Supporting Information

Human constitutive androstane receptor represses liver cancer development and hepatoma cell proliferation by inhibiting erythropoietin signaling

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The file includes:

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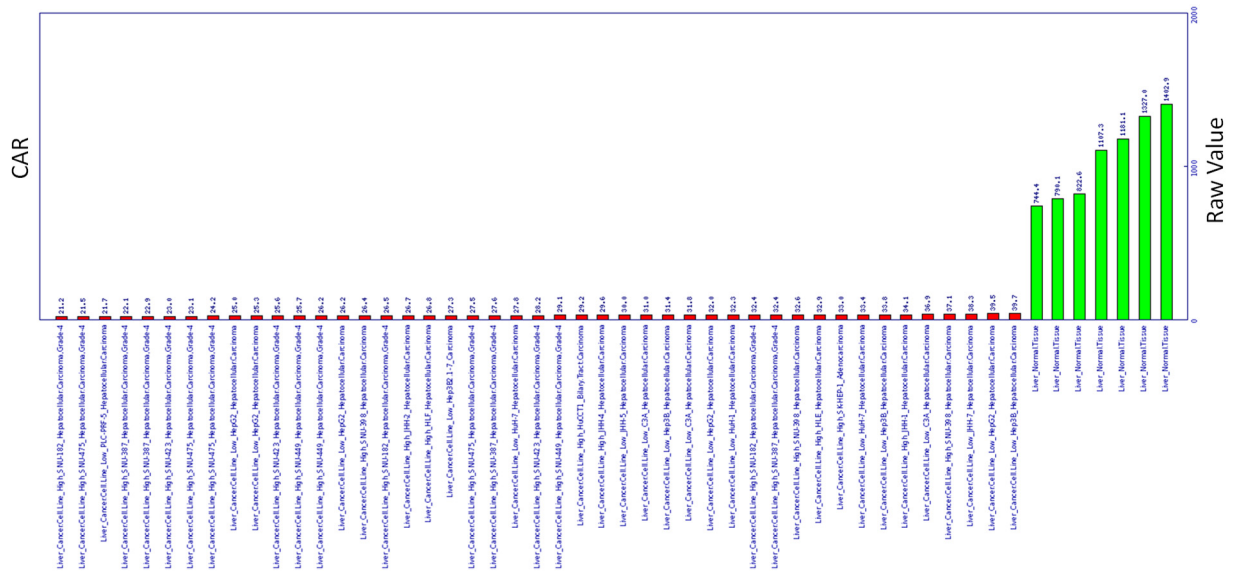


Figure S1. Expression of CAR in different human liver cancer cell lines and normal human liver tissues. The mRNA expression levels of CAR in 45 liver cancer cell lines (red bar) and 7 human normal liver tissues (green bar) were extracted from MERAV (Metabolic gEne RApid Visualizer) database.

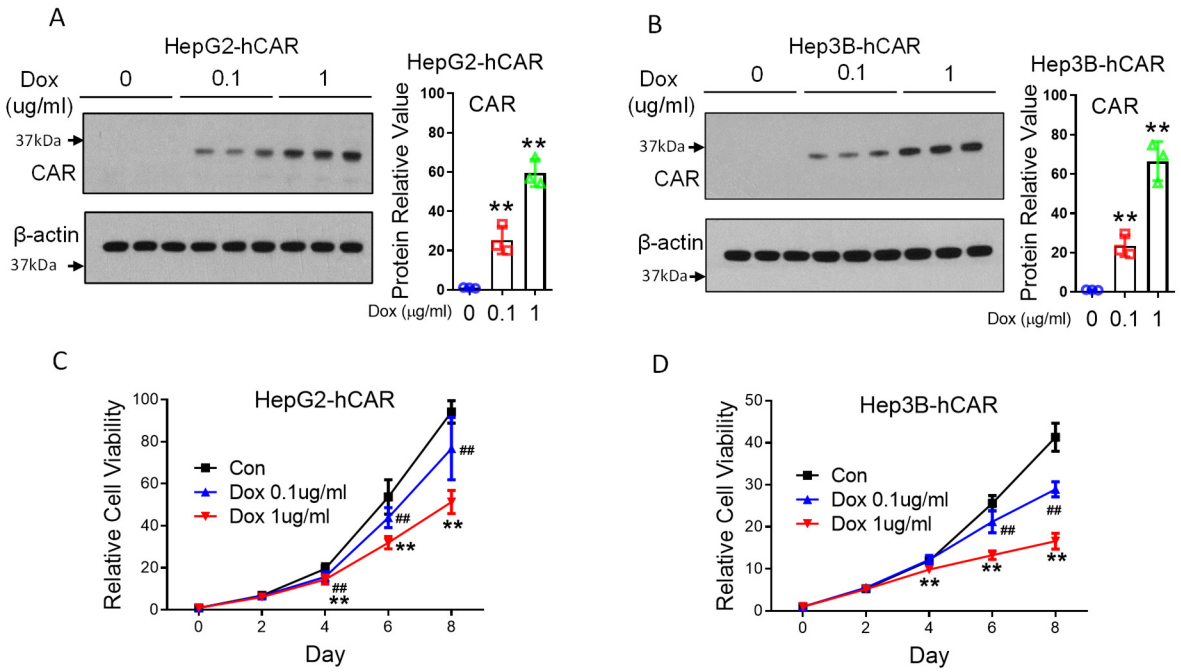


Figure S2. The levels of hCAR overexpression correlate with the suppressed growth of hepatoma cells *in vitro*. (A, B) The protein levels of hCAR in HepG2-hCAR and Hep3B-hCAR cells after Dox treatment at 0.1 μg/ml and 1 μg/ml for 72 h. (C, D) The relative growth rates of HepG2-hCAR and Hep3B-hCAR cells under the treatment with vehicle control or Dox at 0.1 μg/ml and 1 μg/ml were measured on day 2, 4, 6, and 8 using CCK8 reagents. Results are expressed as mean ± S.D. from at least three independent experiments in (C) and (D). ** and ##, $p < 0.01$.

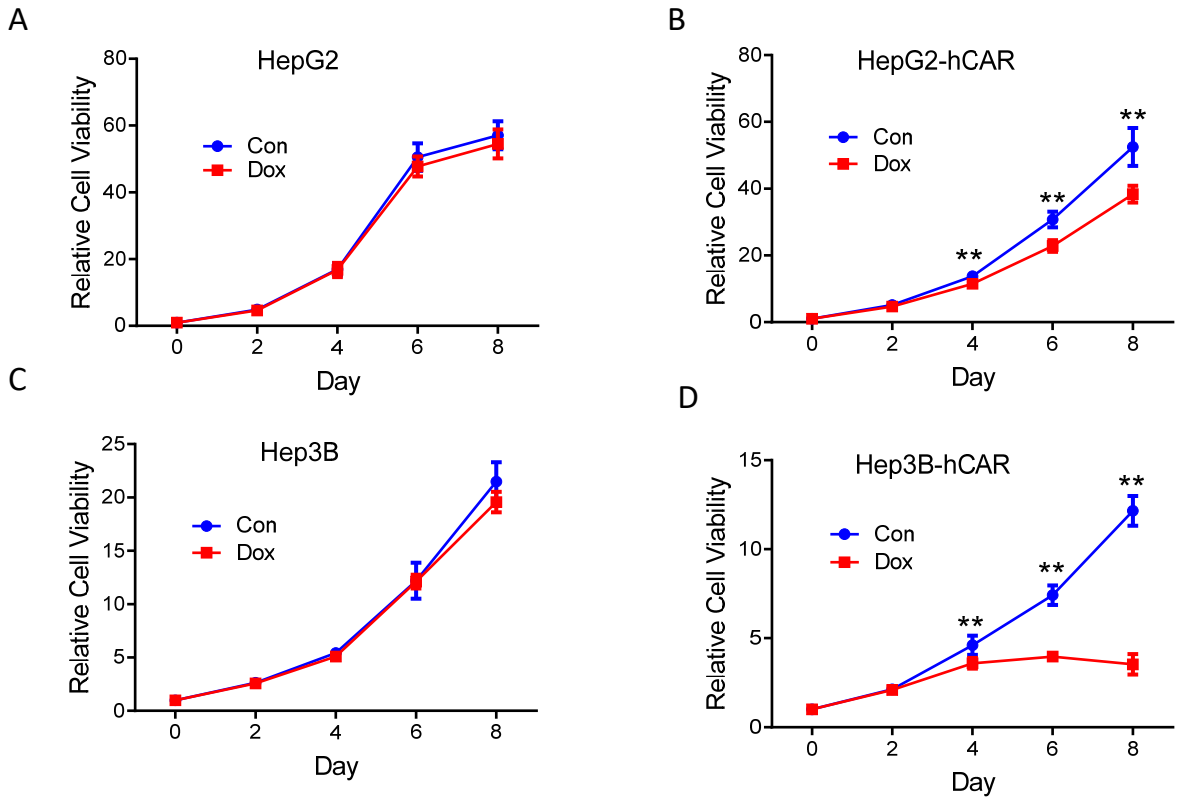


Figure S3. Cell-Titer-Glo assay confirmed overexpression of CAR suppresses the growth of hepatoma cells. Cultured HepG2, HepG2-hCAR (A, B) and Hep3B, Hep3B-hCAR (C, D) cells were treated with vehicle control or Dox (1 μg/ml) for 2, 4, 6, and 8 days as detailed in *Supplementary Materials and Methods*. Relative cell growth rate was measured using the CellTiter-Glo assay following the manufacturer's instructions. Data were collected from at least three independent experiments and expressed as mean ± S.D. **, $p < 0.01$.

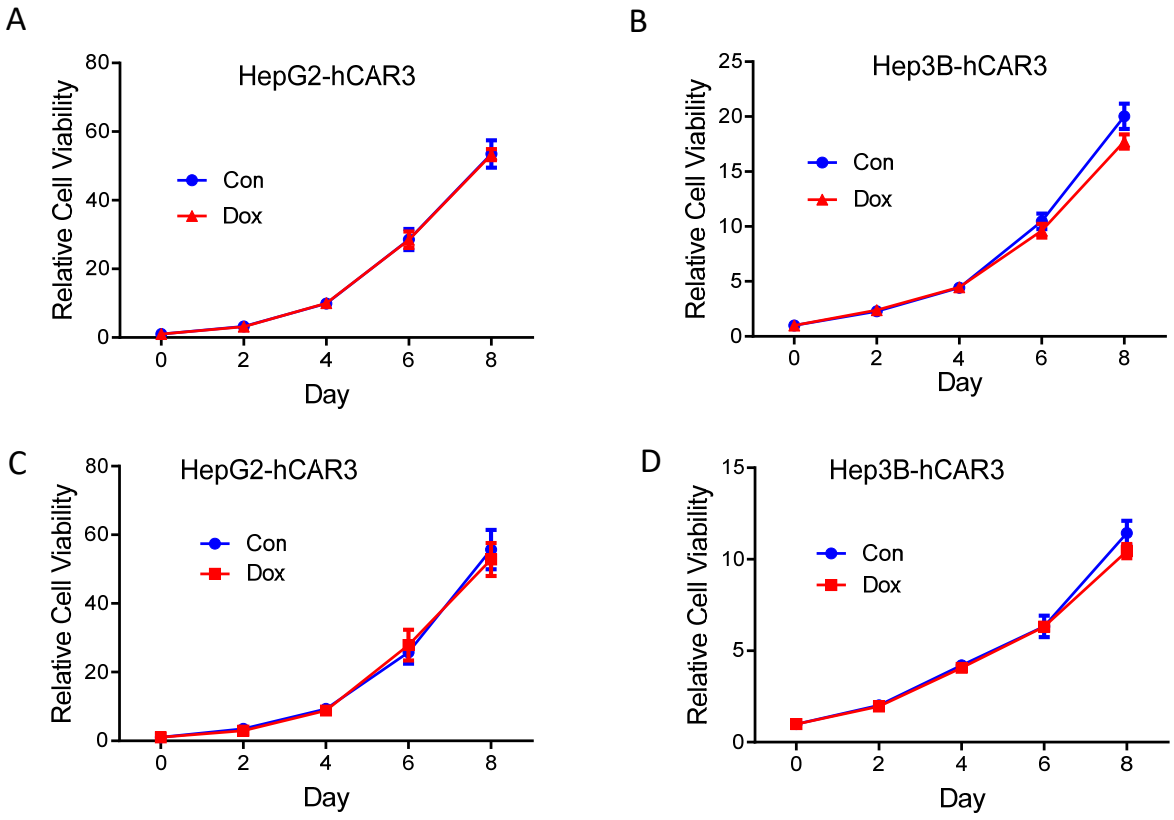


Figure S4. Overexpression of hCAR3 in HepG2 and Hep3B cells does not alter cell growth rates. The relative growth rate of HepG2-hCAR3 and Hep3B-hCAR3 cells was measure on day 0, 2, 4, 6, and 8 after vehicle control or Dox (1 μg/ml) treatment. Relative cell growth rate was analyzed using the CCK8 assays (A, B) and CellTiter-Glo assays (C, D). Results are expressed as mean ± S.D. from at least three independent experiments.

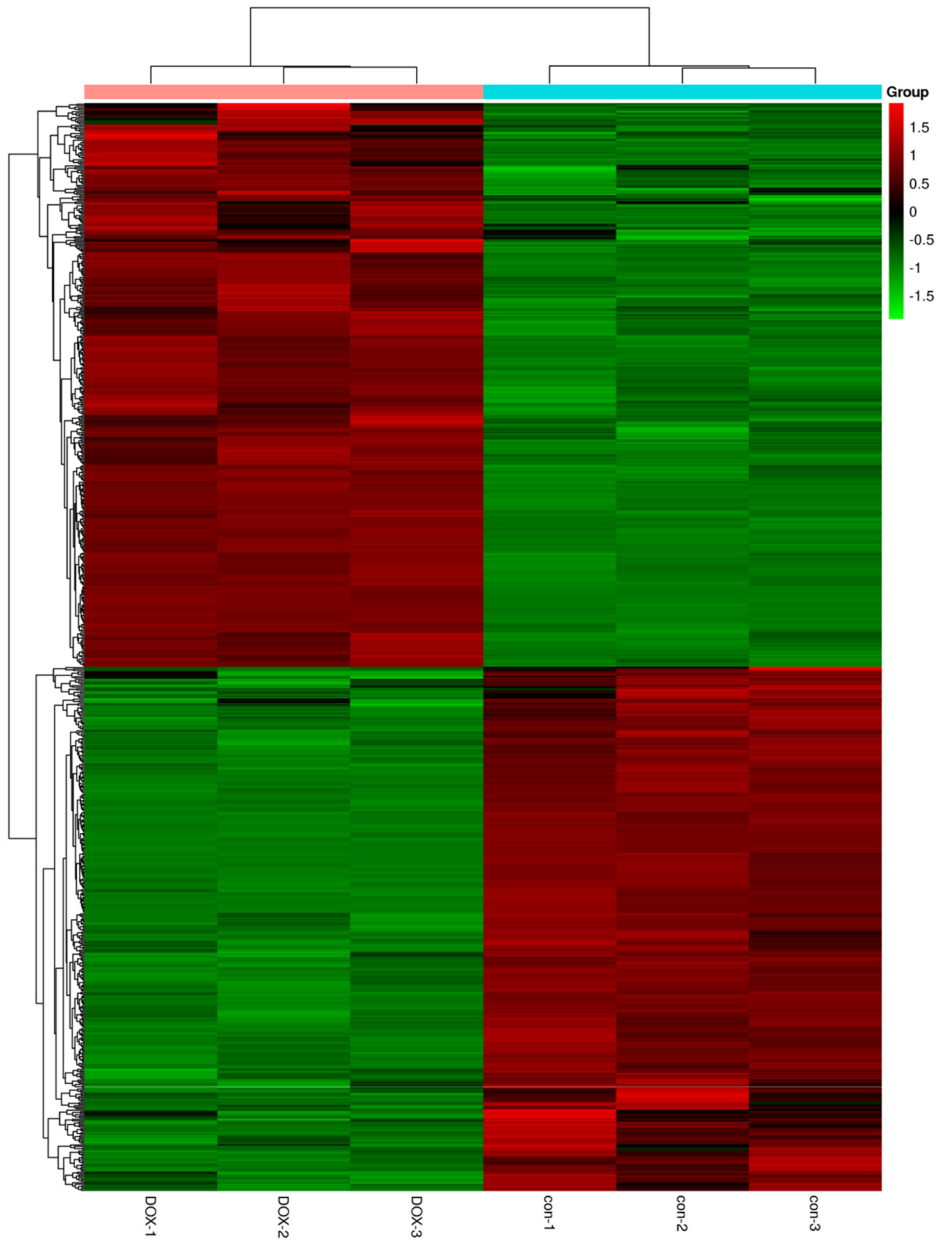


Figure S5. The hierarchical clustering heatmap of RNA-seq data in HepG2-hCAR cells after hCAR induction. The Hierarchical Clustering was performed using significant expressed genes (Group-DOX vs Group-con). The group-DOX (DOX-1, DOX-2 and DOX-3) were HepG2-hCAR cells treated with Dox; and the group-con (con-1, con-2 and con-3) were HepG2-hCAR cells treated with vehicle control.

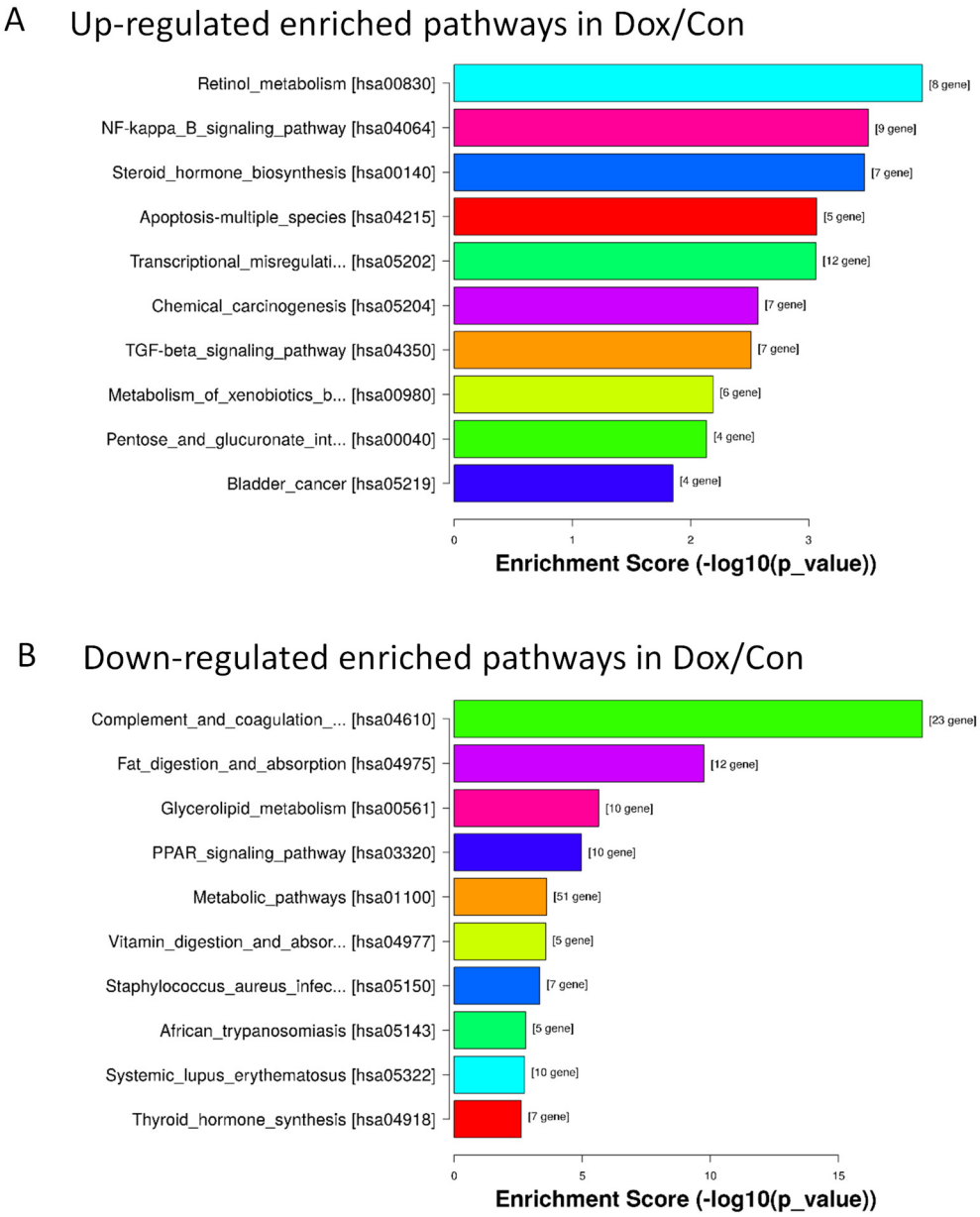


Figure S6. The significantly enriched pathways of RNA-seq data in HepG2-hCAR cells treated with Dox or vehicle control. Ordered from top to bottom by p-value, with the most significant pathway on the top. (A) The significant up-regulated pathways in HepG2-hCAR cells treated with Dox. (B) The significant down-regulated pathways in HepG2-hCAR cells treated with Dox.

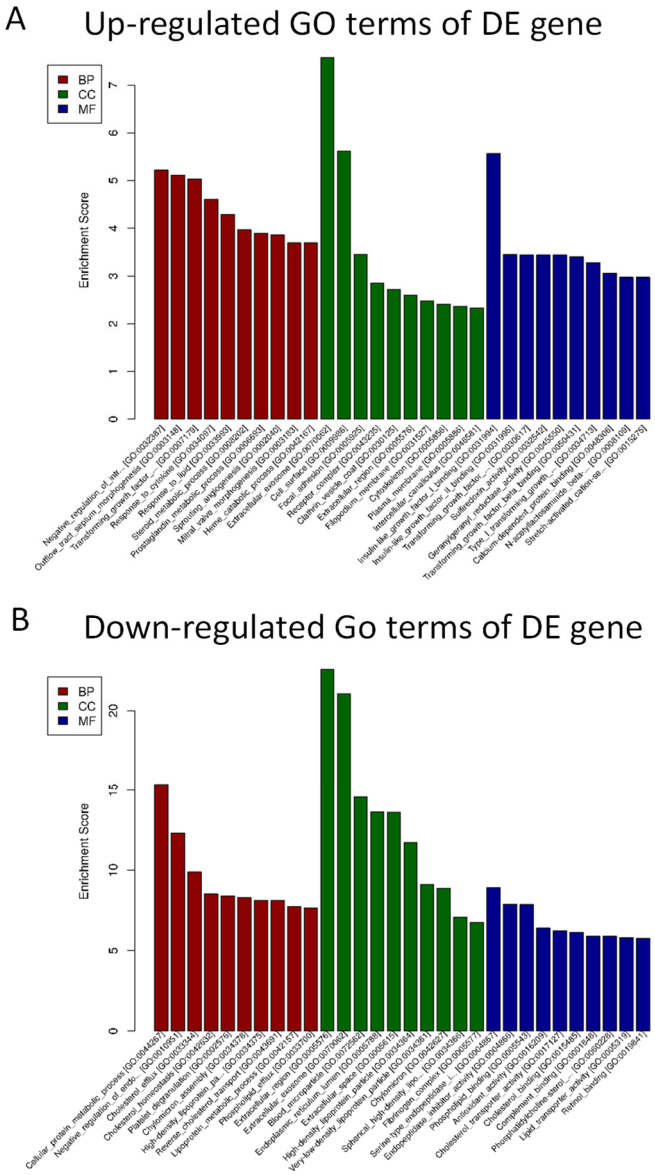


Figure S7. Gene ontology (GO) analysis of RNA-seq data in HepG2-hCAR cells treated with Dox or vehicle control. We conducted GO annotation and enrichment analyses to identify the biological process (BP), molecular function (MF), and cellular component (CC) of differentially expressed (DE) genes in RNA-seq data of HepG2-hCAR cells treated with Dox or vehicle control. (A) The up-regulated GO terms of DE gene in HepG2-hCAR cells treated with Dox. (B) The down-regulated GO terms of DE gene in HepG2-hCAR cells treated with Dox.

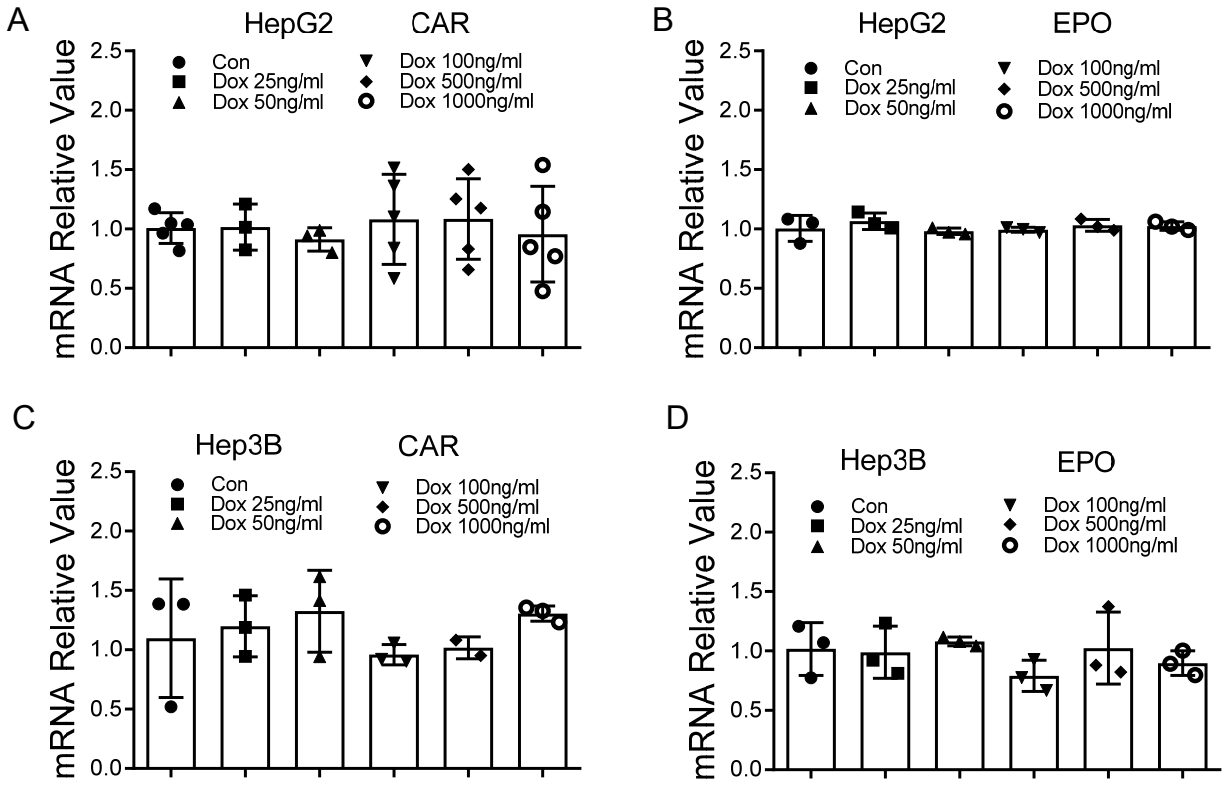


Figure S8. The expression of hCAR and EPO was not significantly affected by Dox treatment in normal HepG2 and Hep3B cells. (A, B) mRNA expression of hCAR and EPO was analyzed in normal HepG2 cells after treatment with vehicle control or Dox at indicated concentrations for 72 h. (C, D) mRNA expression of hCAR and EPO was analyzed in normal Hep3B cells after treatment with vehicle control or Dox at indicated concentration for 72 h. Data were collected from at least three independent experiments and expressed as mean ± S.D.

Table S1. List of PCR Primer sequences

Primer sequences for RT-PCR		
Gene name	**Forward primer sequence 5'-3'**	**Reverse primer sequence5'-3'**
CAR	GAGCTGAGGAACTGTGTGGTA	CTTTTGCTGACTGTTCTCCTGAA
EPO	AGGCCCTGTTGGTCAACTCT	GCAGTGATTGTTCGGAGTGGA
HNF4α	TGAGAATGTGCAGGTGTTGAC	CGGCTAAATCTGCAGGAGTA
p21	CTGGAGACTCTCAGGGTCGAAA	GATTAGGGCTTCCTCTTGGAGAA
GATA-2	CATCAAGCCCAAGCGAAGACT	CAGCTCCTCGAAGCACTCCG
CYP24A1	CAAACCGTGGAAGGCTATC	AGTCTTCCCCTTCCAGGATCA
TNS4	TTCCTCATCGAGTCTTCCGCCAAA	CCATGATGGAATGCTGGCACACAA
ISM1	CTTCCCCAGACCGCGATTC	CGACCACCTCTATGGTGACCT
LOXL4	GTCTAATGGCTGGGGAGTCA	GTCACTGGGCTATGCTGCTT
ANGPTL1	AGGAAACTGCGCCCACTTTCATAAA	ATTCCATCTTGGTGCTTGCTTCTGT
CXCL8	AGGGTTGCCAGATGCAATAC	AAACCAAGGCACAGTGGAAC
CDH1	CCGCCGGCGTCTGTAGGAA	AGGGCTCTTTGACCACCGCTCTC
CD3D	ACTGGCTACCCTTCTCTCG	CCGTTCCCTCTACCCATGTGA
FABP1	GCTGGGTCCAAAGTGATCCA	TGTCACCTTCCAACTGAACCA
APOC3	GTTACATGAAGCACGCCACC	CACGGCTGAAGTTGGTCTGA
AHSG	TCCTTGGGGATACAAACACACC	TACCACGGAAAACTTGCCATC
AFP	AAATACATCCAGGAGAGCCA	CTGAGCTTGGCACAGATCCT
MTTP	GGTGCAATGGAGTTTAGCTTG	GGCCAGCTTTCACAAAAGAG
IGSF1	CTTGGGAGAACATCACGCTTT	CCTGCATTGGACTCAGTAAGG
UPK3A	GCCTCTCTGCATGTTTGACA	CCCACCCTCTGTTTGTAGGA
APOH	CCCAAGCCAGATGATTTACCAT	ACAGTCCTGTGAGAGGGCA
PLA2G12B	ATTCCGATGGTGTCTCCACTCG	CAAGGTCCACACGGTGTTGAAC
ADH6	CAATACTGCCAAGGTGACTCC	GCTCCTGCTGCTTTACAACC
GAPDH	CCCATCACCATCTTCCAGGAG	GTTGTCATGGATGACCTTGGC
DUSP5	ACAGCCCTGCTGAATGTCTC	GGAGCTAATGTCAGCCGTGT
Primer sequences for the cloning of EPO 3'UTR enhancer region		
Forward primer 5'-3'	CGGGGTACCGGGCCCTACGTGCTGTCTCACACA	
Reverse primer 5'-3'	CCGCTCGAG TTGGCAGCTGCCTTACTGCGGTGA	

Table S2. List of antibodies used in this study

Name	Supplier	Cat no.
anti-CAR	Perseus Proteomics	PP-N4111-00
anti-STAT3	BD Transduction Laboratories	S21320
anti-phospho-STAT3	Cell Signaling Technology	9145
anti-AKT	Cell Signaling Technology	4691
anti-phospho-AKT	Cell Signaling Technology	4060
anti-ERK 1/2	ProteinTech Group	16443-1-AP
anti-phospho-ERK 1/2	Sigma-Aldrich	M9692
anti-HNF4α	Santa Cruz Biotechnology	sc-374229
anti-p21	Santa Cruz Biotechnology	sc-397
anti-Ki67	Cell Signaling Technology	9449
anti-β-actin	Sigma-Aldrich	A3854

Supplementary Methods

Generation of Doxycycline (Dox) inducible hCAR3 stable cell lines

Human CAR3 expression lentivirus plasmid was generated by subcloning the full-length hCAR3 cDNA from the pCMV2-hCAR3 expression plasmid into the EcoRI site of a pCW57-GFP-2A-MCS lentiviral vector as detailed in *Materials and Methods*. For generation of Dox inducible hCAR3 overexpression stable cell lines, HepG2 and Hep3B cells were infected with Dox inducible hCAR3 lentivirus for 72 h, then refed with fresh medium including the selection drug. Cells were refed every 2 days until uninfected control cells were completely killed, which took 4-5 days for puromycin (1 μg/ml). The positive infection cells were plated 1 cell/well in 96-well plate. The cells grown up from a single cell were selected and Dox induction was tested again. The cell lines with the best Dox induction for overexpression of hCAR3, named HepG2-hCAR3 and Hep3B-hCAR3, were selected for use in this study.

Cell-Titer-Glo Assay

Cell viability was assayed using Cell-Titer-Glo Assay kit (Promega, Madison, WI) according to the manufacturer's instructions. The HepG2, HepG2-hCAR, HepG2-hCAR3, Hep3B, Hep3B-hCAR, and Hep3B-hCAR3 cells were seeded at the density of 0.5×10^3 or 1×10^3 per well in 96-well plates. The cells were treated with vehicle control or Dox (1 μg/ml). At the time points of days 0, 2, 4, 6, and 8, the luminescence was read on a 96 microplate luminometer (Promega, Madison, WI). Relative cell growth rate was normalized against control on day 0. Data were collected from at least three independent experiments and expressed as mean ± S.D. **, $p < 0.01$.

Unedited Gel for Western blotting

Figure 2D

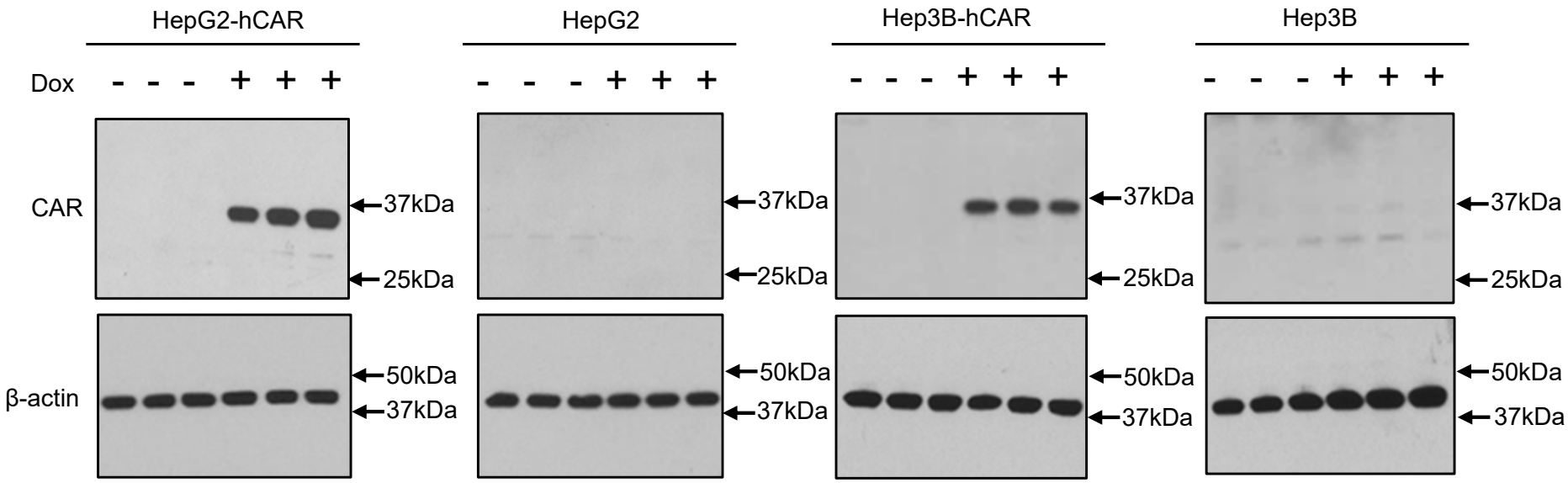


Figure 2G

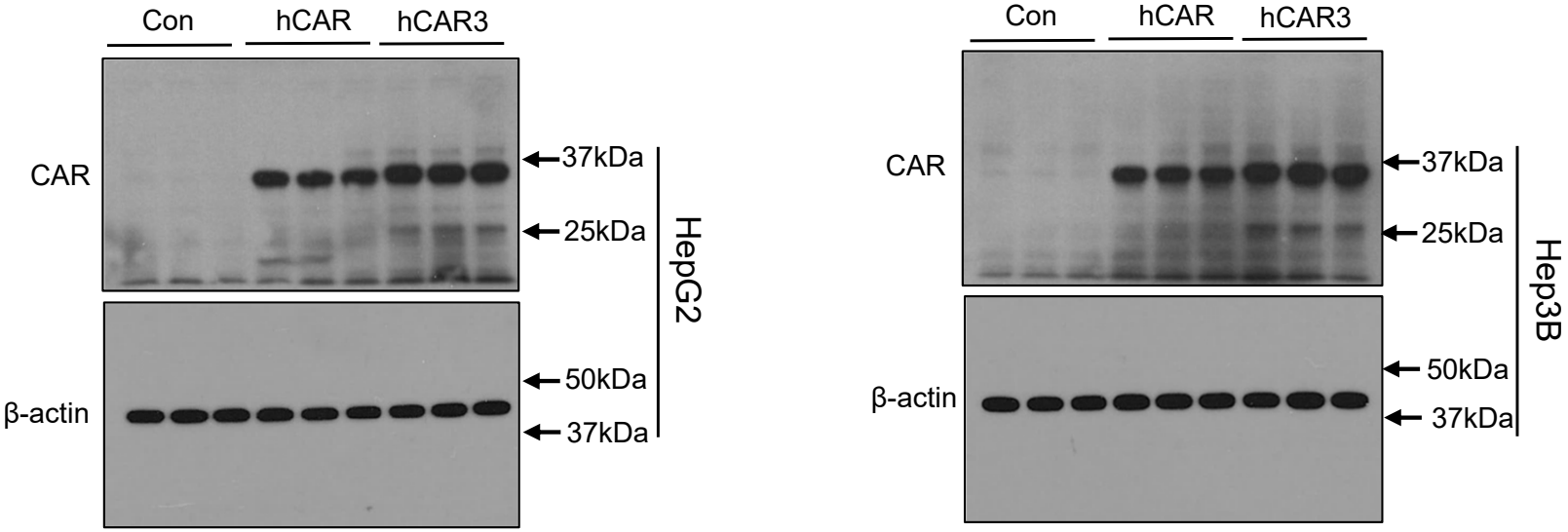
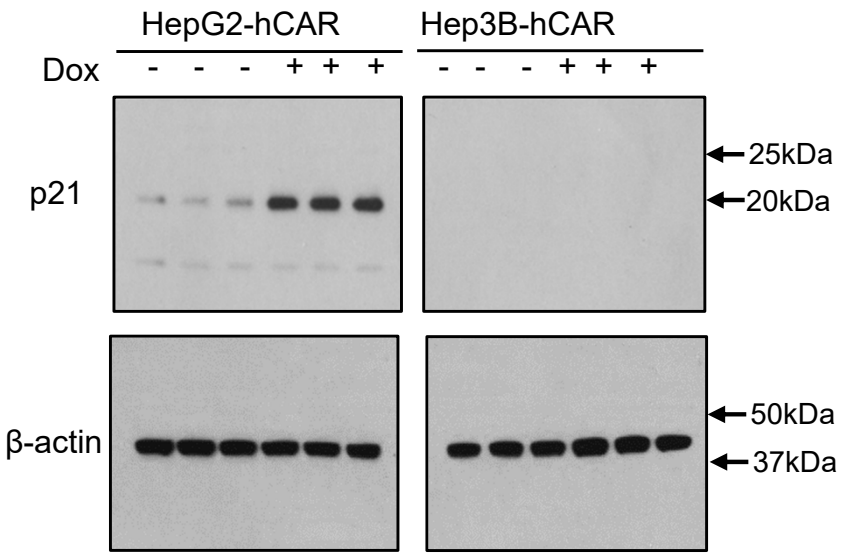


Figure 3D



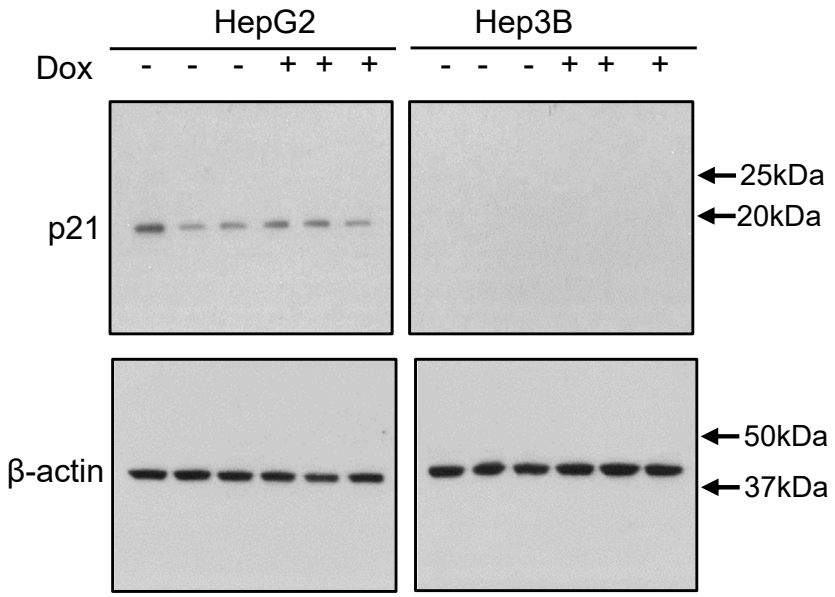


Figure 4D

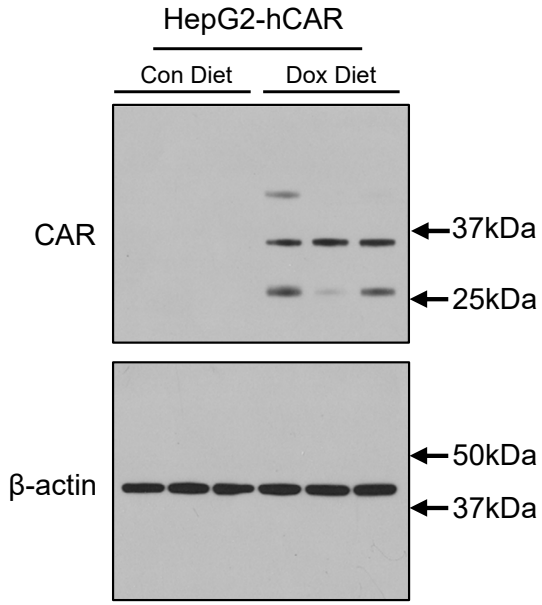


Figure 4G

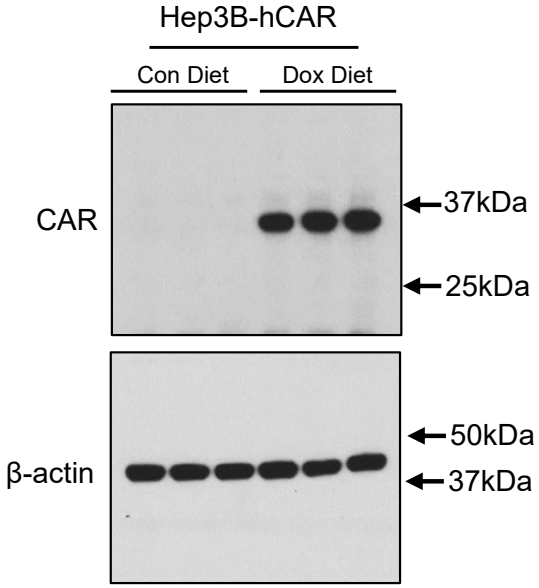


Figure 7B

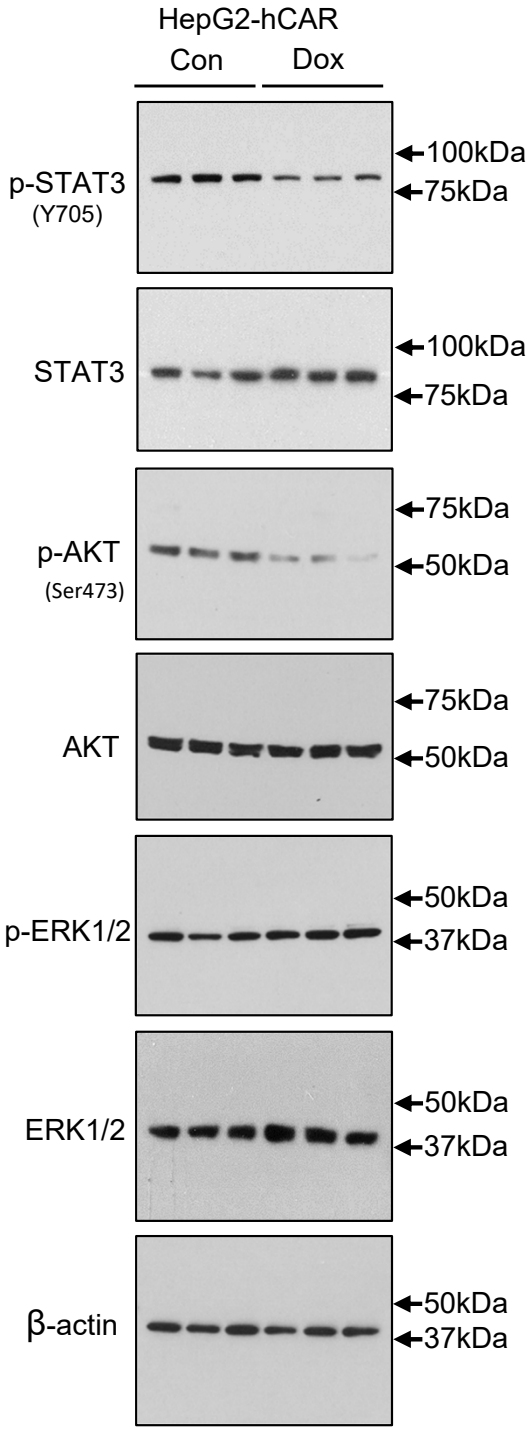


Figure 7C

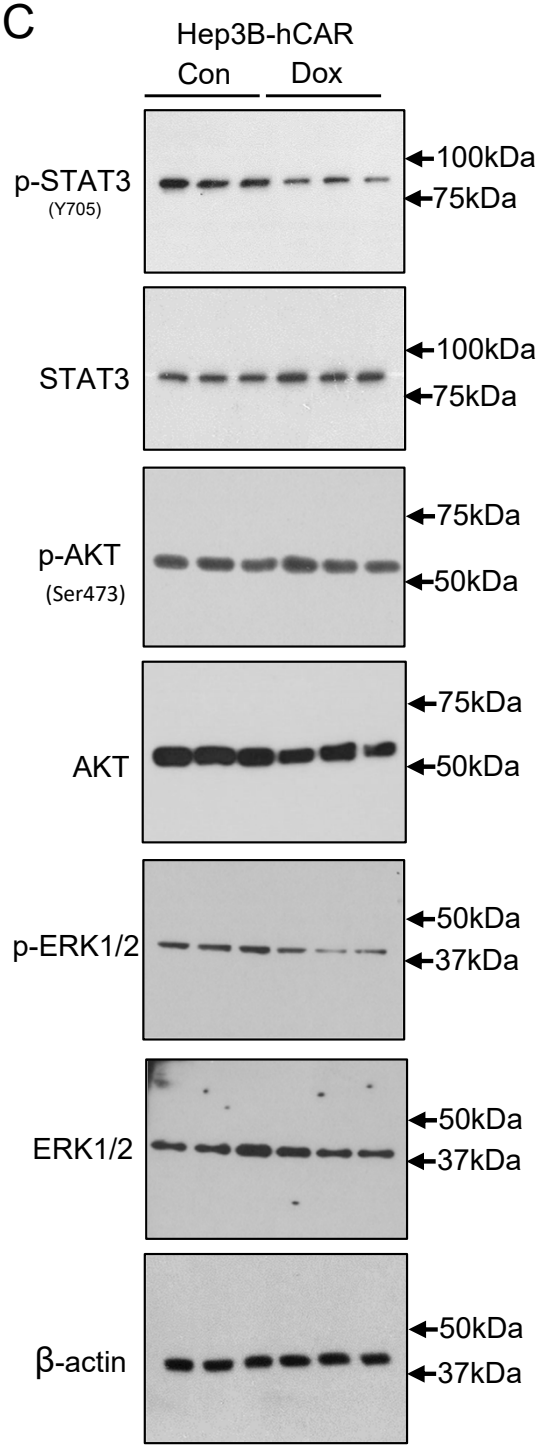
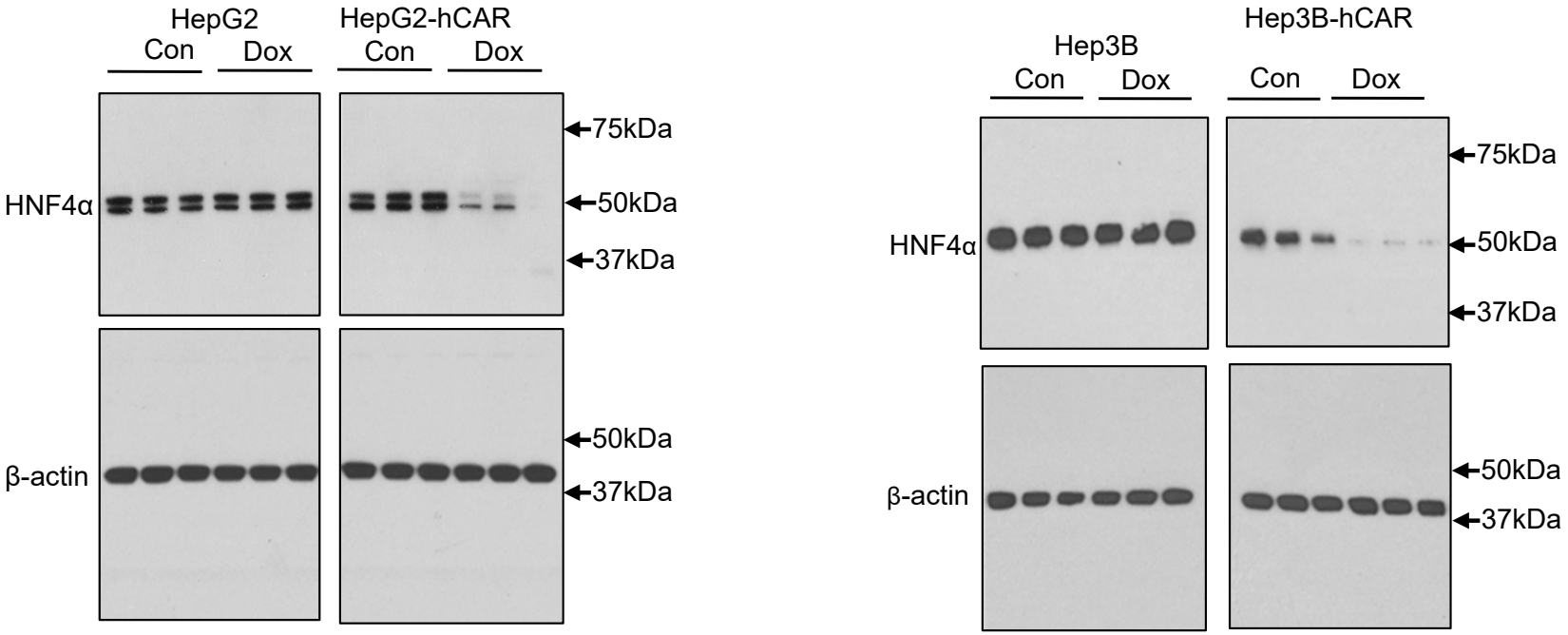


Figure 8B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β-actin
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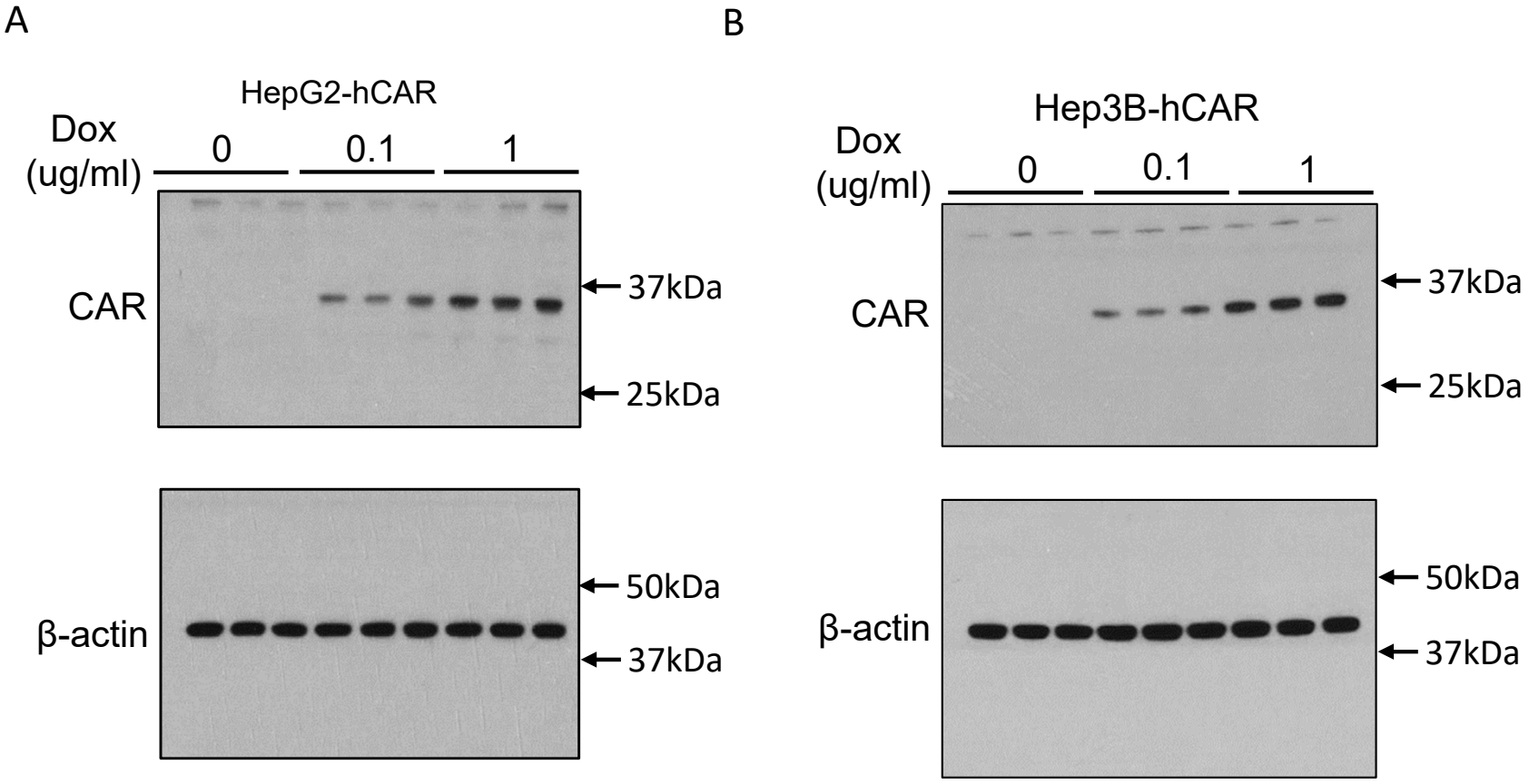
Figure S2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