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Supplemental information

HNRNPA2B1 as a trigger of RNA switch modulates the miRNA-mediated regulation of CDK6

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Supplemental Information

Figure S1.

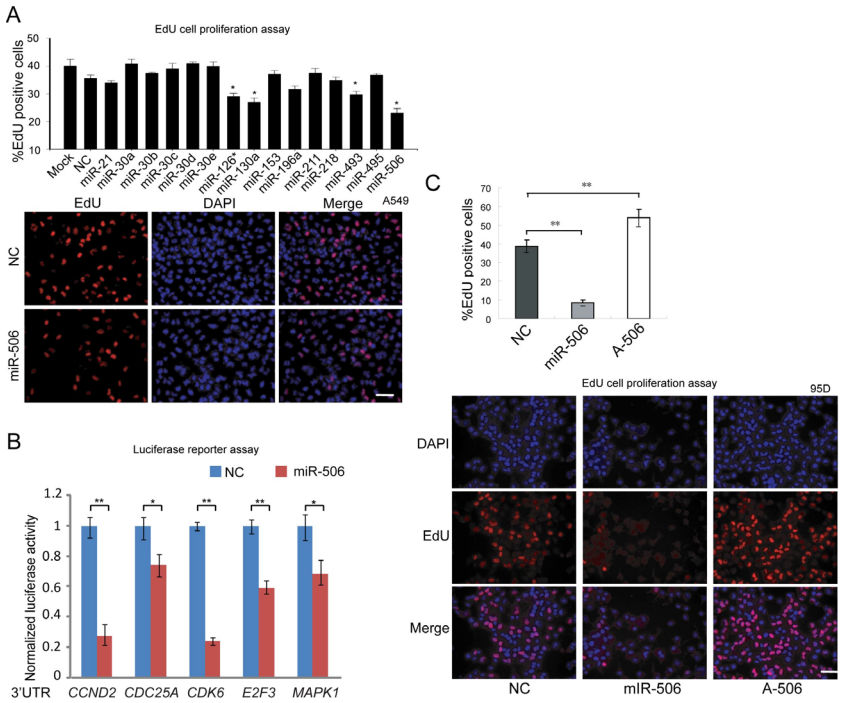

Figure S1. The function and target identification of miR-506, related to Figure 1.

(A) Edu cell proliferation assay of miRNAs result in A549 cells. A549 cells were transfected with 50 nM miRNA mimics. Forty-eight hours after transfection, EdU (50 mM) was added and the cells were cultured for two hours. The white scale bar represents 40 μm. EdU assay was performed as described in method. Data are presented as mean±SD (n = 3). *P* values were determined by Student's t test, * p < 0.05, **p < 0.01.

(B) The luciferase reporter assay of candidate target genes' 3'UTR was performed in 95D lung cancer cells. Data are presented as mean±SD (n = 4). *p* values were determined by Student's *t* test, * *p* < 0.05, ** *p* < 0.01.

(C) The detection of cell proliferation in 95D cell by EdU assay. The 95D lung cancer cells were transfected with No Target Control (NC), miR-506 and Antagomir to miR-506 (A-506) respectively for forty-eight hours. Then the cells were tested with the EdU proliferation assay. The EdU positive percentages were analyzed through BD855 pathway. The white bar presents 40 μm. Data are presented as mean ±SD (n = 3). *p* values were determined by Student's *t* test, * *p* < 0.05, ** *p* < 0.01.

Figure S2

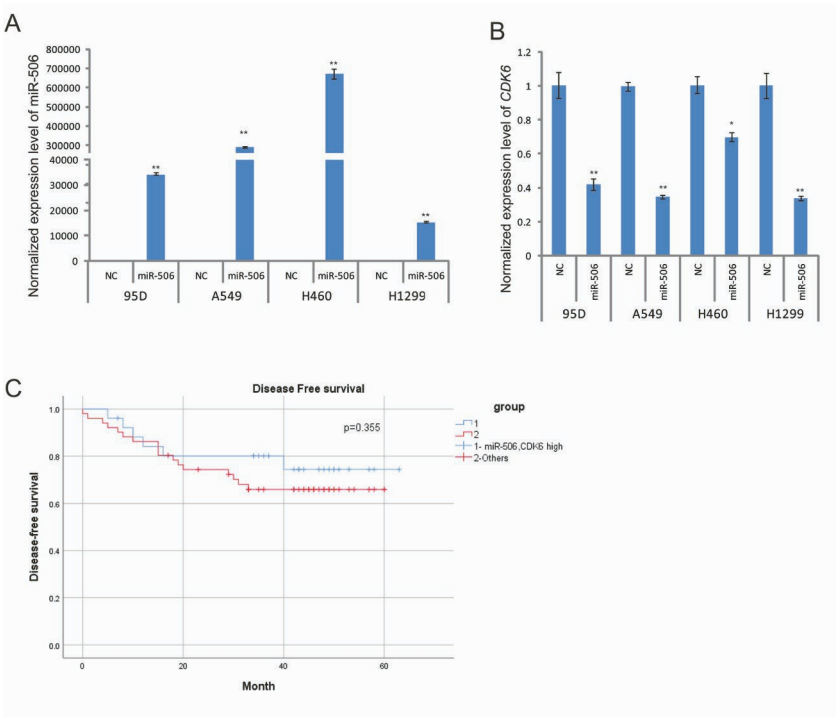

Figure S2. The regulation of miR-506 on CDK6, related to Figure 2.

(A) miR-506 levels in different lung cancer cells transfected with miR-506. Lung cancer cells were transfected with no target control (NC) and miR-506. Forty-eight hours after transfection, the cells were harvested for RT-qPCR analysis. *U6* was used as a reference gene for miR-506 RT-qPCR. Data are presented as mean ±SD (n = 4). * $p < 0.05$, ** $p < 0.01$

(B) *CDK6* mRNA levels in different lung cancer cells. Lung cancer cells were transfected with no target control (NC) and miR-506. Forty-eight hours after transfection, the cells were harvested for RT-qPCR analysis. The mRNA expression was normalized to *β-Actin*. Data are presented as mean ±SD (n = 4). * $p < 0.05$, ** $p< 0.01$. (C) Kaplan–Meier survival curves for disease-free survival of the lung cancer patients. The both upregulated expression level of miR-506 and *CDK6* were used for grouping the samples. The data was compared using the log-rank test.

Figure S3

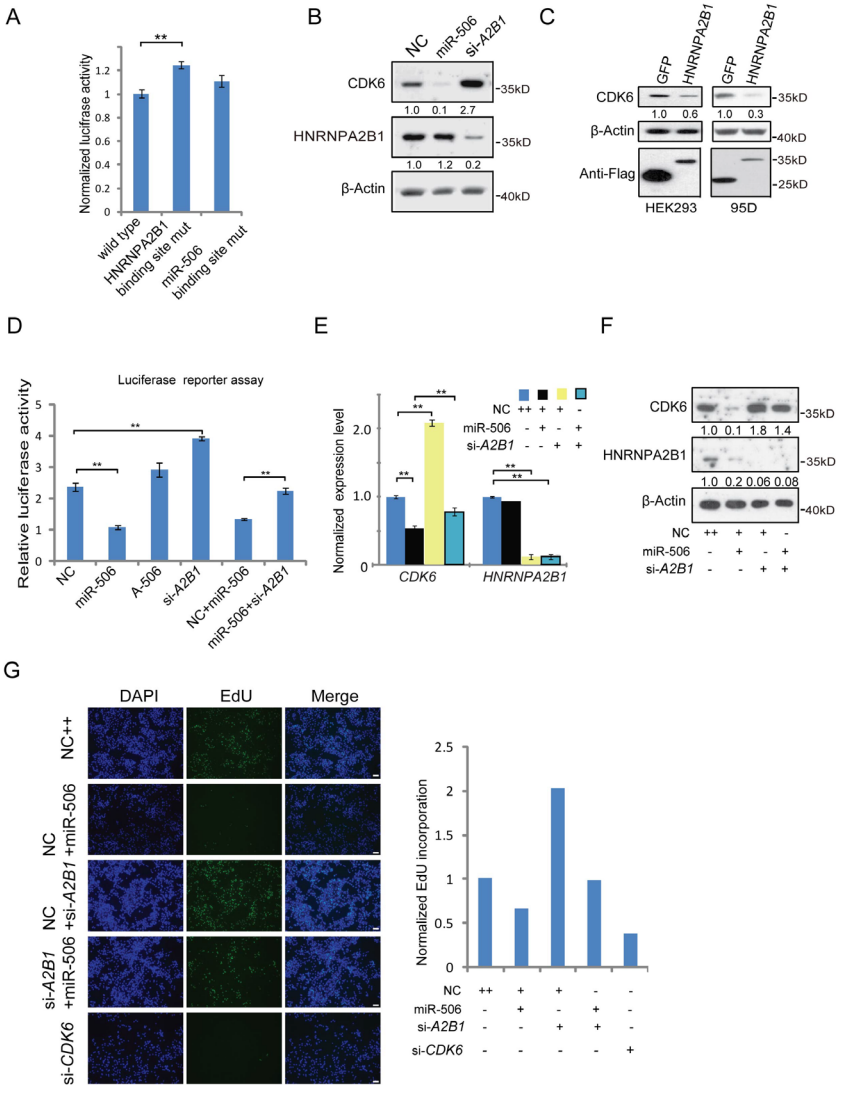

Figure S3. HNRNPA2B1 regulates miR-506-mediated CDK6 suppression, related to Figure 3.

(A) The dual luciferase reporter assay of CDK6 3'UTR reporter vector. The binding site of HNRNPA2B1 in the *CDK6* 3'UTR was mutated and tested with luciferase reporter assay in 95D cells. Data are presented as mean±SD (n = 4). * $p < 0.05$, ** $p< 0.01$.

(B) The western blot of CDK6 in 95D lung cancer cells. The protein level of CDK6 was detected after transfection with NC, miR-506 and siRNA-*HNRNPA2B1* (si-*A2B1*) in lung cancer cell. Forty-eight hours after transfection, the CDK6 protein levels were measured with western blot. *β*-Actin was used as a reference gene for western blot.

(C) Representative western blot of exogenous HNRNPA2B1-Flag, CDK6 and quantification result in 95D and HEK293 cells. HEK293 and 95D cells were transfected with GFP (control) and HNRNPA2B1-Flag expression vectors. *β*-Actin was used as a reference gene for western blot.

(D) The *CDK6* 3'UTR dual luciferase reporter assay. The wild-type *CDK6* 3'UTR luciferase

reporter vector was transfected with miR-506 and siRNA-*HNRNPA2B1* individual or co-transfected with miR-506 and siRNA-*HNRNPA2B1* (si-*A2B1*) in 95D cells. Data are presented as the mean ± SD (n = 4). * $p < 0.05$, ** $p < 0.01$.

(E) RT-qPCR detection of *CDK6* mRNA levels in HeLa cells after transfection. Forty-eight hours after transfection, the *HNRNPA2B1* and *CDK6* mRNA levels were measured with RT-qPCR. The mRNA expression was normalized to *β-Actin*. Data are presented as the mean SD (n = 4). *p* values were determined by one-way ANOVA test, * $p < 0.05$, ** $p < 0.01$.

(F) Western blot detection of the CDK6 protein level in HeLa cells were treated as in (C). Forty-eight hours after transfection, western blot assays were conducted. *β*-Actin was used as a reference gene for western blot.

(G) The EdU proliferation assay in 95D cells. The EdU proliferation assay was performed forty-eight hours after transfection of mimics and siRNA. Then the cells were tested with the EdU proliferation assay. The proliferation cells were marked with green probe. The white scale bar represents 40 μm.

Figure S4.

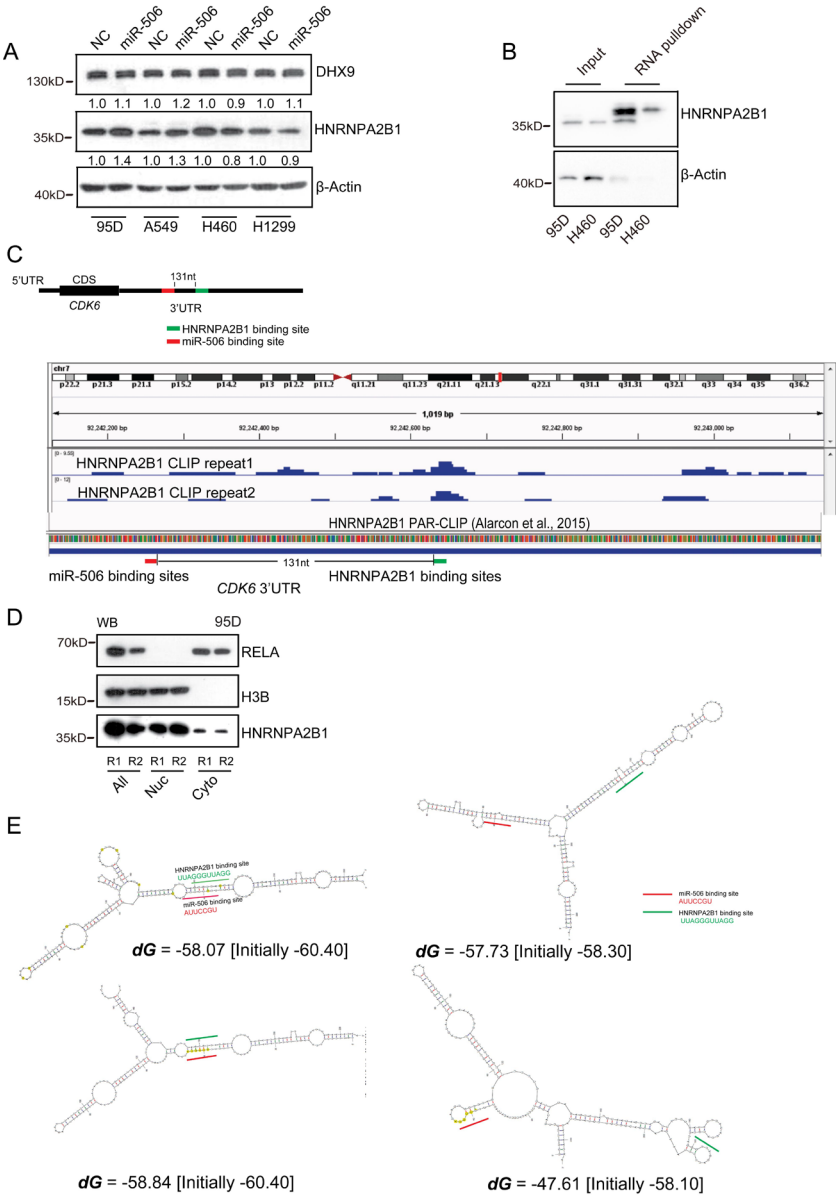

Figure S4. The characteristics of HNRNPA2B1 and *CDK6* 3'UTR, related to Figure 4.

(A). Western blot of HNRNPA2B1 and DHX9 in 95D, A549, H460, H1299 cells. Lung cancer cells were transfected with no target control (NC) and miR-506. Forty-eight hours after transfection, cells were collected for western blot. Cell lysates were probed for the protein levels of HNRNPA2B1. *β*-Actin was used as a reference gene for western blot.

(B) Western blot assay detection of the enrichment of HNRNPA2B1 in 95D and H460 cells RNA pull down samples. 95D and H460 cell were grown to around 80% confluence. Cells were lysed for RNA pull down. RNA pull down assays were performed with the same *CDK6* 3'UTR RNA. Pull down proteins were tested with western blot.

(C) The HNRNPA2B1 binding site in *CDK6* 3'UTR. Bar graph of *CDK6* 3'UTR and sequence analysis of miR-506 and HNRNPs binding site in *CDK6* 3'UTR (up) were shown. IGV (Integrative Genomics Viewer) snapshot shows HNRNPA2B1 (green bar) and miR-506 binding

sites (red bar) in PAR-CLIP data (Alarcon et al., 2015).

(D) The subcellular location of HNRNPA2B1 was tested with western blot. Fractions were analyzed by western blot for protein markers of cytoplasm (RELA/p65), nucleus (histone 3B H3B). R1 (Repeat 1), R2 (Repeat 2).

(E) The representative predicted RNA conformation of *CDK6* 3'UTR. (http://unafold.rna.albany.edu/?q=mfold/RNA-Folding-Form2.3).

Figure S5.

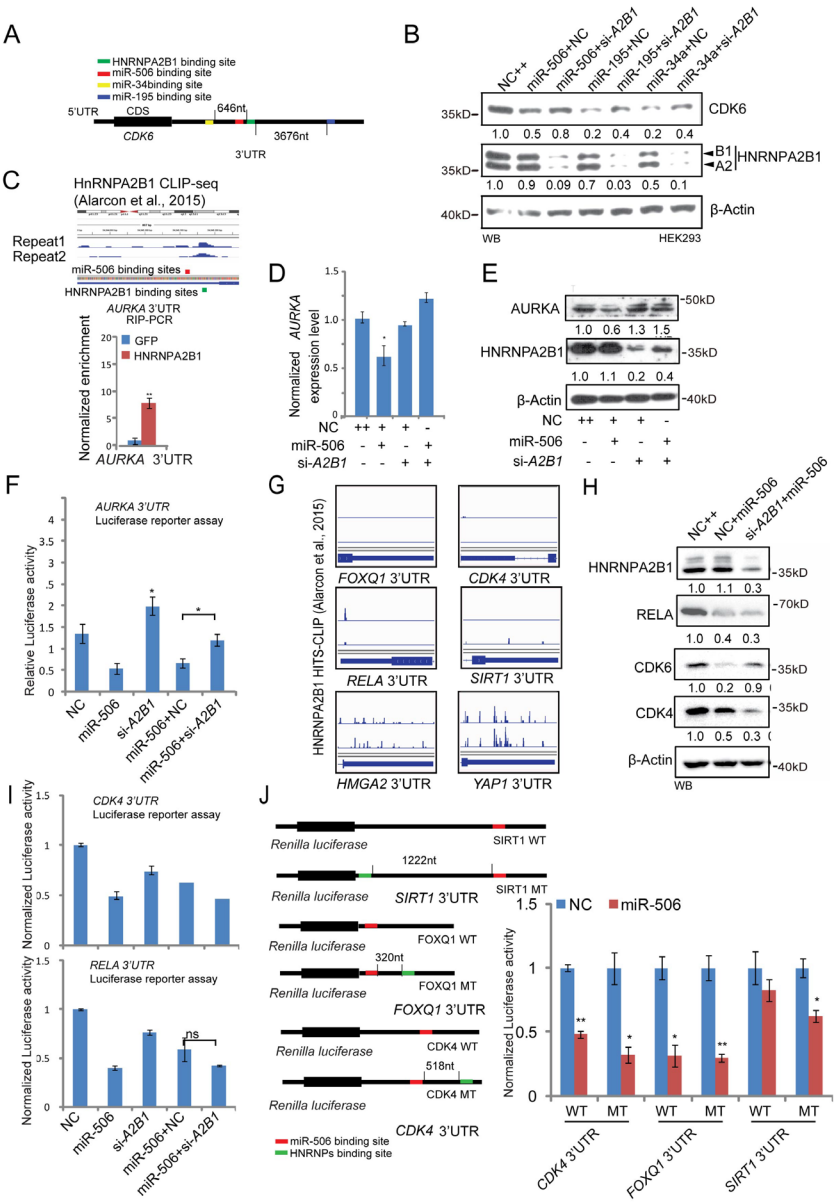

Figure S5. HNRNPA2B1 regulates miRNAs–mediated gene suppression, related to the text results-"**HNRNPA2B1 relieves other miRNA-mediated suppression of different genes**".

(A) The bar graph of predicted miRNAs binding sites in *CDK6* 3'UTR (TargetScan 7.0). The green bar shows HNRNPA2B1 binding sites, the red, yellow and blue bars present binding sites of miR-506, miR-34a and miR-195 respectively.

(B) The detection of protein levels in HEK293. Cells were transfected with mimics and siRNA. Then cells were harvested forty-eight hours after transfection. The western blot was conducted to test the protein level of CDK6 and HNRNPA2B1. *β*-Actin was used as a reference gene western blot.

(C) Integrative Genomics Viewer (IGV) snapshot of the HNRNPA2B1 (Green bar) and miR-506 (Red bar) binding sites in *AURKA* 3'UTR (up). The data was referred form PAR-CLIP-seq

(Alarcon et.al, 2015). The enrichment of the *AURKA* 3'UTRs in HNRNPA2B1 was tested by RIP-PCR with an exogenous HNRNPA2B1-Flag expression sample (down). RIP of HNRNPA2B1 was performed using a Flag monoclonal antibody. (Left) RIP-PCR. Data are presented as the mean ±SD (n = 3). *p* values were determined by Student's t test, * $p < 0.05$, ** $p < 0.01$.

(D) The RT-qPCR detection of *AURKA* mRNA levels in Hela. Cells were transfected with mimics and siRNA. Forty-eight hours after transfection the *AURKA* mRNA levels were tested. mRNA expression was normalized to *β-Actin*. Data are presented as mean ±SD (n = 3). *P* values were determined by Student's *t* test, * $p < 0.05$, ** $p < 0.01$.

(E) The western blot detection of HNRNPA2B1 and AURKA protein levels in Hela cell. The cells were treated as same as (D.) Forty-eight hours after transfection the western blot assay was conducted. *β*-Actin was used as a reference gene for western blot.

(F) The *AURKA* 3'UTR reporter assay in 95D cells. The 95D cells were cotransfection of NC, miR-506, si-*HNRNPA2B1* (si-*A2B1*) and reported vectors. Data are presented as mean SD (n = 4), * $p < 0.05$, ** $p < 0.01$.

(G) Integrative Genomics Viewer (IGV) snapshot depicting HNRNPA2B1 enrichment (HIST-CLIP-seq data, Alarcon et.al, 2015) in *FOXQ1, CDK4, RELA, SIRT1, HMGA2 and YAP1*.

(H) Western blot detection of CDK6, CDK4 and RELA in 95D lung cancer cell. Forty-eight hours after transfection with NC++, miR-506+NC, siRNA-*HNRNPA2B1*+miR-506 (si-*A2B1*+miR-506). CDK6, CDK4 and RELA protein levels were tested by western blot. *β*-Actin was used as a reference gene for western blot.

(I) The 3'UTR of CDK4 and *RELA* reporter assay in 95D cells. The 95D cells were cotransfection of NC, miR-506, si-*HNRNPA2B1* (si-*A2B1*) and reported vectors. Data are presented as mean SD (n = 4), ns, no significance.

(J) The 3'UTR of *SIRT1*, FOXQ1 and CDK4 reporter assay in 95D cells. The bar graph showed wild type (WT) and binding sites of HNRNPA2B1 insertion mutation (MT) of CDK4,FOXQ1 and *SIRT1* 3'UTR reporter vectors. The 95D cells were cotransfection with NC, miR-506, and reported vectors. Luciferase activity was tested forty-eight hours after transfection. *Firefly* luciferase activity was normalized to the *Renilla* luciferase internal control. Data are presented as the mean ± SD (n = 4). *P* values, * $p < 0.05$, ** $p < 0.01$, Mann-Whitney *U* test.

Figure S6.

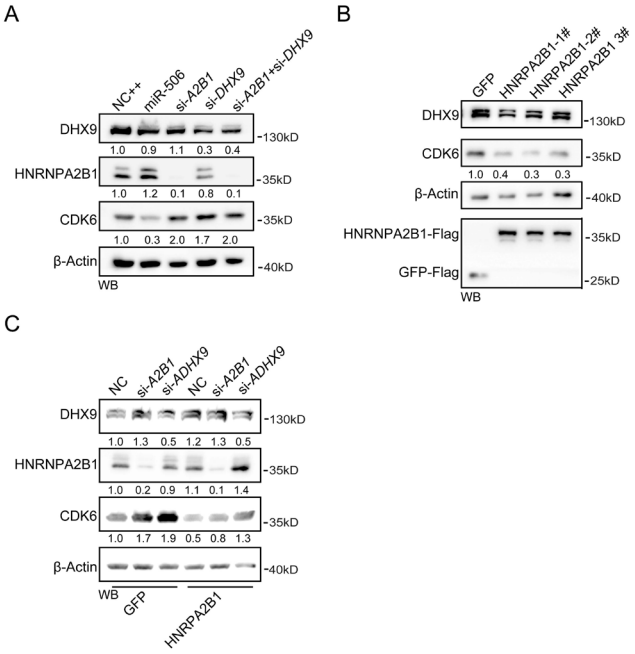

Figure S6. HNRNPA2B1 and DHX9 involve in miR-506 mediated gene regulation, related to Figure 5.

(A) Western blot detection of DHX9 and HNRNPA2B1. HeLa cells were transfected with NC, miR-506, siRNA-*HNRNPA2B1* (si-*A2B1*) and siRNA-*DHX9* (si-*DHX9*). Forty-eight hours after transfection, protein levels were analyzed by western blot. *β*-Actin was used as a reference gene for western blot.

(B) Western blot detection of CDK6 in H460 cells. Forty-eight hours after transfection with vector expressing GFP and HNRNPA2B1, CDK6 protein levels were tested by western blot. *β*-Actin was used as a reference gene for western blot.

(C) Western blot detection of CDK6 in H460 cells. Forty-eight hours after co-transfection with vector expressing GFP and HNRNPA2B1 and NC, siRNA-*DHX9* (si-*DHX9*), CDK6 protein levels were tested by western blot. *β*-Actin was used as a reference gene for western blot.

Figure S7

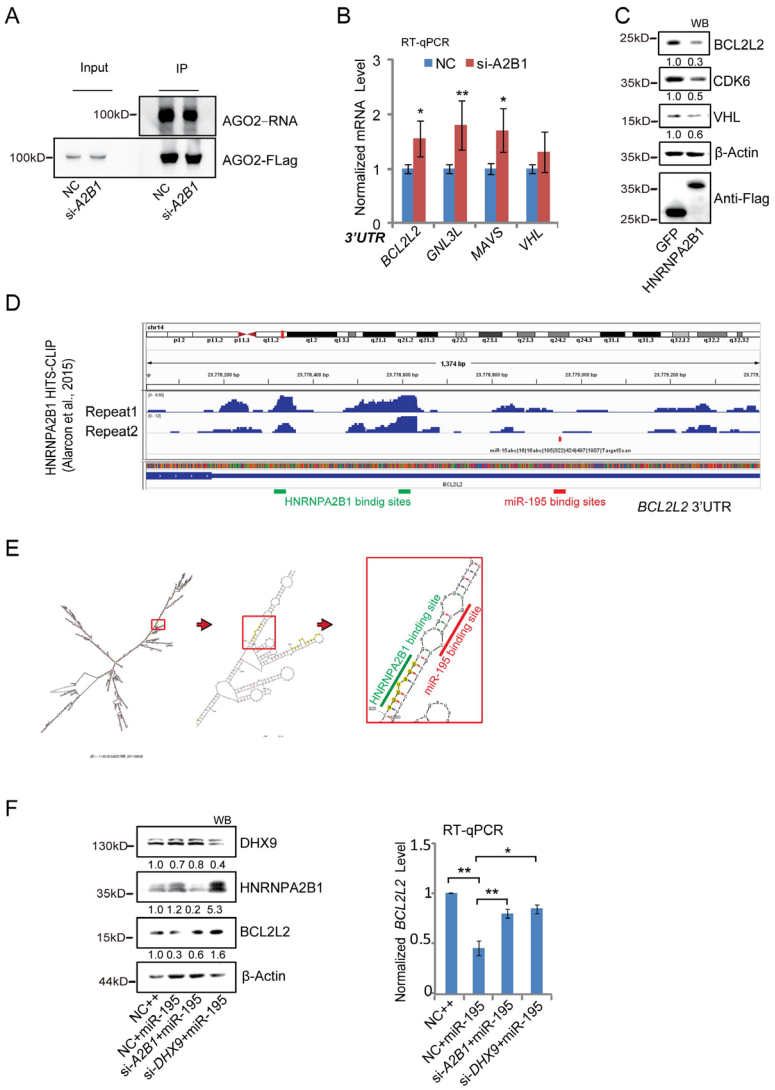

Figure S7. HNRNPA2B1 regulates the expression of other genes, related to Figure 6.

(A) HEK293 cells were co-transfected with NC or siRNA-*HNRNPA2B1* (si-*A2B1*) and AGO2-Flag for 48 hours as indicated. The cell lysates were then subjected to PAR-CLIP using anti-Flag. The pulled down RNA products in the RNA-AGO2 complex were labeled by Biotin and detected by biotin chemiluminescent nucleic acid kit. immunoprecipitate(IP), immunoblotting(IB).

(B) The RT-qPCR detection of *BCL2L2*, *GNL3L*, *MAVS* and *VHL* mRNA levels in 95D cells. The 95D cells were treated with siRNA-*HNRNPA2B* (si-*A2B1*). mRNA expression was normalized to *β-Actin*. Data are presented as mean ±SD (n = 3). *p* values were determined by Student's *t* test,* $p < 0.05$, ** $p < 0.01$.

(C) The western blot detection of BCL2L2, CDK6 and VHL in HEK293 cells. The HEK293 cells were transfected with expression vectors of HNRNPA2B1-Flag and GFP-Flag. Forty-eight hours after transfection, BCL2L2, CDK6 and VHL protein levels were tested by western blot. *β*-Actin was used as a reference gene for western blot.

(D) Integrative Genomics Viewer (IGV) snapshot depicting HNRNPA2B1 (HIST-CLIP-seq data, Alarcon et.al, 2015) in *BCL2L2* 3'UTR. The blue peaks mean the HNRNPA2B1 binding sites.
(E) The graph of predicted structure of *BCL2L2* predicted by Mfold soft (http://unafold.rna.albany.edu/?q=mfold/RNA-Folding-Form2.3). The red tangle presents the predicted stem structure in *BCL2L2* 3'UTR.
(F) Western blot (left) detection of expression level of BCL2L2, HNRNPA2B1 and DHX9 in HEK293 cells. HEK293 cells were transfected with mimics and siRNAs. Forty-eight hours after transfection, the cells were harvested for protein expression analysis by western blot. *β*-Actin was used as a reference gene for western blot. RT-qPCR (right) detection of the expression levels of *BCL2L2* in HEK293 cells. Forty-eight hours after transfection, the RNA was extracted for RT-qPCR analysis. The mRNA expression was normalized to *β-Actin*. Data are presented as the mean ±SD (n = 4). * $p < 0.05$, ** $p < 0.01$, one-way ANOVA test.

Table S3. Information of the qPCR primer sequences, related to Key Resourse Table.

Primer name	Forward sequence (5'-3')	Reverse sequence (5'-3')
CDK6 RIP-PCR	AGTGCTCAGTTGGCTCTAGTAAC	GCAGCAACCTCCATTCTTTGAAA
AURKA RIP-PCR	CAATGCTCAGAGAAGTAC	CACGATTCCTAAGACTGTT
BCL2L2 RIP-PCR	GTAGGCGATTGGAAGAGT	CCTGGAACGACTACATCT
VHL RIP-PCR	GGTTCCTTCCTTAGTTTCAAA	CACCACCTTCTCCTGATAA
MAVS RIP-PCR	TTGGGCAAGGGATTTATCT	ACTCATCCTAAGACCTACATC
GNL3 RIP-PCR	CCAAGTCCATAGGTCTTCA	ATTCCTCCAATTCACAATCTG
CDK6 RT-qPCR	CCAGATGGCTCTAACCTCAGT	AACTTCCACGAAAAAGAGGCTT
HNRNPA2B1 RT-qPCR	GGTTATGGAGGAGGAAGAG	TAGTTAGAAGGTTGCTGGTTA
ACTB RT-qPCR	CATGTACGTTGCTATCCAGGC	CTCCTTAATGTCACGCACGAT
miR-506 RT-qPCR	TA*AGGC*ACCCTTCTG (LNA substitutions are preceded by a '*')	CCTGACTGACTGGGGCAAGA
miR-506 RT primers	CCTGACTGACTGGGGCAAGATCTACTCAGAAG	

Table S4. Information of the FISH probe sequences, related to Key Resourse Table.

Probe name	Sequence (5'-3')	3' labeling
CDK6_01	AGTTAGTTTGGTTTCTCTGTC	Cy3
CDK_02	TGCACTACTCGGTGTGAATGAAG	Cy3
CDK6_03	ACTGAGGTTAGAGCCATCTGG	Cy3
CDK6_04	AGGCTTTCTACGAAACATTTC	Cy3
CDK6_05	ACTTCAGAAGTAGGTCTTTGC	Cy3
CDK6_06	ATTGGTTGGGCAGATTTTG	Cy3