:1067–77.
- [34] Jamal-Hanjani M, Wilson GA, McGranahan N, Birkbak NJ, Watkins TBK, Veeriah S, et al. Tracking the evolution of non-small cell lung cancer. *N Engl J Med* 2017;376:2109–21.
- [35] Vasan N, Baselga J, Hyman DM. A view on drug resistance in cancer. *Nature* 2019;575:299–309.
- [36] El Yacoubi B, Lyons B, Cruz Y, Reddy R, Nordin B, Agnelli F, et al. The universal YrdC/Sua5 family is required for the formation of threonylcarbamoyladenine in tRNA. *Nucleic Acids Res* 2009;37:2894–909.
- [37] Murphy FV, Ramakrishnan V, Malkiewicz A, Agris PF. The role of modifications in codon discrimination by tRNA^{Lys}_{TUU}. *Nat Struct Mol Biol* 2004;11:1186–91.
- [38] Lescrinier E, Nauwelaerts K, Zanier K, Poesen K, Sattler M, Herdewijn P. The naturally occurring N⁶-threonyl adenine in anticodon loop of *Schizosaccharomyces pombe* tRNA_i causes formation of a unique U-turn motif. *Nucleic Acids Res* 2006;34:2878–86.
- [39] Guo J, Zhu P, Ye Z, Wang M, Yang H, Huang S, et al. YRDC mediates the resistance of lenvatinib in hepatocarcinoma cells via modulating the translation of KRAS. *Front Pharmacol* 2021;12:744578.
- [40] Hinman MN, Lou H. Diverse molecular functions of Hu proteins. *Cell Mol Life Sci* 2008;65:3168–81.
- [41] Rotow J, Bivona TG. Understanding and targeting resistance mechanisms in NSCLC. *Nat Rev Cancer* 2017;17:637–58.
- [42] McGranahan N, Swanton C. Clonal heterogeneity and tumor evolution: past, present, and the future. *Cell* 2017;168:613–28.
- [43] Shi B, Heng J, Zhou JY, Yang Y, Zhang WY, Koziol MJ, et al. Phase separation of Ddx3xb helicase regulates maternal-to-zygotic transition in zebrafish. *Cell Res* 2022;32:715–28.
- [44] de Silanes IL, Zhan M, Lal A, Yang X, Gorospe M. Identification of a target RNA motif for RNA-binding protein HuR. *Proc Natl Acad Sci U S A* 2004;101:2987–92.
- [45] Abdelmohsen K, Gorospe M. Posttranscriptional regulation of cancer traits by HuR. *Wiley Interdiscip Rev RNA* 2010;1:214–29.
- [46] Schultz CW, Preet R, Dhir T, Dixon DA, Brody JR. Understanding and targeting the disease-related RNA binding protein human antigen R (HuR). *Wiley Interdiscip Rev RNA* 2020;11:e1581.
- [47] Fabbri L, Chakraborty A, Robert C, Vagner S. The plasticity of mRNA translation during cancer progression and therapy resistance. *Nat Rev Cancer* 2021;21:558–77.
- [48] Crooke ST, Baker BF, Crooke RM, Liang XH. Antisense technology: an overview and prospectus. *Nat Rev Drug Discov* 2021;20:427–53.
- [49] Roberts TC, Langer R, Wood MJA. Advances in oligonucleotide drug delivery. *Nat Rev Drug Discov* 2020;19:673–94.
- [50] Heng J, Lv P, Zhang Y, Cheng X, Wang L, Ma D, et al. Rab5c-mediated endocytic trafficking regulates hematopoietic stem and progenitor cell development via Notch and AKT signaling. *PLoS Biol* 2020;18:e3000696.

-
- [51] Calviello L, Mukherjee N, Wyler E, Zauber H, Hirsekorn A, Selbach M, et al. Detecting actively translated open reading frames in ribosome profiling data. *Nat Methods* 2016;13:165–70.
- [52] Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 2014;30:2114–20.
- [53] Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet J* 2011;17:10–2.
- [54] Langmead B, Trapnell C, Pop M, Salzberg SL. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biol* 2009;10:R25.
- [55] Chen T, Chen X, Zhang S, Zhu J, Tang B, Wang A, et al. The Genome Sequence Archive Family: toward explosive data growth and diverse data types. *Genomics Proteomics Bioinformatics* 2021;19:578–83.