SUPPLEMENTARY MATERIAL

E2F7 is a potent inhibitor of liver tumor growth in adult mice.

Eva Moreno[1], Mathilda J.M. Toussaint[1], Saskia C. van Essen[1], Laura Bongiovanni[1] , Elsbeth A. van Liere[1], Mirjam H. Koster[4], Ruixue Yuan[1,5], Jan van Deursen[2,3], Bart Westendorp[1], Alain de Bruin[*1,4].

Affiliations:

Departments of [1]Pathobiology, Faculty of Veterinary Medicine, Utrecht University, The Netherlands; [2]Biochemistry and Molecular Biology, Mayo Clinic, Rochester, Minnesota; [3]Pediatric and Adolescent Medicine, Mayo Clinic, Rochester, Minnesota; [4]Division Molecular Genetics, Department Pediatrics, University Medical Center Groningen, University of Groningen, The Netherlands; [5]Department of Pathology, Academic Medical Center, Amsterdam, The Netherlands.

TABLE OF CONTENTS

1. SUPPLEMENTAL METHODS

Animal experiments

Animal experiments were approved by the Utrecht University Animal Ethics Committee (approval number: AVD108002016626) and performed according institutional and national guidelines. Mice were housed under standard conditions of temperature and housing. Doxycycline (2g/kg) was administrated ad libitum in pellets to all experimental mice (Bio Services). All lines were generated by prof. Dr. Jan van Deursen (Mayo Clinic, USA) according to a previously described method (38) and maintained on a mixed genetic background 129/Sv x C57Bl/6 x FVB. Genotyping was performed on

DNA extracted from ear tissue and from liver tissue using standard PCR techniques; used primer sets are listed in Supplemental Table 1. Young males and females (postnatal day 21) were used in experiments to generate data in Figure 1-4. Only male mice were used in the tumor study (Figure 5-6). Dissected organs were flash-frozen in liquid nitrogen and stored at -80^{0}C for further analysis. For tumor initiation, male mice received a single intraperitoneal injection with diethylnitrosamine (DEN; 20 µg/g body weight dissolved in PBS) (Sigma-Aldrich) at 14 days of age. BrdU (858811, Sigma-Aldrich) was injected intraperitoneally 2 hours prior to euthanasia in doses of 30 µg/g for young animals (3-4weeks old) and 100 µg/g for adults (>9months old).

Generation of inducible cell lines and cell culture

Mouse E2F7-EGFP and E2F8-EGFP and EGFP cDNA was amplified during a touch-down PCR using Phusion polymerase (New England Bioloabs). The fragments were then cloned into the pLenti CMV/TO plasmid using the Gibson Assembly® Cloning Kit (New England Biolabs E5510S) according to the manufacturer's protocol. The newly assembled plasmids were then transformed XL1-Blue MR Supercompetent cells (Agilent) according to the manufacturers protocol. Plasmids were purified by mini- and maxi-prep (QIAGEN). Correct formation was checked by restriction enzyme digestion (BamHI and XbaI) and Sanger sequencing (Macrogen).

Inducible cell lines were created by introducing consecutively the pLenti CMV TetR and our newly synthesized E2F7-containing plasmids into RPE-hTert cells using a third generation lentiviral packaging system. pLenti CMV TetR Blast (716-1) and pLenti CMV/TO Puro DEST (670-1) were a gift from Eric Campeau & Paul Kaufman (Addgene plasmids #17492; http://n2t.net/addgene:17492; RRID:Addgene_17492; and # 17293 ; http://n2t.net/addgene:17293 ; RRID:Addgene_17293). One day prior to the transfection, 4,0x10^6 HEK293T cells were seeded into 100 mm cell culture dishes. To package lentivirus, HEK293T cells at 70% confluence were transfected with 10ug packaging plasmids, pMDLg pRRE, pCMV-VSV-G, pRSV Rev, mixed in a ratio 1:1:1, together with the pLenti constructs using Polyethylenimine (PEI). After 48 hours, the virus-containing cell culture medium was transferred to cell culture dishes in which RPE cells had been pre-seeded. To facilitate the infection, 4 µg/mL polybrene was added. After 16 hours, medium was removed, cells were washed with fresh DMEM containing 1% Pen-Strep and 10% Tet System Approved FBS was added to the cells.

Cells with stable integration of the constructs were selected with 20 µg/mL Blasticidine and 20 µg/mL Puromycine. To ensure high Tet-Repressor expression (RPE-TetR), single-cell derived colonies were picked and analyzed by qPCR and screened for Tet-Repressor expression before proceeding to infection with E2F7/8-EGFP.

MTT assay and siRNA transfections

$5x10^3$ cells were seeded in 96-well plates in triplicate for each condition. Transfection was performed 24 hours after plating with 10nM of siRNA according to manufacturer's protocol utilizing RNAiMax (13778075, Thermo Fisher Scientific). The following siRNA products were used: Dharmacon LQ-003329-00-0002 (si*TP53*), LQ-003296-02-0002 (si*RB1*) and D-001210-02-05 (scrambled). Doxycycline was added to the medium 24h post-transfections and maintained for 48h. MTT (5mg/ml) was added to the wells and incubated for 1.5 hours at 37ºC while shaking. Finally DMSO 0.005mM HCl was added to the wells to dissolve the crystals and absorbance was measured by spectrophotometry.

Immunohistochemistry and quantification of IHC stainings.

Immunohistochemical staining was performed on formalin-fixed and paraffin embedded tissues with a thickness of 4 µm. Endogenous peroxidase activity was blocked with 1% H_2O_2 . 10mM Citrate buffer (pH 6) was used for heat-induced antigen retrieval. GFP, Ki67, γ-H2AX and Caspase 3 IHC staining sections were stained with the corresponding antibody (Supplemental Table 3) and incubated with biotinylated secondary antibodies. Vectastain Elite ABC reagents (Vector Labs) were used according to the manufacturer's instructions. Slides were counterstained with hematoxylin. For BrdU IHC, tissues were treated with 2N HCl 30 min at 37ºC immediately after the Citrate buffer incubation.

Quantifications in the small intestines sections of ki67 and BrdU IHC were done as following. Total number of cells per crypt was counted manually using the 40x objective. Five different regions along the jejunum were randomly selected and three crypts per region were used for quantification per mouse (n= 3 mice/ genotype/ time-point). Quantification of γ-H2AX and Caspase-3 in the small intestines was done by manually counting of number of positive nuclei in 10 randomly selected fields using the 40x objective (n=5 mice/ genotype/condition). IHC quantification in the liver sections was performed by manually counting the numbers of positive hepatocyte

nuclei in 10 randomly selected fields using the 40x objective (n=5 mice / genotype / condition). Microscopic tumor nodules were manually counted on HE slides obtained from 7 different sections of the liver for each mouse by an observer unaware of the genotypes of the slides (number of mice per genotype indicated in figure legends). Histopathology analysis of liver tumors was performed on HE-stained sections by a board-certified veterinary pathologist (L.B) unaware of the genotypes of the slides at the moment of analysis.

Distinction between EGFP-positive or negative nodules was based on the presence of at least 1 positive cell. Only nodules without a single positive nucleus were considered negative. The quantification for Figure 5C was obtained calculating the percentage of negative nodules over the total number of microscopic nodules previously counted. The graph represents average percentages of EGFP-negative nodules per mouse (n= at least 5 mice/ condition). The average numbers of EGFP-positive cells per nodule were obtained by manually counting positive cells within positive nodules in 5 randomly selected fields (40x objective) of at least 3 different positive nodules. At least 9 animals were used per genotype.

In Figure S6B the total number of BrdU positive cells per nodule was obtained by manually counting positive cells within nodules of the different malignancy in 5 random selected fields (40x objective) of at least 10 nodules/ grade of malignancy/ genotype (when less than 10 nodules indicated in figure legend). This analysis was performed by a board certificate pathologist unaware of the genotypes.

Isolectin B4 positive endothelial blood vessels were quantified in EGFP negative and positive nodules in 5 random selected fields (40x objective) of at least 6 nodules (neg or pos) per genotype.

Immunofluorescence staining on cell lines and tissue

Cells were seeded over cover slips (5mm) and treated with desired condition as indicated (Figure S4B). Cells were first treated with ice-cold 0.2% Triton X-100 for 1 minute, and then fixed with 4% PFA for 20min and lastly with 0.1% Triton X-100 for 10min at room temperature. For the IF staining, cells were first block with 5% goat serum in PBS for 20min, followed by incubation with 1st antibody diluted in 10% goat serum in PBS(rabbit anti- γ-H2AX, Cell Signaling 2577, 1:200) for 2 hour at room temperature. Next, cover slips were incubated with 2nd antibody diluted in 10% goat

serum in PBS (Goat anti-rabbit Alexa 568, 1:250) for 1 hour at room temperature. Nuclei were stained with DAPI (Sigma D9542, 1:4000) and cover slips mounted using Fluoroshield™. γ-H2AX dots per nuclei were analyzed in an automated manner using ImageJ software. The macro used for quantification is available upon request.

Immunofluorescence staining of E-cadherin was performed on formalin-fixed and paraffin embedded intestines with a thickness of 4 µm. Endogenous peroxidase activity was blocked with 0.3% H_2O_2/methanol . 10mM Citrate buffer (pH 6) was used for heat-induced antigen retrieval. Slides were pre-incubate with 5% serum in 1%BSA/PBS. Incubation of 1st antibody (mouse anti E-cadherin, #610181, BD Biosciences, 1:100) was done overnight at 4^{o}C in 1%BSA/PBS. The fluorochrome-coupled secondary antibody was diluted in 1%BSA/PBS (1:200) and incubated for 1h at RT in the dark. After washing 3 times with PBS for 2 minutes, slides were incubated with Hoeschst (Sigma #B-2883) in PBS (1:1000) for 3 minutes at RT in the dark. Finally, slides were mounted with Fluorshield and analyzed on the next day on a Leica LMD7000 fluorescence microscope equipped with a DFC 7000T camera. Pictures were taken with a 63x objective in representative areas using LasX software.

Flow cytometry

Pepsin (0.5mg/ml 0.1N in HCl) was used to generate the nuclei suspensions from frozen livers. Afterwards, the nuclei were washed twice with TBS and then stained with anti-BrdU-FITC (Becton Dickinson; 347583), and propidium iodide (20µg/ml propidium iodide, 250µg/ml RNase A and 0.1% bovine serum albumin). All samples were measured on a BD FACS Canto II (BD Biosciences) and further analyzed using FlowJo software.

DNA fiber analysis

Cells were pulse-labelled with 25µM CldU followed by 250µM IdU for 20 min each. Afterwards cells were trypsinized and lysed in pre-warmed (300C) spreading buffer (200mM Tris-HCl pH 7.4, 50 mM EDTA and 0,5% SDS). A droplet (2µl) was placed on the edge of a microscope slide and the slide was tilted at an angle of approximately 15 degrees to let the suspension spread over the slide for 15 minutes. Slides were fixed in freshly-made methanol: acetic acid (3:1) for 10 minutes. Before immunodetection, slides were treated with 2.5M HCl for 75min and subsequently with

blocking solution (1% BSA, 0.1% Tween20, PBS) for 60 minutes. Subsequently, slides were incubated for 60 minutes with primary antibodies to detect CldU and IdU (rat-anti BrdU (OBT0030G, 1:100), mouse-anti-BrdU (BD 347580, 1:100), respectively). Then, slides were fixed with 4% paraformaldehyde for 10 minutes and incubated with Alexa 488-labeled goat anti-mouse (Molecular Probes A21121, 1:300) and Alexa 594-labeled goat anti-rat (Molecular probes A11007 1:300) for 90 minutes.

TCGA Data analysis

All available RNA-sequencing and clinical metadata data from the liver cancer dataset (LIHC) were downloaded as level 3 data. Subsequent analysis was performed in Rstudio version 3.3.0 using the packages “rjson”, “parallel”, “GenomicRanges”, and “DESeq2” in combination with a number of custom R scripts and functions to merge all data, remove non-tumor samples, and normalize RNA-seq counts. Cumulative counts from a previously identified panel of E2F7/8 target genes were used to determine an E2F7/8 target expression score [1-3]. For survival analysis, patients were divided into low (below median) versus high (above median) E2F7/8 target gene expression, for tumor progression stage separately. Patients with no reported tumor stage and stage IV patients (too few) were excluded from the analysis. Log-rank analysis was used to test for differences in survival between patients with low versus high E2F7/8 target gene expression. Heatmaps were generated using the R package “Pheatmap”, and survival plot were created using “Survminer”. All used R code is available on request.

References

(1) Westendorp B, Mokry M, Groot Koerkamp, Marian J A, Holstege FCP, Cuppen E, de Bruin A. E2F7 represses a network of oscillating cell cycle genes to control S-phase progression. Nucleic Acids Res 2012;40(8):3511-3523.

(2) Kent L, Rakijas J, Pandit S, Westendorp B, Chen H, Huntington J, et al. E2f8 mediates tumor suppression in postnatal liver development. J Clin Invest 2016;126(8):2955-2969.

(3) Yuan R, Vos H, van Es R, Chen J, Burgering B, Westendorp B, et al. Chk1 and 14-3-3 proteins inhibit atypical E2Fs to prevent a permanent cell cycle arrest. EMBO J 2018;37(5).

2. SUPPLEMENTAL TABLE 1: PCR primers for transgene detection

Primer	Sequence (5'-3')
TA Tg_ for 1	GCG AAG AGT TTG TCC TCA ACC
TA Tg_ rev1	AAA GTC GCT CTG AGT TGT TAT
TA Tg_ rev2	AAA GTC GCT CTG AGT TGT TAT
E2f7, E2f8 Tg and E2f8^{DBDmut} Tg_ for1	CCC TCC ATG TGT GAC CAA GG
E2f7, E2f8 Tg and E2f8^{DBDmut} Tg_ rev1	GCA CAG CAT TGC GGA CAT GC
E2f7, E2f8 Tg and E2f8^{DBDmut} Tg_ rev2	GCA GAA GCG CGG CCG TCT GG

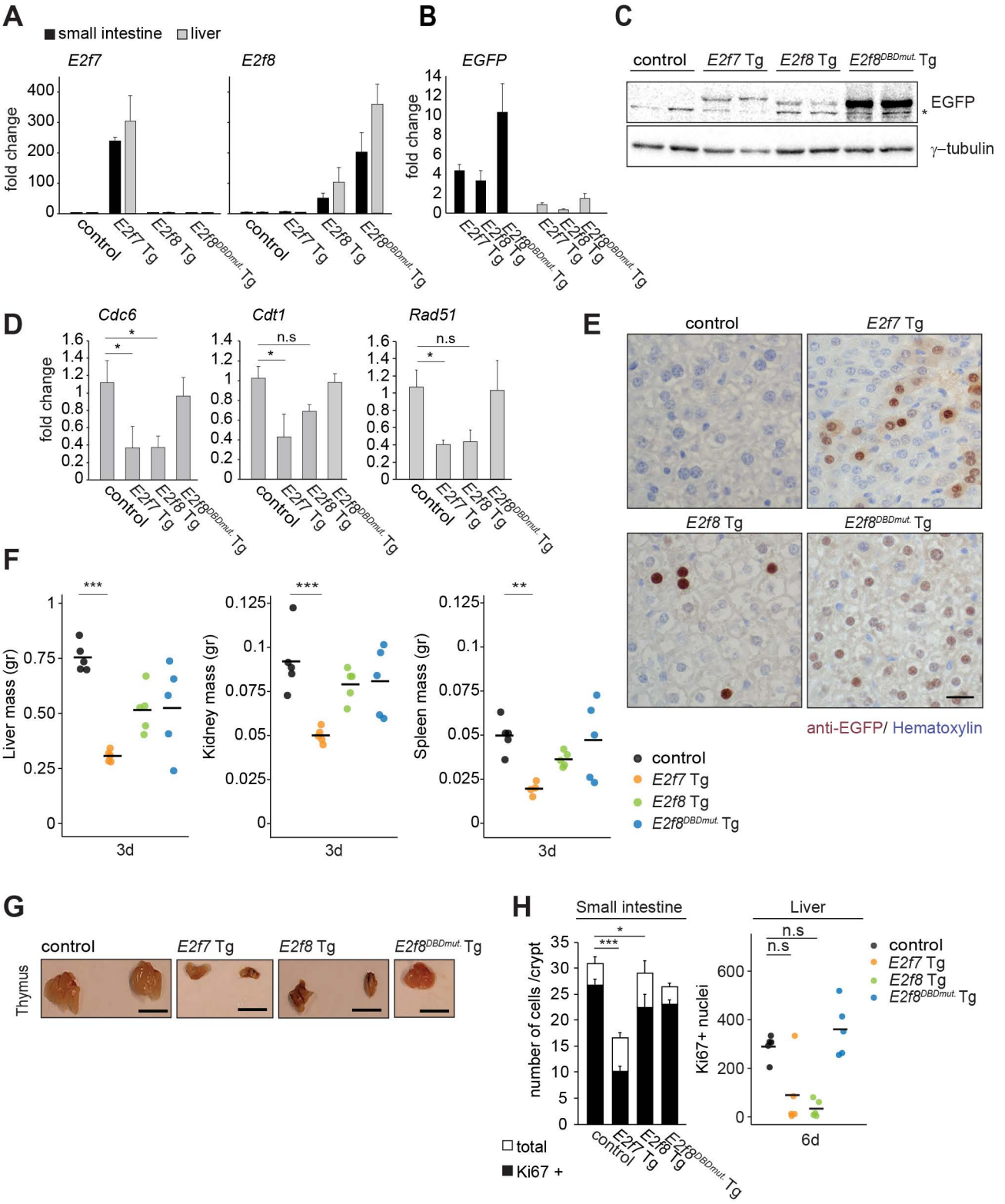
3. SUPPLEMENTAL TABLE 2: qPCR primers

	Forward primer (5'-3')	***Reverse primer (3'-5')***
E2F7; mouse	GATGCGTTCGTGAACTCCCTG	AGAAACTTCTGGCACAGCAGCC
E2F7; human	CTCCTGTGCCAGAAGTTTC	CATAGATGCGTCTCCTTTCC
E2F8; mouse	GAGAAATCCCAGCCGAGTC	CATAAATCCGCCGACGTT
E2F8; human	AATATCGTGTTGGCAGAGATCC	AGGTTGGCTGTCGGTGTC
GAPDH; mouse	GAAGGTCGGTGTGAACGG	TGAAGGGGTCGTTGATGG
GAPDH; human	CTCTGCTCCTCCTGTTCG	GCCCAATACGACCAAATCC
EGFP	CACTACCAGCAGAACACCCC	GTCACGAACTCCAGCAGGAC
CDC6; mouse	AGTTCTGTGCCCGCAAAGTG	AGCAGCAAAGAGCAAACCAGG
CDC6; human	AAACCCGATCCCAGGCACAG	AGGCAGGGCTTTTACACGAGGAG
CDT1; mouse	ACAGCCGGGCAAGATCCCCT	GGCTCCCAACTTCCGTGCCC
M2-rtTA;mouse	CTGGGAGTTGAGCAGCCTAC	AGAGCACAGCGGAATGACTT
TP53; human	GTTCCGAGAGCTGAATGAGG	TCTGAGTCAGGCCCTTCTGT
RB1; human	GAGACACAAGCAACCTCAGC	GCTCAGACAGAAGGCGTTC
RAD51; mouse	CTCATGCGTCAACCACCAG	GCTTCAGGAAGACAGGGAGAG
RAD51; human	TGCTTATTGTAGACAGTGCCACC	CACCAAACTCATCAGCGAGTC
E2F1; human	GACCACCTGATGAATATCTG	TGCTACGAAGGTCCTGAC
CCNE1; human	GACACCATGAAGGAGGACGG	ATTGTCCCAAGGCTGGCTC
RSP18; human	AGTTCCAGCATATTTTGCGAG	CTCTTGGTGAGGTCAATGTC

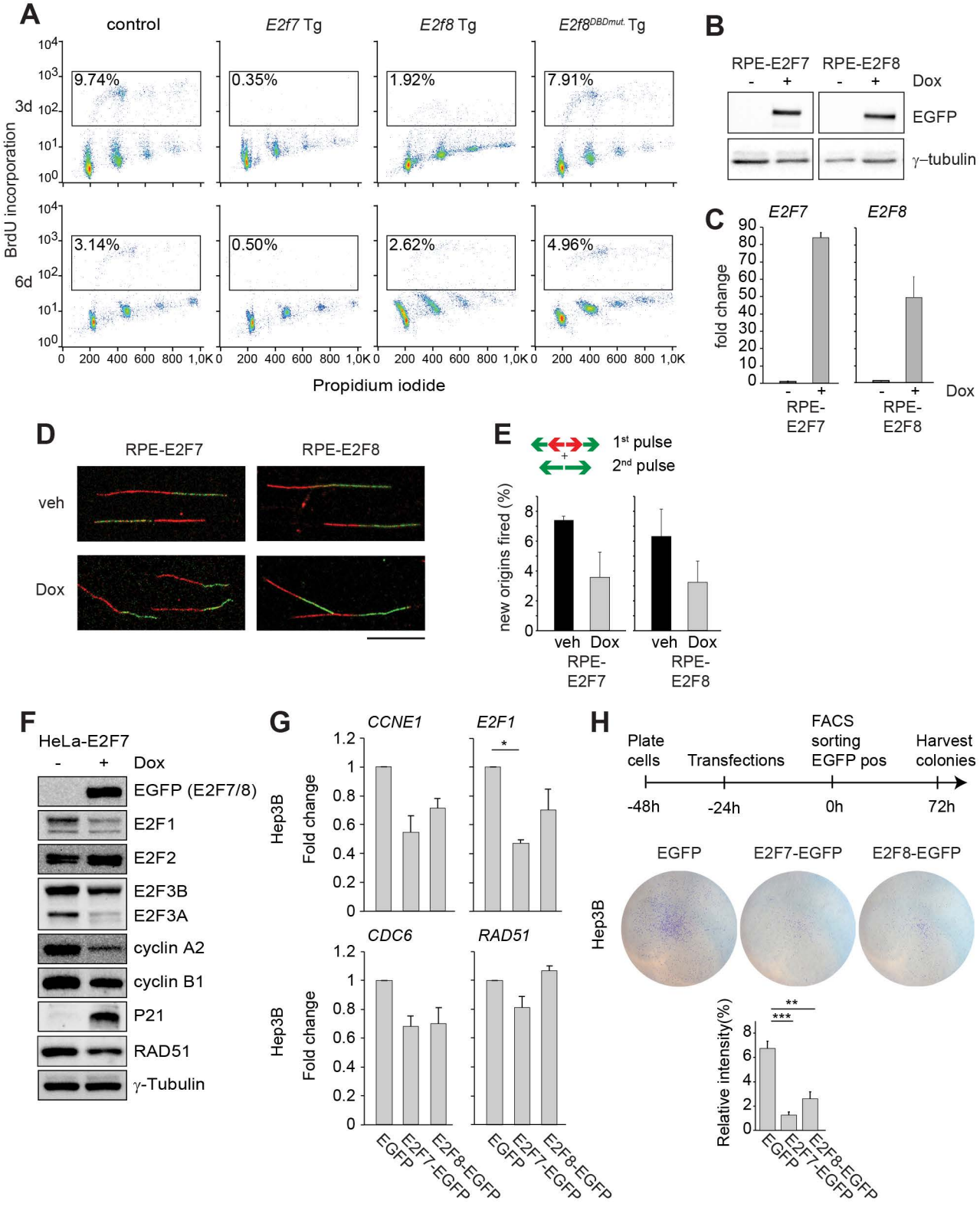
4. SUPPLEMENAL TABLE 3: Antibodies for immunoblots and immunohistochemistry

Application	***Name***	***Company***	***Cat #***	***Dilution***
Immunoblot	GFP	Abcam	AB6673	1:1000
	E2F1	Santa Cruz	Sc-193	1:1000
	E2F2	Santa Cruz	(C-20) sc-633	1:1000
	E2F3	Santa Cruz	(C-18) sc-878	1:5000
	cyclin A2	Santa Cruz	Sc-751	1:1000
	cyclin B1	Santa Cruz	Sc-245	1:1000
	P21	Santa Cruz	(M-19) sc-471	1:1000
	RAD51	Santa Cruz	(H-92) sc-8349	1:1000
	P53	Calbiochem	OP03	1:500
	RB	Santa Cruz	(C-15) sc-50	1:1000
	γ-tubulin (clone GTU-88)	Sigma	T6557	1:1000
Immunohistochemistry	GFP	Abcam	AB6673	1:800
	Ki67	Thermo Scientific	RM-9106	1:75
	BrdU	DAKO	M0744	1:50
	γ-H2AX	Cell Signaling	S139	1:500
	Caspase-3	R&D systems	AF835	1:400
	Isolectin B4	Vector Labs	B-1205	1:100

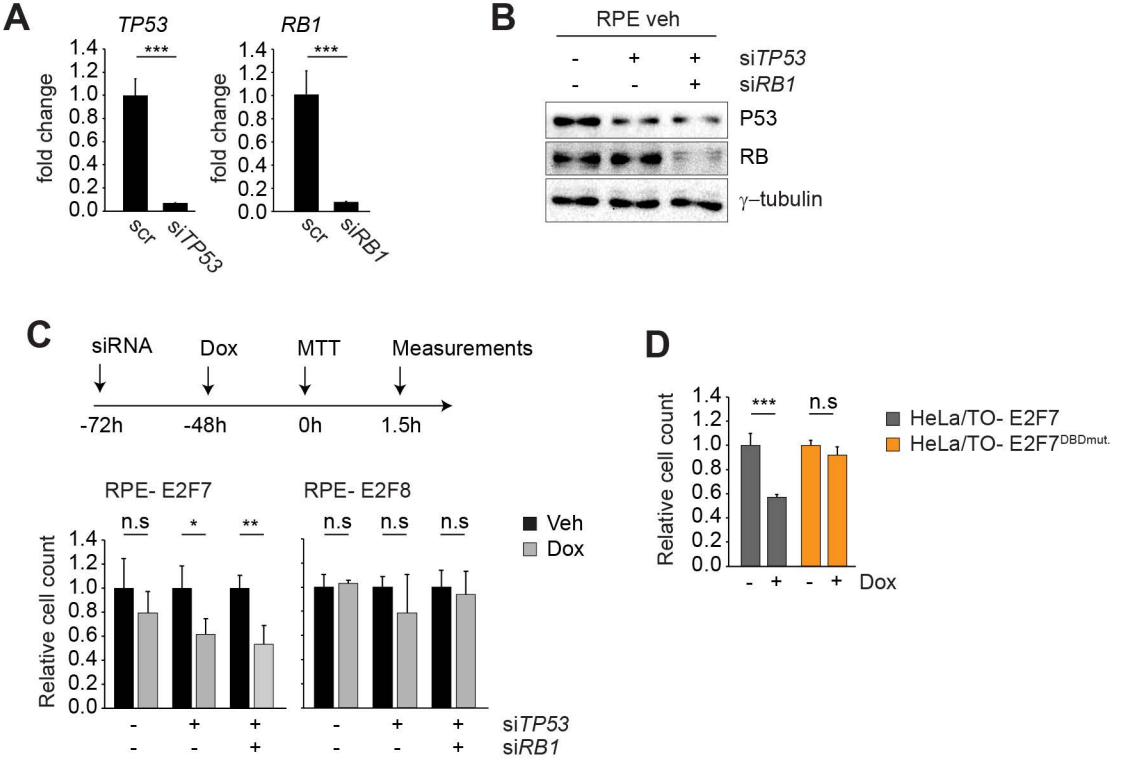
Supplemental Figure 1



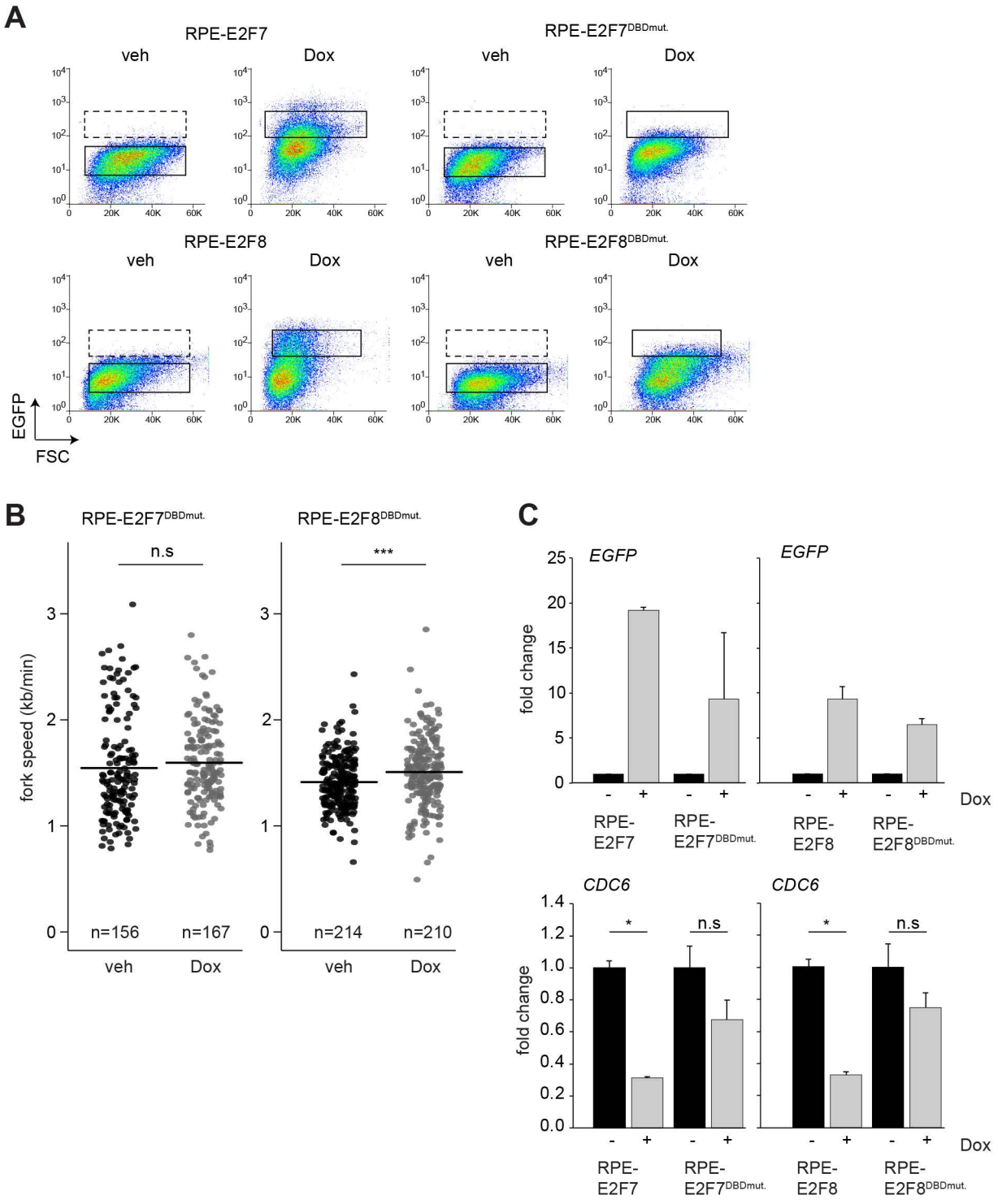
Supplemental Figure 2



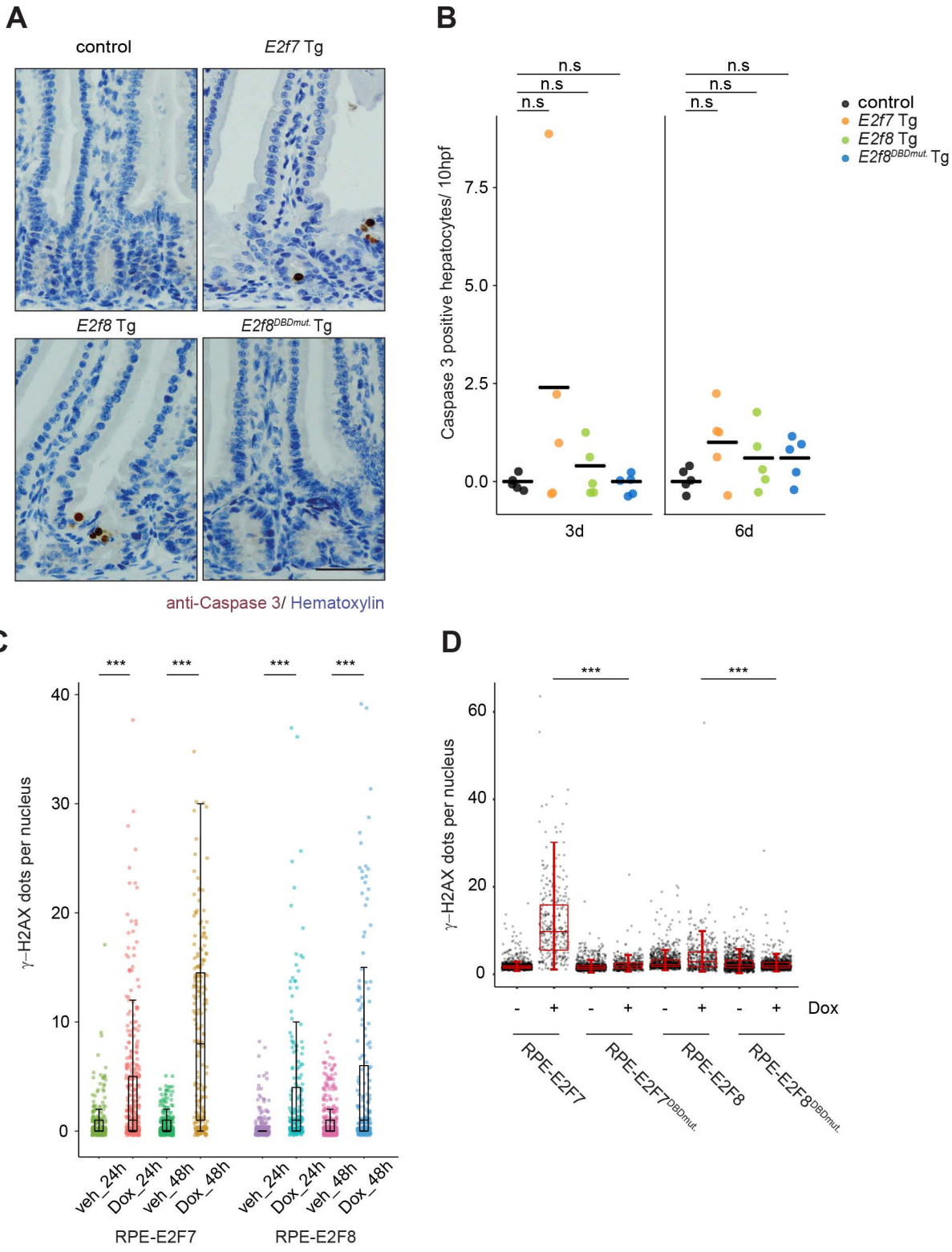
Supplemental Figure 3



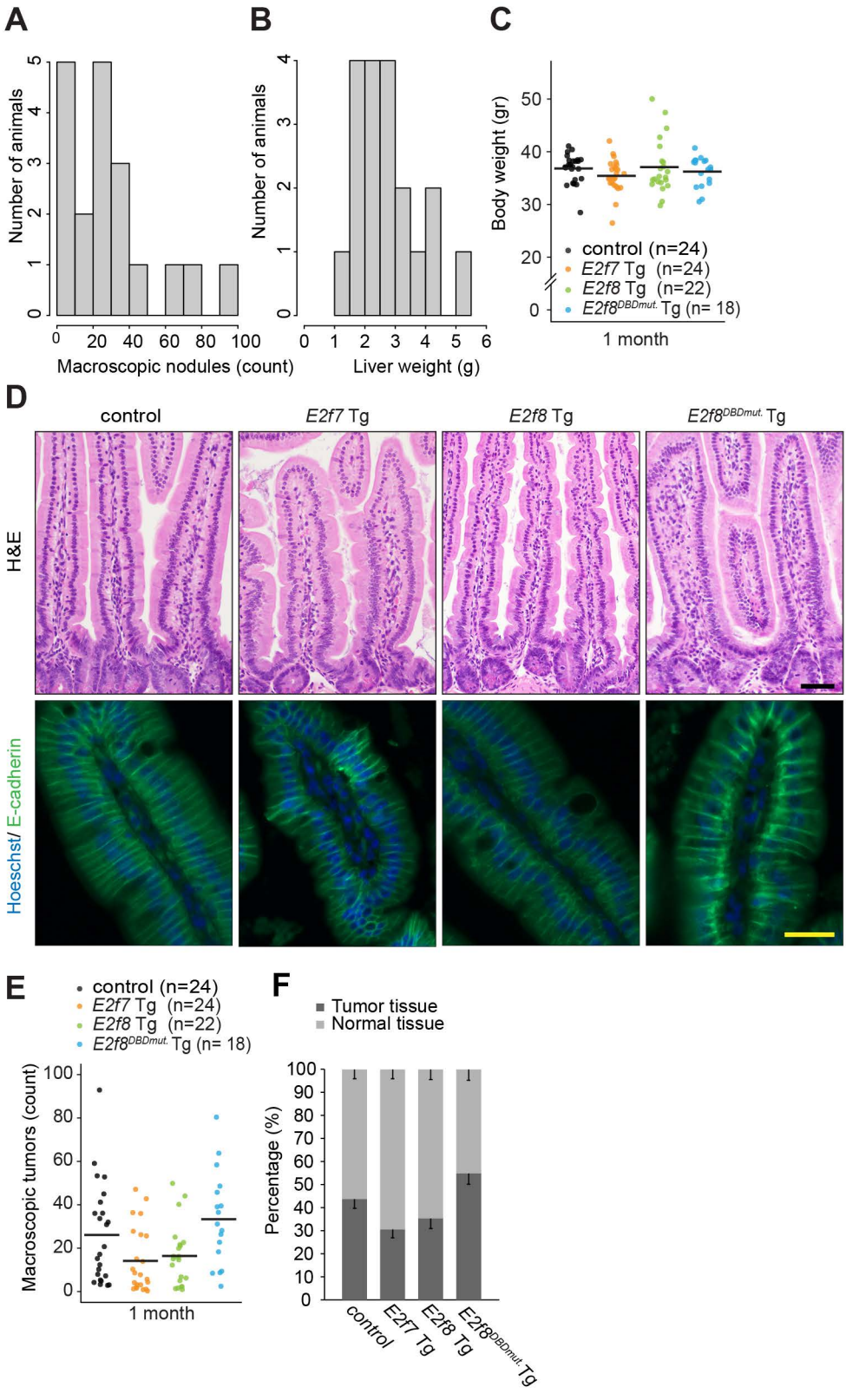
Supplemental Figure 4



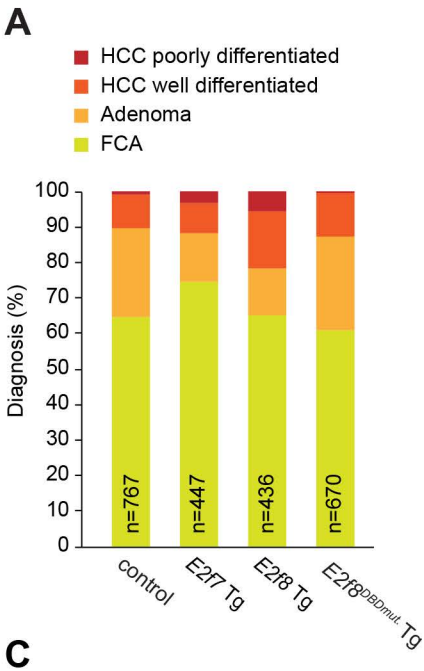
Supplemental Figure 5

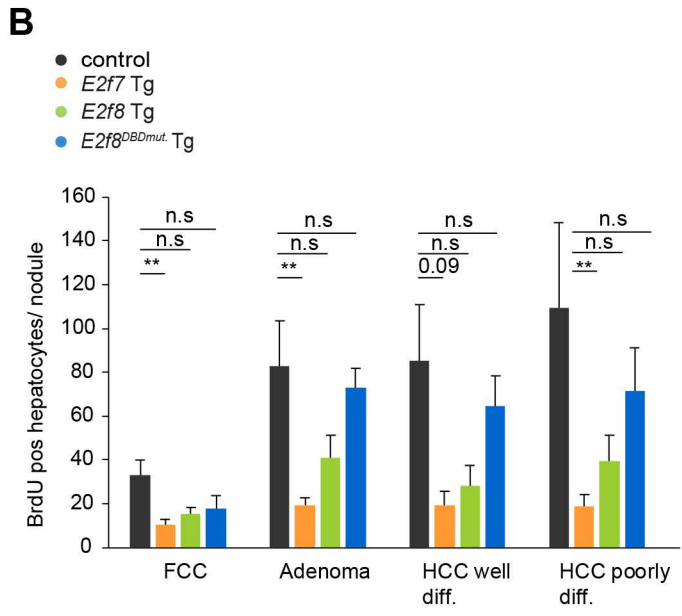


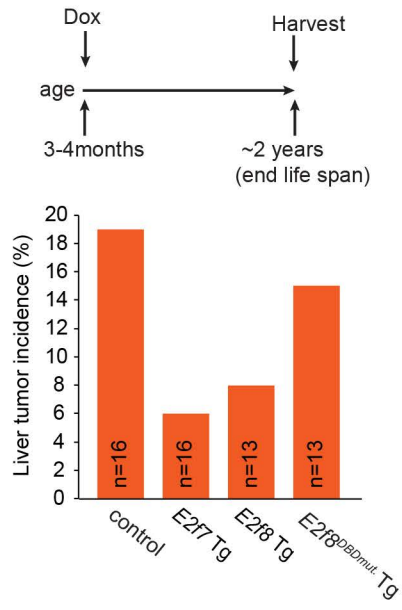
Supplemental Figure 6



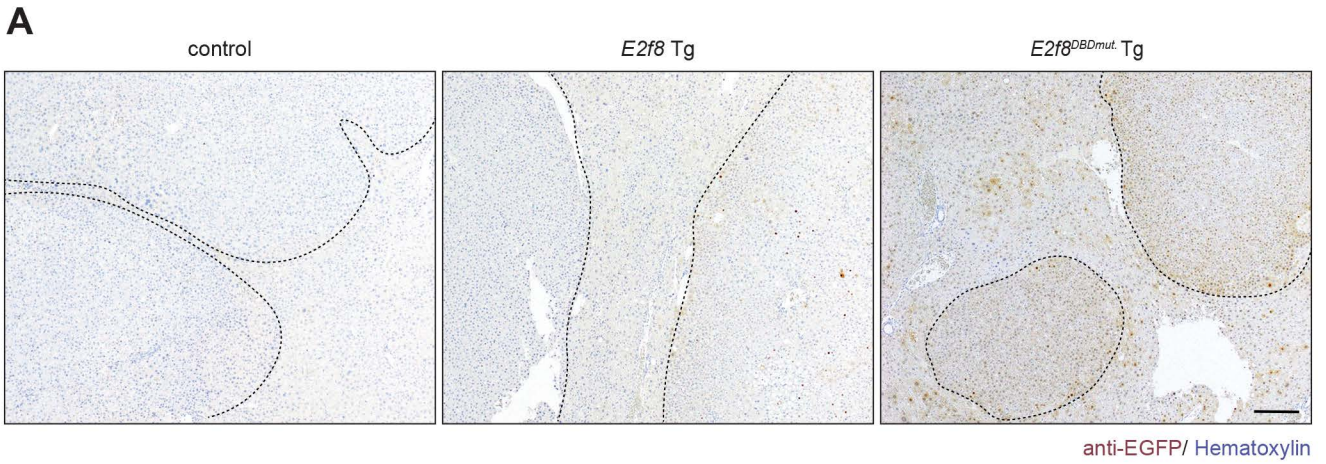
Supplemental Figure 7



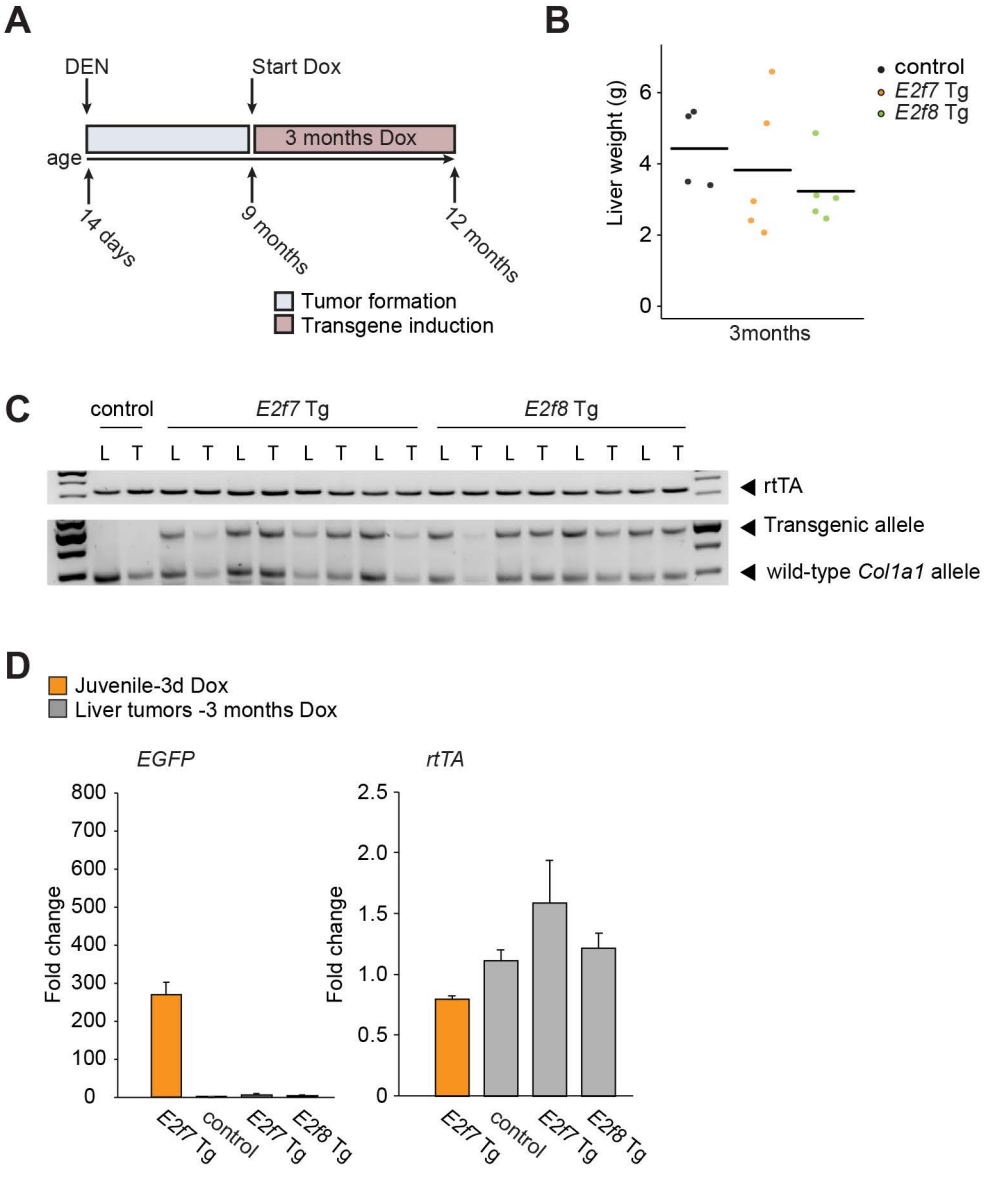




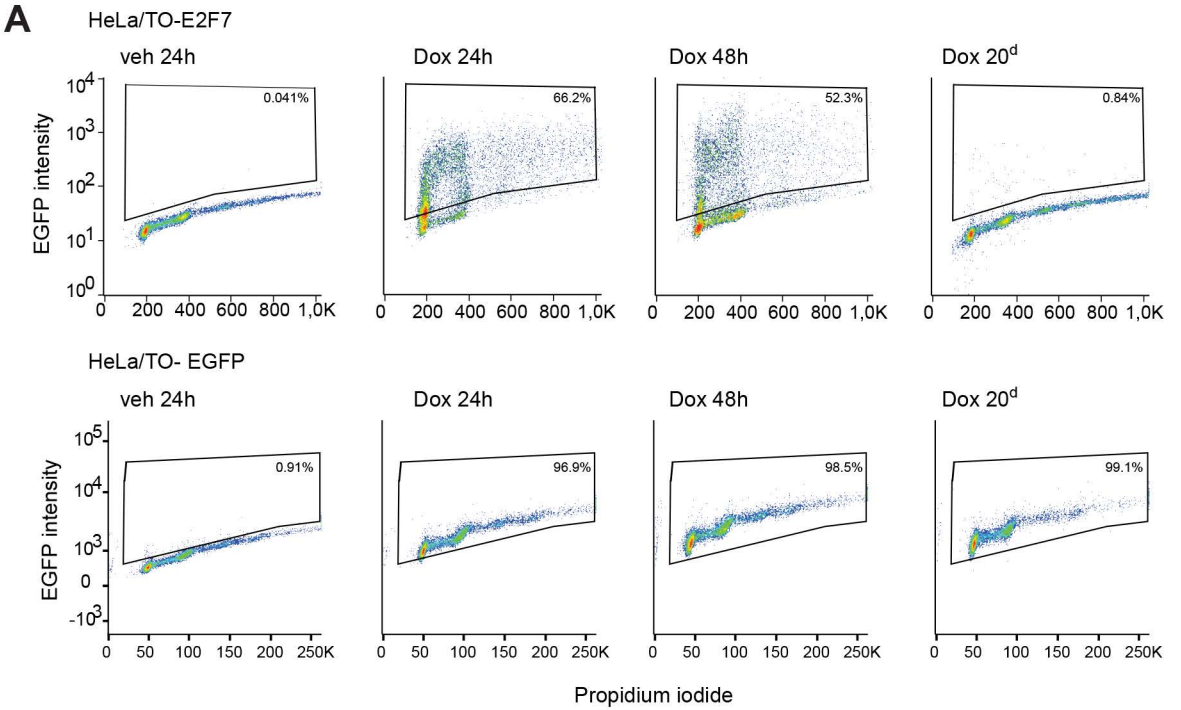
Supplemental Figure 8

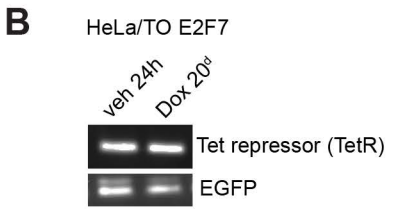


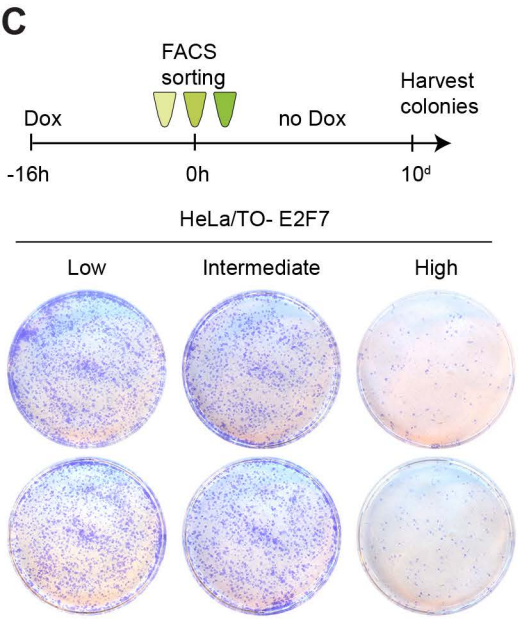
Supplemental Figure 9

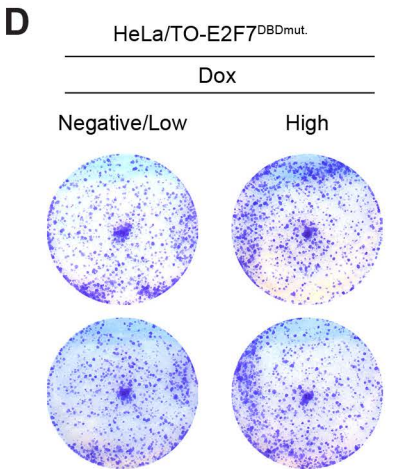


Supplemental Figure 10









Supplemental Figure 11