MDM2 overexpression deregulates the transcriptional control of RB/E2F leading to DNA methyltransferase 3A overexpression in lung cancer

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ONLINE DATA SUPPLEMENT

Supplementary figures

Supplementary Figure S1. RB represses the activity of promoter containing E2F1-binding site.

Dual luciferase assays were performed using the empty vector (pTA-Luc) and pE2F-luciferase construct (pE2F-Luc), which contains four repetitive E2F1-binding sites.
pE2F-Luc promoter activity was downregulated by ectopically expressed RB in 5637 cells (A) and upregulated by RB knockdown (si-RB) in H1299 cells (B) compared to control. All experiments were performed at least three times. Quantitative data are presented as mean ± SD. $P$ values are as indicated.
Supplementary Figure S2. RB downregulates DNMT3A protein expression in four human cancer cell lines.

(A) Western blot analysis showed the repression of DNMT3A protein expression by ectopically expressed RB in A549, H1299, 5637, and Saos2 cells. (B) Quantitative analysis of relative DNMT3A protein expression in cells transfected with RB expression plasmid comparing to control vector pCMVSPORT6 of three independent experiments is shown. Quantitative data are presented as mean ± SD. P values are as indicated.
Supplementary Figure S3. RB represses DNMT3A protein levels.

The quantitative analyses of DNMT3A protein levels in cells overexpressing RB or with RB knockdown from Fig. 1C are presented as mean ± SD. $P$ values are as indicated.
Supplementary Figure S4. The validation and primary data of qMSP assays.

(A) Quantitative MSP assays were performed using DNA of normal lung cell IMR90 (IMR90) and SssI enzyme-treated IMR90 DNA (IMR90-SssI), which serve as positive control for unmethylated and methylated alleles, respectively. The methylation levels of SssI-treated IMR90 DNA of five tumor suppressor genes were significantly higher than untreated IMR90 DNA. All experiments were performed at least three times. Quantitative data are presented as mean ± SD. P values are as indicated. (B) The primary data of the $C_T$ value of $FHIT$ and $ACTIN$ genes generated by ABI PRISM 7000 (version 1.1 software) and the $2^{-\Delta\Delta C_T}$ determination method were shown as an example.
Supplementary Figure S5. Overexpression of RB leads to reduction in promoter methylation of multiple TSGs.

Quantitative MSP assays for methylation status in the RARβ, FHIT, and RASSF1A promoters in 5637 cells overexpressing RB. All experiments were performed at least three times. Quantitative data are presented as mean ± SD. P values are as indicated.
Supplementary Figure S6. RB reduces global 5’-methylcytosine.

Global 5’-methylcytosine was measured at 72 hours by immunofluorescence staining with anti-5-methylcytosine in H1299 (A) and 5637 (B) cells after RB overexpression or knockdown. Experiments were performed at least three times. Results from one representative experiment are shown. DAPI counterstaining was used to justify the cell density, and quantitative data are presented as mean ± SD. P values are as indicated.
Supplementary Figure S7. DAPA assay shows the binding of RB and E2F1 to the DNMT3A P2 region.

DAPA assay using 3A P2 probe with E2F1 binding site was performed for analysis of RB and E2F1 binding status. Binding specificity tests using non-biotin-labeled 3A P2 (competitor) and 3A P2 sequences with mutations at E2F site (mut) are shown. The DNMT1 probe, which has been shown to be bound by E2F/RB complex, was used as a positive control. Experiments were performed at least three times. Results from one representative experiment are shown.
Supplementary Figure S8. E2F1 positively regulates DNMT3A protein expression in 5637 and H1299 cancer cells.

Western blot analysis of DNMT3A protein level in 5637 and H1299 cells with overexpressed E2F1 (A) or with si-E2F1 (B). Quantitative analysis of relative DNMT3A protein expression in cells overexpressing E2F1 or with E2F1 knockdown compared to control cells from three independent experiments is shown. Quantitative data are presented as mean ± SD. $P$ values are as indicated.
Supplementary Figure S9. Model for the transcription regulation mediated by MDM2/RB on DNMT3A promoter. 

(A) RB and E2F1 bind on the DNMT3A promoter and negatively regulate DNMT3A gene expression, lead to the repression of DNMT3A mRNA and protein expression levels. (B) Transcriptional repression of DNMT3A expression by RB/E2F1 pathways can be diminished by MDM2 overexpression through ubiquitination-mediated degradation of RB protein.