RNA editing enzyme ADAR1 governs the circadian expression of P-glycoprotein in human renal cells by regulating alternative splicing of the *ABCB1* gene

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EXPERIMENTAL PROCEDURES

Construction of ADAR1-overexpressing RPTECs

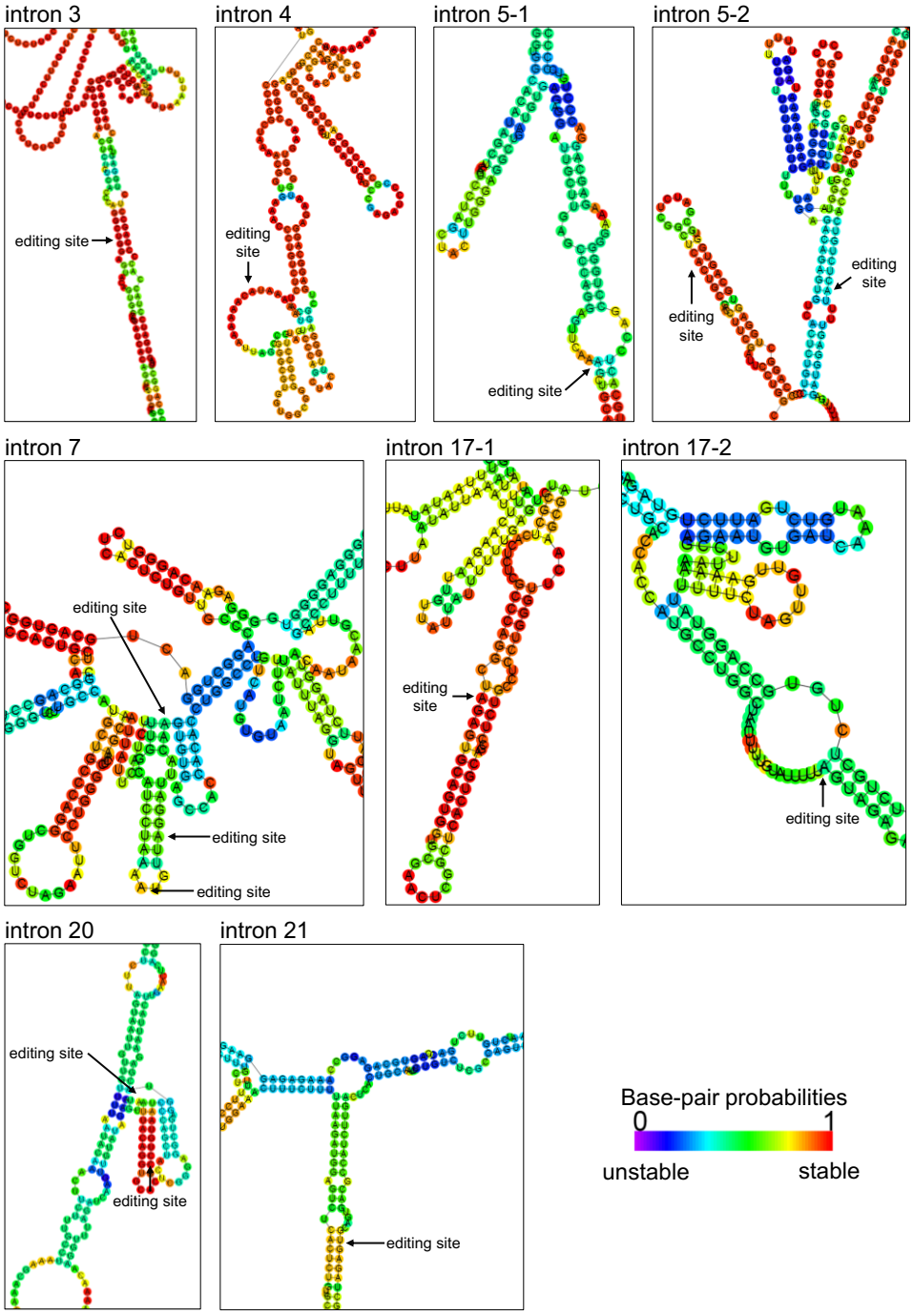
The ORF sequence of human *ADAR1* with C-terminal GFP-tag was amplified with ADAR1 Human Tagged ORF Clone (RG219761; Origene Technologies, Inc., Rockville, MD) as a template, and subcloned into pLVSIN-CMV Pur Vector (Takara Bio Inc.) using In-Fusion HD Cloning Kit (Takara Bio Inc.) and primer sets as follows: forward 5′-CGGTGAATTCCTCGAGCCGCCGCGATCGCCATG-3′, reverse 5′-TAGAACTAGTCTCGATTAAACTCTTTCTTCACCGG-3′. Lentiviral particles were prepared by the Lentiviral High Titer Packaging Mix with pLVSIN series (Takara Bio Inc.) with Lenti-X 293 T cell lines. RPTECs were infected with ADAR1-expressing lentivirus and maintained in a medium containing 5 μg/mL of puromycin (FUJIFILM Wako Pure Chemical Corporation). Overexpression of ADAR1 with GFP-tag was confirmed by Western blotting.

Direct Sequencing

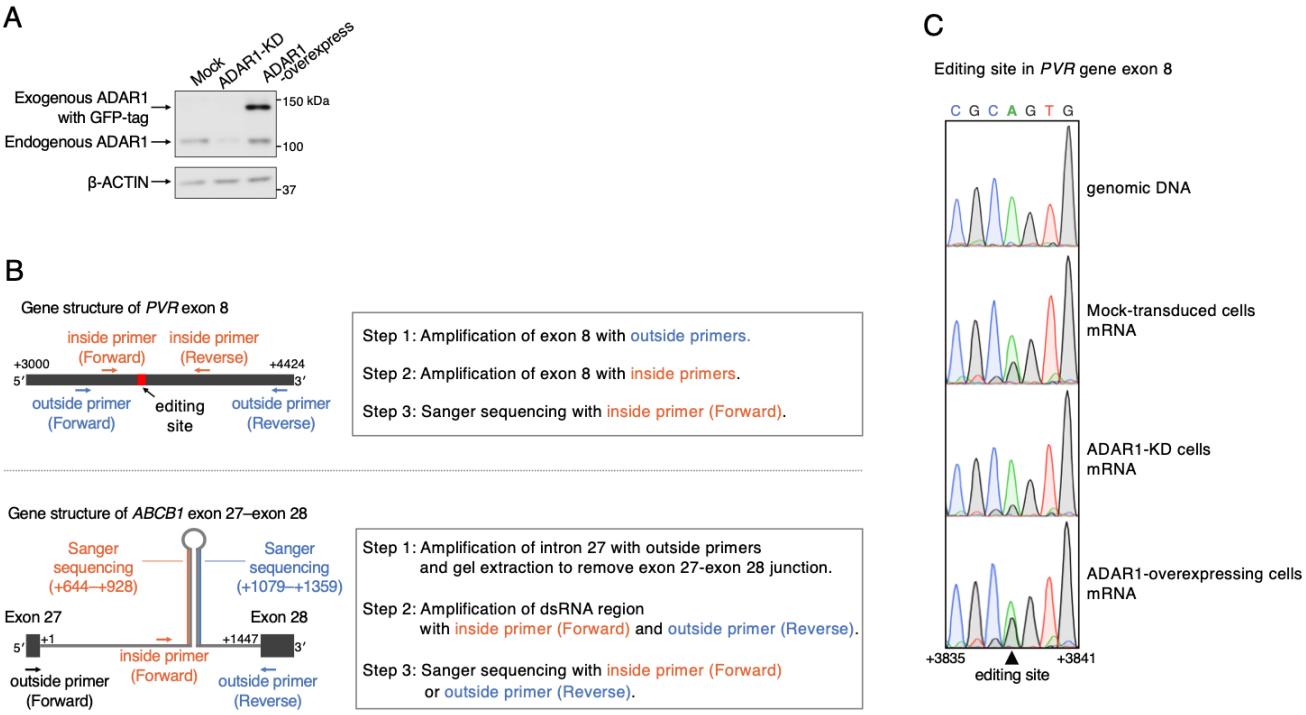
RNA was extracted using the ReliaPrep RNA Cell Miniprep System (Promega) and treated with DNase I on a column. A total of 500 ng of RNA was used for cDNA synthesis with ReverTra Ace qPCR RT Kit (TOYOBO Co., Ltd.). Genomic DNA was extracted using Wizard Genomic DNA Purification Kit (Promega). Direct sequencing for exon 8 of the *PVR* gene was conducted as follows. The cDNA and genomic DNA were amplified by the GoTaq Green Master Mix (Promega) with gene-specific primers (Human *PVR* exon 8 outside; **Supplementary Table S1**). The PCR-amplified products were used for 2nd amplification as templates with another gene-specific primers (Human *PVR* exon 8 inside; **Supplementary Table S1**). The PCR-amplified products were analyzed by Sanger sequencing using the same primers used in 2nd amplification. Direct sequencing for intron 27 of the *ABCB1* gene was conducted as follows. The cDNA and genomic DNA were amplified by the GoTaq Green Master Mix (Promega) with gene-specific primers (Human *ABCB1* intron 27 outside; **Supplementary Table S1**). PCR-amplified products were separated by electrophoresis using 1% agarose gel containing ethidium bromide. PCR-amplified products of intron 27-containing transcripts (maybe both retained intron 27 and pre-mRNA), which were 1,590 bp in length, were extracted and purified using QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). The purified DNA was amplified by the GoTaq Green Master Mix (Promega) with another gene-specific primers (Human *ABCB1* intron 27 inside Forward and outside Reverse; **Supplementary Table S1**). The PCR-amplified products were analyzed by Sanger sequencing using the same primers used in 2nd amplification. Electropherograms were aligned using SnapGene Viewer (https://www.snapgene.com/snapgene-viewer/).

Supplementary Table S1 Primer sets for direct sequencing

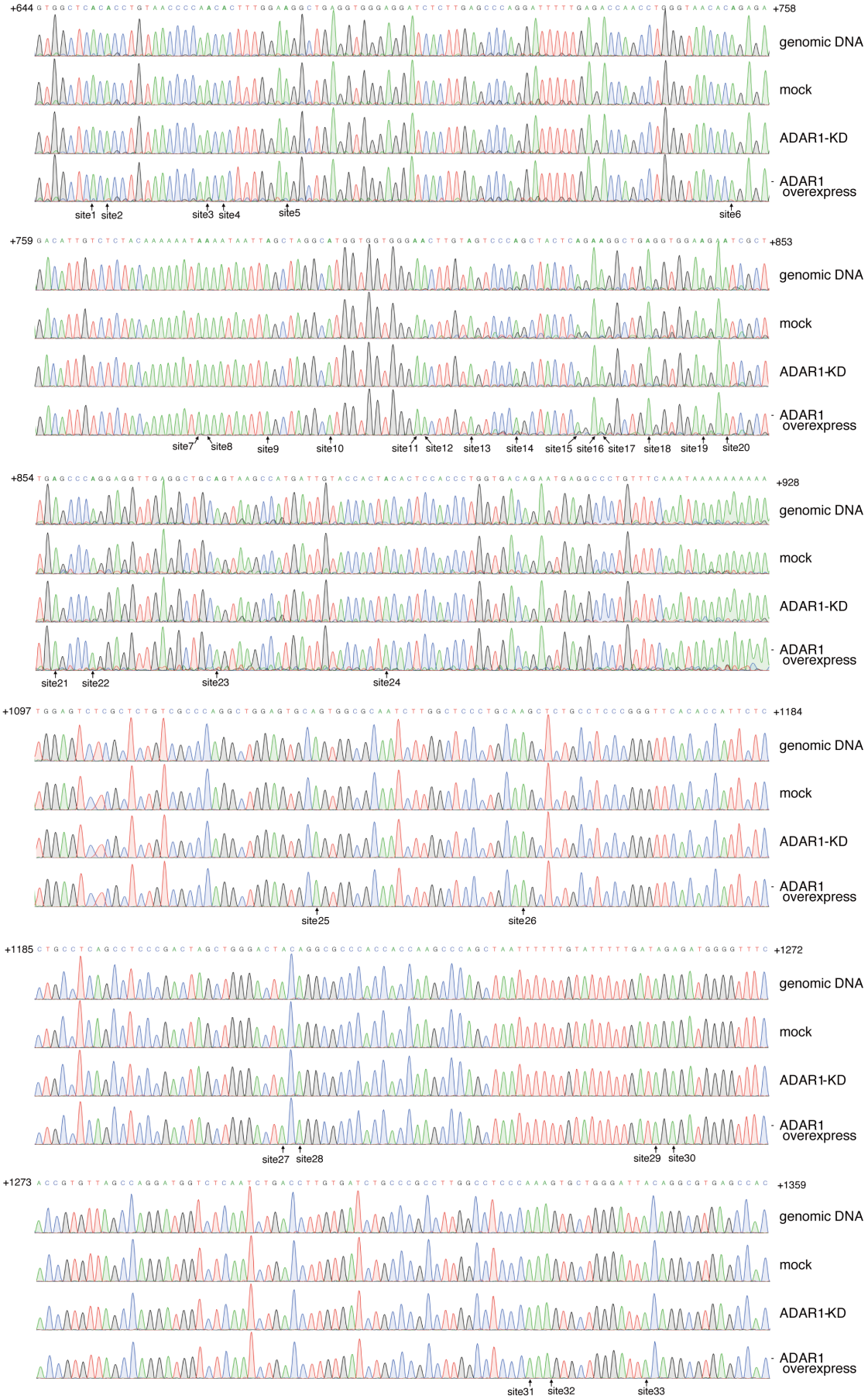
Gene	Primers
Human *PVR* exon 8 outside	
Forward	5′-CACAGAGCCAGGAATGGAGAGTG-3′
Reverse	5′-ATGTTCCAACCACCCAGACAGAGG-3′
Human *PVR* exon 8 inside	
Forward	5′-GGCTAAAACACTCCACACCCTC-3′
Reverse	5′- ACAAACAGCAGAGCAGCAGA-3′
Human *ABCB1* intron 27 outside	
Forward	5′-AGCTCGTGCCCTTGTTAGAC-3′
Reverse	5′-GGCGGTGAGCAATCACAATG-3′
Human *ABCB1* intron 27 inside	
Forward	5′-ATGGAGTCTCGCTCTGTCG-3′



Supplementary Figure S1 *In silico* prediction of RNA secondary structure of introns of the human *ABCB1* gene by RNAfold. The minimum free energy structure with base-pair probabilities calculated to have the lowest value of free energy. Base-pair probabilities are shown by a color spectrum. The RNA editing sites registered in REDIportal are indicated by arrows.



Supplementary Figure S2 Sequence analysis for exon 8 of the human *PVR* mRNA and intron 27 of the human *ABCB1* pre-mRNA. *A,* Expression of endogenous and exogenous ADAR1 protein in mock-transduced, ADAR1-KD, and ADAR1-overexpressing RPTECs. The protein levels of β-ACTIN are shown as loading controls. *B,* Upper panels show schematic image of procedure of amplification of exon 8 region of the human *PVR* gene for direct sequencing. Lower panels show schematic image of procedure of amplification of intron 27 region of *ABCB1* pre-mRNA for direct sequencing. *C*, Electropherograms around A-to-I editing site of the human *PVR* gene exon 8, which is a well-known A-to-I editing site (1). This region was used as positive control for ADAR1-mediated A-to-I editing. The sequence data are obtained from mock-transduced, ADAR1-KD, and ADAR1-overexpressing RPTECs.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genomic DNA
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ADAR1-KD
ADAR1 overexpress
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site3
site4
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ADAR1-KD
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ADAR1-KD
ADAR1 overexpress
site31
site32
site33

Supplementary Figure S3 Electropherograms from direct sequencing of the PCR-amplified product of intron 27 of the human *ABCB1* pre-mRNA. The sequence data are obtained from pre-mRNA extracted from mock-transduced, ADAR1-KD, and ADAR1-overexpressing RPTECs. The RNA editing sites registered in REDIportal are indicated by arrows.

REFERENCE

1. Hong, H., An, O., Chan, T. H. M., Ng, V. H. E., Kwok, H. S., Lin, J. S., Qi, L., Han, J., Tay, D. J. T., Tang, S. J., Yang, H., Song, Y., Bellido Molias, F., Tenen, D. G., and Chen, L. (2018) Bidirectional regulation of adenosine-to-inosine (A-to-I) RNA editing by DEAH box helicase 9 (DHX9) in cancer. *Nucleic Acids Res.* **46**, 7953–7969