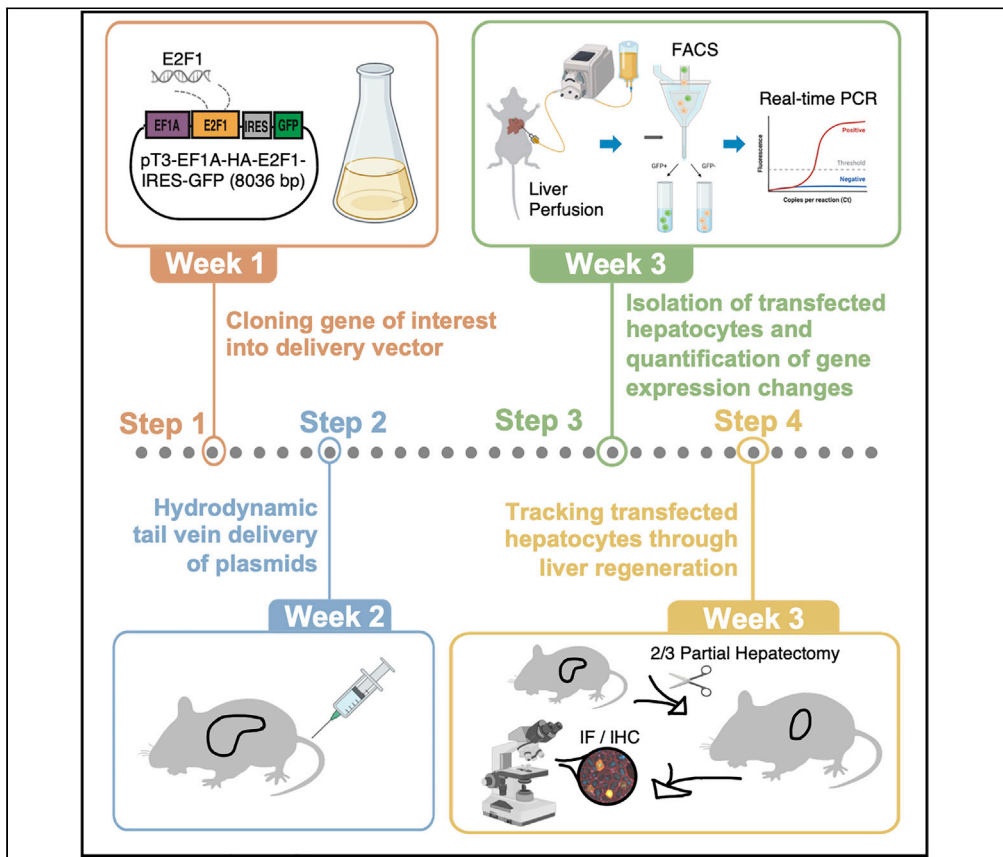


## Protocol

# Manipulating and tracking single hepatocyte behavior during mouse liver regeneration by performing hydrodynamic tail vein injection



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### Highlights

Rapid *in vivo* transfection of hepatocytes adaptable to any gene of interest

Transfected hepatocytes can be tracked with fluorescent reporter over time

Overexpressed E2F1 in hepatocytes and tracked them through liver regeneration

Studies to identify genes relevant to mammalian hepatocyte biology *in vivo* are largely carried out using germline genetic-engineering approaches, which can be costly and time-consuming. We describe hydrodynamic tail vein injection as an alternative approach to introduce genetic elements into hepatocytes. Transfected hepatocytes can then be traced with a GFP reporter enabling the use of immunohistochemistry and FACS sorting to examine the changes in hepatocyte gene expression and proliferation during liver regeneration induced by 2/3 partial hepatectomy (PH).

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## Protocol

## Manipulating and tracking single hepatocyte behavior during mouse liver regeneration by performing hydrodynamic tail vein injection

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## SUMMARY

Studies to identify genes relevant to mammalian hepatocyte biology *in vivo* are largely carried out using germline genetic-engineering approaches, which can be costly and time-consuming. We describe hydrodynamic tail vein injection as an alternative approach to introduce genetic elements into hepatocytes. Transfected hepatocytes can then be traced with a GFP reporter enabling the use of immunohistochemistry and FACS sorting to examine the changes in hepatocyte gene expression and proliferation during liver regeneration induced by 2/3 partial hepatectomy (PH).

For complete details on the use and execution of this protocol, please refer to Wang et al. (2019).

## BEFORE YOU BEGIN

This protocol involves the use of mice. Protocols for ethical use of animals must be obtained prior to starting these experiments. The research administration infrastructure in all institutions that are authorized to carry out animal experimentation have committees to approve such protocols (such as the Institutional Animal Care and Use Committee in the United States, Korea, and China, or the Animal Welfare and Ethical Review Body in the United Kingdom). In addition, technical proficiency in the surgical approaches for partial hepatectomy (PH), tail vein injection, and tissue harvest should be achieved prior to starting this protocol. A step-by-step description of PH can be found in a detailed protocol by Mitchell and Willenbring (Mitchell and Willenbring, 2008).

## Design infusion cloning primers

© Timing: 1–4 h

1. Design primers that are complementary to 20 bp of carrier plasmid (transposon-based vector) at the site of insertion and 15 bp of the target gene (in this case human E2F1 (<https://www.addgene.org/24225/>) at the gene boundary.



2. As an example, infusion primers used in this study are as shown below with part of the sequence that matches the carrier plasmid shown in non-bold and part of the sequence that matches the target gene shown in bold. The forward primer (primer 1) overlaps 20 bp of the carrier plasmid, upstream to the insertion site and the first 15 bp of the target gene, at the 5' end. The reverse primer (primer 2) overlaps 20 bp of the carrier plasmid, downstream to the insertion site (reverse complementary) and the last 15 bp of the target gene, at the 3' end (reverse complementary).

E2F1 infusion primer 1: AGCTGGGGGGATCTCTACCCATACGATGTTCCAGA

E2F1 infusion primer 2: GGGGGGGCGGAATTCAGAAATCCAGGGGGGTGA

3. Primers are synthesized as custom DNA oligos at Sigma or other vendor using standard conditions (purification by desalt). The primers are resuspended in Tris Acetate EDTA buffer (TE) at a final concentration of 100  $\mu$ M for stock solutions which are stored at  $-20^{\circ}$ C. Stocks are diluted in water to the working concentration of 25  $\mu$ M by diluting at a ratio of 1:4 (100  $\mu$ M primer solution : water).

## KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Antibodies</b>		
Chicken Polyclonal Anti-GFP	Abcam	Cat#ab13970
Rabbit Polyclonal Anti-Ki67	Abcam	Cat#ab15580
<b>Bacterial and virus strains</b>		
pCMVHA-E2F1	Addgene	plasmid #24225
pCMV-SB13	Dr. Scott Lowe	N/A
pT3-EF1a-NRAS-IRES-GFP	Dr. Scott Lowe	N/A
Stellar Competent Cells	Takara Bio	Part of In-Fusion® HD Cloning Plus kit
<b>Chemicals, peptides, and recombinant proteins</b>		
2-Propanol, ACS Reagent Grade	Sigma	Cat#190764
10% Neutral Buffered Formalin	Sigma	Cat#HT501128
Agarose	Denville	Cat#CA3510-8
Ampicillin sodium salt	Sigma	Cat#A9518
Bovine Serum Albumin	Sigma	Cat#05470
Chloroform, ACS Reagent Grade	Sigma	Cat#472476
Collagenase B from <i>Clostridium histolyticum</i>	Roche	Cat#11088831001
DAPI Fluoromount-G	SouthernBiotech	Cat#0100-20
DAPI for nucleic acid staining	Sigma	Cat#P4864
DNase/RNase-free water	Thermo Fisher	Cat#10977015
Ethanol, ACS Reagent Grade	Sigma	Cat#1009831011
Hepatocyte Wash Media	Gibco	Cat#17704024
GeneRuler 1 kb Plus DNA Ladder	Fisher Scientific	Cat#SM1331
Glycerol	Sigma	Cat#D9542
LB Agar Plates	Sigma	Cat#L7533
Liver Perfusion Media	Gibco	Cat#17701038
OCT Compound	Tissue-Tek	Cat#4583
Phosphate Buffered Saline, pH 7.4	Thermo Fisher	Cat#10010031
Pronase from <i>Streptomyces griseus</i>	Sigma	Cat#11459643001
SOC Medium	Sigma	Cat#S1797
Sodium chloride 0.9%	Intermountain	Cat#Z1377
SYBR® Safe DNA Stain, 10,000 $\times$	VWR	Cat#470193-138
Tris Acetate EDTA buffer, pH 8.4 (i.e., TE buffer)	Fisher Scientific	Cat#FERB49
TRizol®	Invitrogen	Cat#15596026
Trypan Blue Solution 0.4%	Gibco	Cat#15250061

(Continued on next page)

### Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Critical commercial assays</b>		
Alkaline Phosphatase, Calf Intestinal (CIP)	New England Biolabs	Cat#M0525S
BstXI Restriction Enzyme	Thermo Fisher	Cat#ER1021
CloneAmp Hifi PCR kit	Takara Bio	Cat#639298
EcoRI Restriction Enzyme	New England Biolabs	Cat#R0101S
EcoRV Restriction Enzyme	New England Biolabs	Cat#R0195S
In-Fusion® HD Cloning Plus	Takara Bio	Cat#638910
iQ SYBR Green Supermix	Bio-Rad	Cat#1708884
Plasmid Plus Midi Kit	QIAGEN	Cat#12945
QIAprep Spin Miniprep Kit	QIAGEN	Cat#27106
RNA to cDNA EcoDry Premix	Takara Bio	Cat#639549
XhoI Restriction Enzyme	New England Biolabs	Cat#R0146S
<b>Experimental models: organisms/strains</b>		
C57BL/6J wild-type mice (or any other wild-type or mutant inbred strains). For hydrodynamic injections, mice between 6–8 weeks of age are used to maximize transfection efficiency. For PH, male mice are typically used since the estrus cycle can alter the regenerative response in female mice.	The Jackson Laboratory	Stock#000664
<b>Oligonucleotides</b>		
E2F1 Infusion 1: AGCTGGGGGGATCTCTACCCATACGATGTTCCAGA	Designed in this study	N/A
E2F1 Infusion 2: GGGGGGGCGGAATTCAGAAATCCAGGGGGGTGA	Designed in this study	N/A
Sanger Sequencing: EF-1a_fwd TCAAGCCTCAGACAGTGGTTC	Addgene	N/A
Sanger Sequencing: IRES_fwd TGGCTCTCCTCAAGCGTATT	Addgene	N/A
Sanger Sequencing: IRES_rvs CCTCACATTGCCAAAAGACG	Addgene	N/A
<b>Software and algorithms</b>		
ImageJ		<a href="https://imagej.nih.gov/">https://imagej.nih.gov/</a>
Prism 6	GraphPad	<a href="https://www.graphpad.com/scientific-software/prism/">https://www.graphpad.com/scientific-software/prism/</a>
<b>Other</b>		
3 mL Syringe	VWR	Cat#BD309657
50 mL Falcon Tubes	Corning	Cat#352070
26-Gauge 5/8 Inch Needle	VWR	Cat#305115
BDSyringes without needle, 50 mL	Fisher Scientific	Cat#14-823-43
Falcon™ Round-Bottom Polystyrene Test Tubes with Cell Strainer Snap Cap, 5 mL	Fisher Scientific	Cat#352235
Sterile Syringe Filter 0.22 µm	Fisher Scientific	Cat#09-720-511
Ultracentrifuge Tubes	Beckman Coulter	Cat#344059
9mm EZ Clip (Braintree EZC- KIT) (applier and staples for closing outer skin after PH)	Braintree	Item#EZC- KIT
FACS Aria™ II	BD	N/A
TAKE3 Spectrophotometer	BioTek	N/A
Gel Doc™ EZ System	Bio-Rad	Cat#1708270
Heat Block	Denville	Cat#IncuBlock D1100
Hemocytometer	Fisher Scientific	Cat#Fisher 02-671-6
PowerPac™ Basic Power Supply, Gel Box, Gel Casters, and Combs	Bio-Rad	N/A
LightCycler® 480 (for real-time PCR)	Roche	N/A
Tailveiner Restraint for Mice	Braintree	Cat#TV-150 SM
Thermocycler (MJ Research PTC-200)	PCR, RT-PCR	
Ultracentrifuge	Beckman Coulter	Cat#LE-80K
Invitrogen Safe Imager (UV box)	Invitrogen	Cat#S37102
Water Bath	Cole-Parmer	Cat#EW-12105-93
Zeiss Observer 7 Light/Fluorescent Inverted Microscope equipped with monochrome camera for fluorescent imaging. Imaging liver sections stained with fluorescence-labelled antibody with the ZEN 2.3 (blue edition) software. Filters used in this study are Cy5, GFP, and DAPI. Images generated using 10X objective on multi-color imaging mode. Images were quantified using the ImageJ “cell counter” add-on ( <a href="https://imagej.nih.gov/ij/plugins/cell-counter.html">https://imagej.nih.gov/ij/plugins/cell-counter.html</a> )	Zeiss	Item#491917-0001-000

## MATERIALS AND EQUIPMENT

**Table 1: PCR conditions. Steps 1-3 were repeated 35 times.**

Step	Temperature	Time
1	98 °C	10 s
2	55 °C	5 s
3	72 °C	10 s

**Table 2: Real-time PCR conditions. Steps 2-4 were repeated 45 times.**

Step	Temperature	Time
1	95 °C	5 minutes
2	95 °C	10 s
3	60 °C	10 s
4	72 °C	10 s

## STEP-BY-STEP METHOD DETAILS

The steps in this protocol can be carried out in immediate succession over the course of 4 weeks or there can be a stopping point after the cloning step or after the tissue harvest step.

### Cloning gene of interest into delivery vector

⌚ Timing: ~ 4 days

Genes to be overexpressed in hepatocytes *in vivo* are cloned into the carrier plasmid in this step.

1. Prepare agarose gel.
  - a. A 1% agarose gel is prepared by dissolving 1.2 g of agarose in 120 mL of TAE buffer (40 mM tris base, 20 mM acetic acid, 1 mM of EDTA, pH 8) using microwave at median power for 1–2 min.
  - b. Wait ~ 10 min for the gel to cool down a bit before adding 12  $\mu$ L (1 : 10,000) of SYBR® Safe DNA Stain.
  - c. Gently swirl to mix and try to avoid bubbles.
  - d. Pour gently into gel casters with combs.

**Note:** make sure no bubbles are trapped under the combs. If bubbles are suspected, lift combs quickly, pop bubbles with pipette tip, and re-insert combs. This can only be done right after the gel is poured and before it begins to solidify.

- e. Once poured, the gel will rapidly solidify at 20°C–24°C (~20 min).

**Note:** Can prepare several days in advance and store at 4°C wrapped in plastic.

2. PCR amplify gene of interest (in this case E2F1) from parent plasmid (pCMVHA-E2F1, Addgene) using Infusion cloning primers.
  - a. Add 12.5  $\mu$ L of Hifi PCR Premix.
  - b. Add 2.5  $\mu$ L of Infusion primer 1.
  - c. Add 2.5  $\mu$ L of infusion primer 2.
  - d. Add 0.1–100 ng of DNA (see **Note** below).
  - e. Add DNase/RNase-free water to 25  $\mu$ L of total volume.
  - f. Run PCR using conditions in [table 1](#) on the thermocycler.

**Note:** Although the E2F1 transgene is overexpressed in hepatocytes in this protocol, it can be replaced with any other gene of the interest. In theory even multiple genes can be cloned into the same plasmid using the In-Fusion cloning kit and delivered to hepatocytes. It is recommended that for testing any new primer/plasmid pair the experimenter set up a few different reactions in parallel using different amounts of template DNA (e.g., 0.1 ng, 1 ng, 10 ng, 100 ng) as the amount of input can impact the amount of product obtained as well as the presence of nonspecific PCR products.

**Alternatives:** Even though the Hifi PCR reagents are included with the Infusion cloning kit, any high-fidelity DNA polymerase can be used at this stage to amplify the gene of interest. The sequence of the cloned gene will be verified via Sanger sequencing in step 7.

3. Gel purify PCR fragment.
  - a. Carry out electrophoresis of PCR products on a 1% agarose gel to purify the amplicon.
  - b. Load 10  $\mu$ L of a 1 kb ladder in the first lane to accurately assess the size of the PCR products.
  - c. Visualize DNA bands using a UV box and use a scalpel to cut the PCR band at 1383 bp containing E2F1 gene flanked by carrier-overlapping sequences.
  - d. Column purify the PCR fragment from the gel slice using the QIAquick Gel Extraction Kit:
    - i. Add 5 volumes of Buffer PB to 1 volume of the gel containing the PCR band.
    - ii. Apply mix to QIAquick spin column in collection tube.
    - iii. Centrifuge and discard flow through.
    - iv. Wash column by adding 0.75 mL of Buffer PE to the top of the column, repeat centrifugation, and discard flow through.
    - v. Centrifuge empty column in a new collection tube to remove excess buffer.
    - vi. Place the column into a new 1.5 mL Eppendorf tube.
    - vii. Elute the DNA by pipetting 50  $\mu$ L of buffer EB to the top of the column and centrifuge.

**Note:** Centrifugation steps are carried out by standard tabletop centrifuge at 10,000  $\times$  g for 1 min at 20°C–24°C unless otherwise stated.

4. Double digest carrier plasmid (pT3-EF1-Nras-IRES-GFP) with restriction enzymes XhoI and EcoRI.
  - a. Grow bacteria from glycerol stock to amplify the plasmid so it can be isolated for further manipulation.
    - i. Pre-warm LB agar plate with appropriate antibiotic (in this case ampicillin) to 37°C.
    - ii. Scratch surface of glycerol stock with a sterile pipette tip and return glycerol stock to –80°C.

**Note:** To prevent the glycerol stock from thawing, bring agar plate to the –80°C freezer for this step.

- iii. Gently streak the LB agar plate with appropriate antibiotic to select for plasmid used.
    - iv. Incubate the plate up-side-down at 37°C for 16 h.
    - v. Next day pick 4 colonies from plate and grow up each in 5 mL of LB media with appropriate antibiotic (in this case ampicillin), incubate for 8 h (day culture) in 37°C incubator shaking at 225 rpm.
  - b. Add 100  $\mu$ L of the day culture to 30 mL of LB with appropriate antibiotic and incubate for 16 h in 37°C incubator shaking at 225 rpm.
  - c. Next day, isolate plasmid DNA using Qiagen Midiprep EndoFree kit:
    - i. Harvest bacterial culture by centrifuging at 6000  $\times$  g for 15 min

**Note:** ultracentrifuge and special ultracentrifuge-friendly tubes need to be used here.

**Note:** 4 cultures are prepared to ensure that at least one will yield the plasmid with no mutations.

- ii. Resuspend bacterial pellet in 2 mL of Buffer P1.
- iii. Add 2 mL of Buffer P2, invert several times to mix, and incubate at 20°C–24°C for 3 min
- iv. Add 2 mL of Buffer S3 to mix and invert several times to mix.
- v. Load mix into QIAfilter Cartridge in tube and incubate at 20°C–24°C for 10 min
- vi. Gently insert the plunger into the QIAfilter Cartridge and filter the cell lysate into tube.
- vii. Add 2 mL of Buffer BB to filtered lysate and invert several times to mix.
- viii. Transfer lysate to spin column on QIAvac 24 plus.
- ix. Apply vacuum until liquid has drawn through all columns.
- x. Add 0.7 mL Buffer ETR to wash columns and apply vacuum until liquid has drawn through all columns.
- xi. Add 0.7 mL of Buffer PE and apply vacuum until liquid has drawn through all columns.
- xii. Centrifuge the columns at 10000 × *g* for 1 min in tabletop microcentrifuge to remove residual wash buffer.
- xiii. Place columns into clean 1.5 mL tube, add 200 μL Buffer EB, and incubate for 3 min at 20°C–24°C to elute DNA.
- xiv. Determine DNA concentration using the BioTek TAKE3 spectrophotometer.

**Note:** Any generic spectrophotometer that can measure absorbance at 260 nm can be used.

- xv. DNA can be stored at –20°C until use.
- d. Premix 400 ng of carrier plasmid (in 10 μL) + 25 pmol of each sequencing primer (in 5 μL) to Genewiz (or any other commercial sequencing service provider) for Sanger sequencing to verify sequence. For universal sequencing primers that are available with vendor, it is possible to submit carrier plasmid only and instruct the vendor to add the specified sequencing primer.
- e. Add 5 μg of plasmid DNA in 16 μL or less.
- f. Add 1 μL (20 units) of XhoI.
- g. Add 1 μL (20 units) of EcoRI.
- h. Add 2 μL of CutSmart buffer.
- i. Bring volume of reaction to 20 μL with DNase/RNase-free water.
- j. Incubate at 37°C for 4 h.
- k. Stop reaction by heat inactivation at 65°C for 20 min.
- l. Dephosphorylate DNA by adding 1 μL of alkaline phosphatase and incubate at 37°C for 1 h in heat block.
- m. Carry out electrophoresis on the digested plasmid on 1% agarose gel with 1 kb ladders and cut out the DNA band at 6683 bp that represent the linearized carrier plasmid; column purify the linearized plasmid using QIAquick Gel Extraction Kit as in step 3d.

**Note:** Different heat blocks maintained at these temperatures (37°C and 65°C) are useful for these experiments but an incubator, PCR machine, or water bath can be used as alternatives.

5. Insert PCR fragment or control insert (provided by the In-Fusion HD Cloning Plus Kit) into carrier plasmid via InFusion cloning:
  - a. Add 100 ng of gel purified PCR fragment
  - b. Add 50 ng of gel purified linearized vector.
  - c. Add 2 μL of 5× InFusion Premix
  - d. Top to 10 μL total volume with DNase/RNase-free water.
  - e. Incubate the reaction for 15 min at 50°C in the thermocycler, stop reaction by putting on ice.

**Note:** Amount of PCR fragment and linearized vector to add per reaction depends on the length of these vectors. According to manufacturer's instructions for PCR fragment: < 0.5 kb use 10–50 ng; 0.5 to 10 kb use 50–100 ng; > 10 kb use 50–200 ng; for linearized vector < 10 kb use 50–100 ng; > 10 kb use 50–200 ng.

△ **CRITICAL:** It is important to include both negative control (no PCR fragment) and positive control (2 µL of the control insert and 1 µL of the control vector included in the InFusion kit) at this step.

▮▮ **Pause point:** DNA at the end of each step can be stored at –20°C indefinitely.

6. Propagate the plasmid along with controls by transforming into Stellar competent bacteria:
  - a. Warm SOC medium to 37°C.
  - b. Thaw Stellar Competent cells in an ice bath (wet ice to ensure even cooling) just before use.
  - c. After thawing, mix gently to ensure even distribution.
  - d. Transfer 50 µL of competent cells into a 14 mL round-bottom tube (falcon tube). Do not vortex.
  - e. Add 2.5 µL of the In-Fusion cloning reaction from step 5.
  - f. For negative control: add 2.5 µL of the In-Fusion cloning negative control reaction from step 5.
  - g. For positive control: add 2.5 µL of the In-Fusion cloning positive control reaction from step 5.
  - h. Place tubes on ice for 30 min
  - i. Heat shock the cells for exactly 45 s at 42°C in a water bath.
  - j. Place tubes on ice for 1–2 min
  - k. Add pre-warmed SOC medium to bring the final volume to 500 µL.
  - l. Incubate by shaking (160–225 rpm) for 1 h at 37°C.
- m. Make 1/100 dilution and 1/5 dilution (into 100 µL of pre-warmed SOC).
- n. Concentrate the remaining bacteria by centrifuging at 2000 × g for 5 min and resuspend in 100 µL of pre-warmed SOC media.
- o. Plate bacteria in SOC media onto LB agar plates.
- p. Incubate plates upside down at 37°C in an incubator 16 h.

**Note:** Use the agar plate with the appropriate antibiotic resistance: for the pT3-EF1A-HA-E2F1-IRES-GFP plasmid the correct antibiotic is ampicillin.

**Note:** Normally we do observe some colonies even on the negative control plate. Cloning may be successful as long as the number of colonies in experimental plate is greater than the number of colonies in negative control plate, which will be verified in step 7.

- q. Next day, pick 4 colonies from each experimental plate and grow up each in 5 mL of LB media with appropriate antibiotic (in this case ampicillin), and incubate for 16 h in 37°C incubator shaking at 225 rpm.

△ **CRITICAL:** It is important to remember to store glycerol stocks for the transformed bacteria before validation of plasmid is done, otherwise one will need to restart the entire protocol from InFusion cloning (step 5).

- r. In the morning, after bacterial growth has reached saturation, retain 800 µL of transformed bacteria to prepare as glycerol stock: 800 µL bacteria + 200 µL glycerol, mix by vortexing and directly store at –80°C.
- s. Isolate plasmid DNA from E. coli culture using Miniprep Kit:
  - i. Centrifuge LB media at 2000 × g for 10 min at 4°C to pellet bacteria.
  - ii. Resuspend bacteria pellet in 250 µL of buffer P1.
  - iii. Add 250 µL of buffer P2, mix.
  - iv. Add 350 µL of buffer N3, mix.
  - v. Centrifuge for 10 min at 20°C–24°C.
  - vi. Add 800 µL of supernatant to QIAprep spin column.
  - vii. Centrifuge and discard flow through.
  - viii. Wash column with 0.5 mL of buffer PB, discard flow through.
  - ix. Wash column with 0.75 mL of buffer PE, discard flow through.
  - x. Elute DNA with 50 µL of buffer EB.



**Note:** Centrifugation is performed at 10000 × g unless otherwise specified.

7. Verify cloned plasmid by restriction digest and Sanger sequencing
  - a. Mix 400 ng of plasmid DNA (in 10 μL) + 25 pmol of each sequencing primer (in 5 μL) to Genewiz (or any other commercial sequencing service provider) for Sanger sequencing as described in step 4d.
  - b. For restriction digest:
    - i. Add 1 μg of plasmid DNA.
    - ii. Add 1 μL of XhoI.
    - iii. Add 1 μL of BstXI.
    - iv. Add 1 μL of EcoRV.
    - v. Add 10 μL of NEB 3 buffer.
    - vi. Add DNase/RNase-free water to 100 μL.
    - vii. Incubate at 37°C for 1 h, stop reaction by placing reaction on ice.
    - viii. Run reaction on 1% agarose gel along with 1 kb DNA ladder.
    - ix. Check for expected digest pattern (For the pT3-EF1A-HA-E2F1-IRES-GFP plasmid, expect 3 bands at 5348, 1891, and 797 bp).

△ **CRITICAL:** It is important to verify by both restriction digest, to confirm the preparation contains only the expected plasmid, and Sanger sequencing, to verify no mutations were introduced during cloning. It is not sufficient to only carry out Sanger sequencing at this verification step as Sanger sequencing will only pick up the most abundant sequence, therefore masking other potentially contaminating plasmids. If undesirable cloning products are observed in the restriction digest (i.e., bands are detected at unexpected locations in the agarose gel), then the cloning reaction will need to be repeated from step 5 with a few different PCR fragment to linearized vector ratios.

### Hydrodynamic tail vein delivery of plasmids

⌚ **Timing:** ~ 4 days

Hydrodynamic delivery of plasmids into mice will result in the expression of plasmid-encoded transgene in hepatocytes (Liu et al., 1999, Carlson et al., 2005). This protocol involves injecting a large volume of liquid containing the plasmid of interest into the mouse tail vein; the high colloidal pressure results in the transfer of the plasmid from the serum into the intracellular space of the liver through the fenestrated endothelium of the hepatic capillary bed (Chen and Calvisi, 2014).

8. Prepare plasmid for injections
  - a. Pre-warm an LB agar plate with appropriate antibiotic to 37°C to select for plasmid used.
  - b. Scratch surface of glycerol stock stored in step 6r or glycerol stock for pCMV-SB13 quickly with a sterile pipette tip and return glycerol stock to –80°C.

**Note:** Never allow the glycerol stock to thaw, bring agar plate to the –80°C freezer.

- c. Surface with the pipette tip without puncturing the agar with ampicillin.
- d. Incubate the plates up-side-down at 37°C for 16 h.
- e. Next day pick 4 colonies from plate and grow up each in 5 mL of LB media with appropriate antibiotic (in this case ampicillin), incubate for 8 h (day culture) in 37°C incubator shaking at 225 rpm.
- f. Add 100 μL of the day culture to 30 mL of LB with appropriate antibiotic and incubate for 16 h in 37°C incubator shaking at 225 rpm.
- g. Next day, isolate plasmid DNA using Qiagen Midiprep EndoFree kit as described previously in step 4c and determine the concentration of the plasmid DNA using BioTek TAKE3 spectrophotometer.

**Note:** If the yield of the Midiprep is very low (e.g., less than 100  $\mu\text{g}$  total yield), retransforming the final vector into One Shot™ Stbl3™ Chemically Competent *E. coli* (Thermo Fisher Cat#C7373-03) can help improve efficiency.

9. Hydrodynamic delivery of plasmids into mice.
  - a. Thaw plasmids to be injected (e.g., pT3-EF1A-HA-E2F1-IRES-GFP and pCMV-SB13) on ice.
  - b. For 1 mL of the injection mix, combine 15  $\mu\text{g}$  of pT3-EF1A-HA-E2F1-IRES-GFP, 3.75  $\mu\text{g}$  of pCMV-SB13, and sterile saline.
  - c. Filter the injection mix through an 0.22  $\mu\text{m}$  filter into a brand new 50 mL Falcon tube to ensure sterility.
  - d. Mouse tail vein hydrodynamic injection (in the biosafety hood of the mouse facility):
    - i. Weigh the mouse and calculate the amount of plasmid solution to administer (10% v/w, i.e., 2 mL plasmid solution per 20 g mouse). Always prepare some extra plasmid solution (~ 10% extra) to account for some volume wasted in the syringe.
    - ii. Draw up the desired amount of plasmid solution into a 3 mL syringe with a 26 gauge 5/8 inch needle.
    - iii. Place the entire mouse body inside of the restrainer with its tail sticking out.
    - iv. Warm the bottom of the mouse tail under heat lamp for 10 s.
    - v. Rapidly inject mouse via the tail vein (within ~ 5 to 7 s) with syringe held at a 10 degree angle to the vein.
    - vi. Take mouse out of strainer and check for vitality signs (normal heartbeat and breathing).
    - vii. Mice can be placed back into the original cage immediately after injection.

**△ CRITICAL:** It is important to use sterile techniques for sample preparation and injection (i.e., use of sterile single-use syringes, needles, Falcon tubes etc. and sterile solutions) to prevent unwanted immune reactions to the procedure.

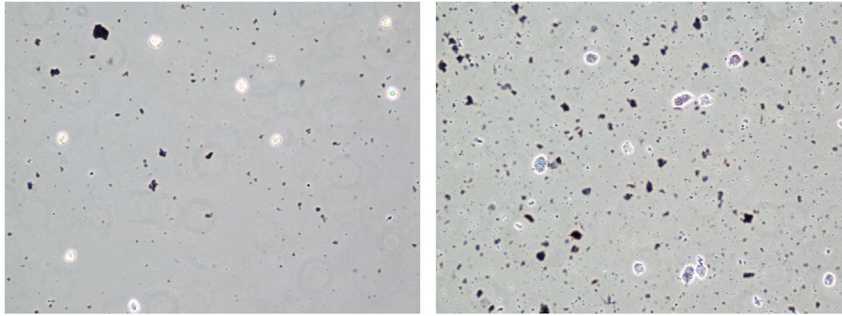
**Note:** The efficiency of plasmid delivery to hepatocytes will likely vary for different vectors in mice with different conditions (e.g., male/female, young/old, healthy/fat etc.) in different laboratory settings. Thus, it is advised to try 2–3 different concentrations ranging from 5  $\mu\text{g}$  to 30  $\mu\text{g}/\text{mL}$  of the target vector to achieve ideal delivery efficiency (i.e., how many hepatocytes get transfected). This can be assessed in a first round optimization experiment whereby the investigator checks the injected mice after 1 week to count the number of GFP positive cells on a tissue section. Then, a second round can be carried out using only the optimized concentration.

**Note:** Typically, after a successful injection, mice will appear “drowsy” for a few minutes up to an hour and return to full alertness/complete normalcy. If the mouse remains drowsy for longer than 1 h, this indicates improper injection technique and fatal injury, in which case the mouse needs to be euthanized immediately. Mice that do recover rapidly need to be monitored at 2, 12, and 24 h post-injection. Any signs of distress requiring euthanasia are to be attended to immediately.

### Isolation of transfected hepatocytes and quantification of gene expression changes

⌚ Timing: 4 days

GFP expressing hepatocytes can be isolated from the livers of hydrodynamically injected mice by in situ perfusion of the liver followed by fluorescence activated cell sorting (FACS). RNA is subsequently extracted from the isolated hepatocytes to quantify the amount of the transgene and any gene expression changes of interest using real-time polymerase chain reaction (real-time PCR).



**Figure 1. Hepatocyte preparations that contain mostly live (left) vs. dead (right) hepatocytes as viewed on light microscope following Trypan blue staining**

White arrows point to individual hepatocytes viewed at 200× total magnification.

10. Collagenase-based liver perfusion technique is used to isolate hepatocytes from mouse livers as described in detail by Li et al. (Li et al., 2010) with the following modifications:
  - a. Perfusion medium I is replaced with Liver Perfusion Medium.
  - b. Perfusion medium II is replaced with Hepatocyte Wash Medium containing 0.05% w/v Collagenase.
  - c. Instead of culturing the isolated hepatocytes, resuspend the final hepatocyte pellet in PBS + 1% BSA + 0.1 μg/mL of DAPI on ice and FACS immediately.

**Note:** The quality of Collagenase can be extremely variable; therefore, it is worthwhile purchasing small tester samples from different lots when optimizing hepatocyte isolation. Once a good lot of collagenase is identified, bulk order collagenase with the same lot number and store in frozen aliquots.

11. Evaluate hepatocyte cell viability using a light microscope
  - a. Mix 10 μL of cell suspension with 10 μL of Trypan Blue solution
  - b. Load 10 μL of the mixture onto a hemocytometer onto a light microscope
  - c. Count the total number of live cells (not stained blue, see Figure 1) from two random square grids out of the 9 square grids
  - d. Concentration of live hepatocytes is determined as follows: total # cells from two square grids × 10<sup>4</sup>/mL

**Note:** A good hepatocyte isolation preparation yields > 80% viable hepatocytes using Trypan Blue staining, in our hands. We found that even dead hepatocytes isolated by this method are not completely blue when stained with trypan blue dye, instead they appear to have some light-colored regions (Figure 1).

12. FACS isolation of transfected hepatocytes by GFP fluorescence on BD FACSAria™ II
  - a. Electronically gate hepatocytes based on their forward and side scatter parameters.
  - b. Single cells are selected based on forward scatter-area and forward scatter-height.
  - c. DAPI positive cells are excluded as dead cells.
  - d. The rest of cells are sorted into GFP positive and GFP negative fractions into 1 mL of TRIzol®.

▢ **Pause point:** Cells can be vortexed then stored in TRIzol® at −80°C indefinitely.

△ **CRITICAL:** Hepatocytes are very large cells; thus, necessary precautions need to be taken including filtering cells into FACS tubes immediately before loading into FACS machine and using a large size nozzle (100 μm used here).

**Alternatives:** Any FACS machine that can resolve GFP and DAPI positive cells can be used here instead of the BD FACSAria™ II.

13. Extract RNA from samples stored in TRIzol®.
  - a. Thaw sample in TRIzol®.
  - b. Add 0.2 mL of chloroform, vortex to mix.
  - c. Incubate for 3 min at RT.
  - d. Centrifuge samples at 12000 × *g* for 15 min at 4°C.
  - e. Carefully transfer the top aqueous phase to new tube.
  - f. Add 0.5 mL of 2-propanol, incubate for 10 min at 4°C.
  - g. Centrifuge samples at 12000 × *g* for 10 min at 4°C.
  - h. Discard supernatant.
  - i. Resuspend the RNA pellet in 75% ethanol.

**Note:** The pellet is very loose at this stage and may be dislodged easily. Be very gentle when removing supernatant.

- j. Centrifuge samples at 7500 × *g* for 5 min at 4°C.
- k. Discard supernatant.
- l. Airdry the RNA pellet for 10 min at RT.
- m. Resuspend RNA pellet in DNase/RNase-free water, pipette up and down to mix.
- n. Incubate RNA at 55°C–60°C for 15 min.

▮▮ **Pause point:** RNA can be stored at –80°C until RT-PCR.

**Alternatives:** RNA isolated here can also be used for RNA sequencing to profile global transcriptomic changes in transfected hepatocytes. It might be necessary to re-purify the TRIzol® isolated RNA through a silicone column (such as the Qiagen RNeasy kit) before samples can be used for library preparations.

14. Reverse-transcribe RNA into cDNA.
  - a. Determine RNA concentration using the BioTek TAKE3 spectrophotometer.
  - b. Add 1 µg RNA in 20 µL DNase/RNase-free water to cDNA EcoDry Premix, pipette up and down for up to 10 s to mix.
  - c. Incubate at 42°C for 60 min and stop reaction by incubating at 70°C for 10 min in PCR machine.

▮▮ **Pause point:** cDNA can be stored at –20°C or –80°C until real-time PCR.

15. Real-time PCR to determine gene expression in transfected hepatocytes.
  - a. Mix forward and reverse primers for each primer pair to make primer stock that contain 25 µM of each primer in DNase/RNase-free water (e.g., for 100 µL of primer stock: mix 25 µL of 100 µM forward primer solution, 25 µL of 100 µM reverse primer solution, with 50 µL DNase/RNase-free water).
  - b. Dilute cDNA to 5 ng/µL with DNase/RNase-free water.
  - c. For each reaction add 0.4 µL of 25 µM primer stock, 4.6 µL of DNase/RNase-free water, and 5 µL of iQ SYBR Green Supermix.

**Note:** To minimize pipetting errors, make master mix for each primer stock with SYBR Green (e.g., total number of samples = number of cDNA samples × triplicates). We normally prepare a few extra reactions to adjust for pipetting errors especially using automatic pipettors as well as for the negative control condition.

- d. Load samples into Axygen 384 Well PCR Microplate (pipette 5 µL of the appropriate cDNA or DNase/RNase-free water (negative control) into each well, then add 5 µL of the appropriate primer master mix).

- e. Seal the plate with Axygen 384 Well PCR Microplate Seals.
- f. Load plate into the Roche LightCycler® 480, real-time PCR program is shown in [Table 2](#).
- g. Calculate Ct values using the “Abs Quant/2<sup>nd</sup> Derivative Max” option in the LightCycler® 480 software.

**Alternatives:** Any real-time PCR machine and compatible SYBR reagents can be used.

- h. Delta Ct values are first generated by normalizing average Ct values (over triplicates) to the endogenous control (e.g., Gapdh):

$$dCt_{\text{sample}} = 2^{(Ct_{\text{endo}} - Ct_{\text{sample}})}$$

- i. Relative gene expression in the transfected hepatocytes is generated by normalizing to a reference sample (i.e., untransfected hepatocytes):

$$ddCt = dCt_{\text{sample}}/dCt_{\text{ref}}$$

### Tracking transfected hepatocytes through liver regeneration

⌚ Timing: 1 week

Hydrodynamically injected mice undergo PH and are sacrificed for liver immunohistochemistry/immunofluorescence to determine the effect of transgene overexpression on hepatocyte behavior during liver regeneration. It is recommended that a separate cohort of mice is used here as it is unclear how PH affects liver perfusion, which in turn represents a terminal procedure.

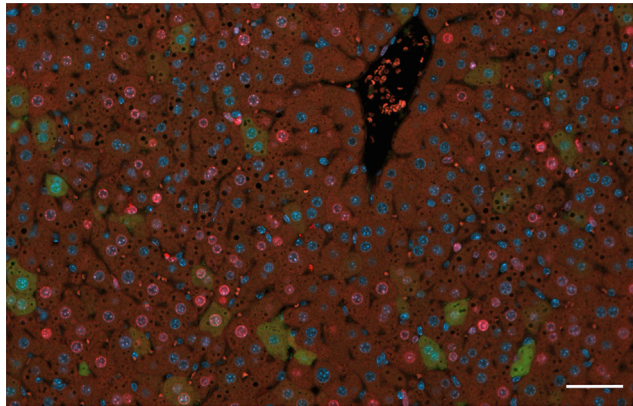
16. Removing 2/3 of liver mass by PH is carried out at 7 days after hydrodynamic vein injection as described in detail by Mitchell et al. ([Mitchell and Willenbring, 2008](#)) with the only modification being instead of sutures, wound clips are used to close the outer skin to increase speed and efficiency of surgery.

**Note:** It is highly recommended that experimenters practice with dead mice until they are capable of performing the surgical and suturing procedures before proceeding to using live mice.

17. To determine the effects on gene expression prior to cell division, mice are sacrificed at 30 h post-surgery. Intact liver lobes were fixed in 10% formalin (16 h, followed by switch to 70% ethanol until paraffin embedding and sectioning) or fixed in 10% formalin (16 h, followed by 10% sucrose/PBS, 20% sucrose/PBS, 30% sucrose/PBS over the next days, embed in OCT compound on dry ice then transferred to storage at  $-80^{\circ}\text{C}$  until sectioning on the cryostat in step 19).

**Note:** It is important to only use the untampered/non-resected lobes for subsequent studies as the lobes that have been resected may contain necrotic tissue.

18. Surgery and mouse sacrifice/tissue collection should be staggered at 30 min intervals to make sure the same 30-h recovery timepoint is measured for each mouse.
19. Immunofluorescence from cryosections:
  - a. OCT embedded tissues are mounted onto a cryostat and sectioned to 10  $\mu\text{m}$  thickness and placed on positively charged slides.



**Figure 2. Regenerating mouse liver at 30 h post-PH with transfected hepatocytes labeled by GFP (green) and stained with Ki67 (red) and DAPI (blue)**

Scale bar denotes 50  $\mu\text{m}$ .

- b. Antigen retrieval is carried out by incubating in Acetone for 10 min at  $-20^{\circ}\text{C}$ .
- c. Rehydrate in PBS for 5 min, then block in 3% BSA in PBS for 10 min at RT.
- d. Apply primary antibody (GFP and Ki67 antibodies 1 : 400 diluted in blocking solution) and incubate for 16 h at  $4^{\circ}\text{C}$ .
- e. Wash 3 $\times$  with PBS, 5 min each at RT.
- f. Apply secondary antibody (1:400 dilution of anti-Chicken Alexa Fluor™ 488 antibody and anti-Rabbit Alexa Fluor™ 647 antibody) in block and incubate for 30 min at RT.
- g. Wash 3 $\times$  with PBS, 5 min each at RT.
- h. Add a drop of DAPI Fluoromount-G
- i. Slowly place a coverslip on top to avoid trapping bubbles and seal with nail polish.

▮▮ **Pause point:** Mounted sections can be stored for a few months at  $4^{\circ}\text{C}$  until imaging.

- j. Image on a fluorescence microscope.
- k. 10 images are taken with the 10 $\times$  objective (100 $\times$  total magnification).
- l. The number of GFP and Ki67 double positive cells are counted and normalized to the number of GFP positive cells to obtain the fraction of transfected hepatocytes initiating cell proliferation at 30 h post-PH.

**Note:** The primary antibodies should be raised in different species to avoid cross-reactivity with the secondary antibodies.

## EXPECTED OUTCOMES

In this experiment, all mice tolerated hydrodynamic injection with no noticeable side effects or signs of morbidity or mortality. Under current conditions we can detect  $\sim 10\%$  GFP positive hepatocytes by immunohistochemistry/immunofluorescence at 7 days following hydrodynamic injection (example shown in Figure 2). Following FACS we recovered 1–2  $\mu\text{g}$  of RNA from 10,000 FACS isolated mouse hepatocytes. This is sufficient for cDNA preparations and for RNA-seq library preparation. We found that even though E2F1 transfected hepatocytes demonstrated an increase in the expression of cell cycle genes, this was not sufficient to initiate cell proliferation as measured by Ki67 staining. Subjecting these mice to PH provided an additional mitogenic stimulus and led to increased proliferation of E2F1 transfected hepatocytes. However, this increase was only significant in mice missing a key epigenetic regulator, Uhrf1, that held cell cycle genes in a primed position. There is no change in mortality or morbidity when we combine hydrodynamic vein injection with PH.

## LIMITATIONS

The system described here is used to overexpress transgenes in hepatocytes and cannot be used for knocking out genes in hepatocytes as it is; however, one can easily envision a system where a gRNA is inserted into the carrier plasmid and hydrodynamically delivered to mice that constitutively express the Cas9 protein (such as this line from The Jackson Laboratory - <https://www.jax.org/strain/024858>) to generate hepatocytes with specific gene knockouts.

To the best of our knowledge, insertion of the plasmids into each hepatocyte genome by transposase is random. This may introduce variability in the expression level of the transgene depending on the surrounding genomic context despite being driven by the EF-1a constitutive promoter.

## TROUBLESHOOTING

### Problem

The sequence or digest pattern of the cloned plasmid at the end of step 7 does not match those intended.

### Potential solution

After failed cloning, the experimenter may pick several more colonies from step 6q and repeat the procedure from there. If this fails to recover the desired sequence again then the experimenter may need to repeat the InFusion cloning (step 5), taking extra precautions to make sure to verify the size of the PCR product (at the end of step 3) and linearized vector (at the end of step 4) match those expected used in this step and test different ratios of PCR fragment to linearized vector as described. If all fails, the experimenter needs to validate the sequence of the parent plasmids used (by Sanger sequencing and restriction digest – see step 7) and restart at the very beginning.

### Problem

Unintended transfection of non-hepatocytes in the liver and in other tissues leading to undesirable biological phenotypes at steps 15 and 19.

### Potential solution

Within the liver, only hepatocytes (large, polygonal cells) get transfected as detected using GFP immunofluorescence (example in [Figure 2](#)). According to literature, the large volume injected briefly leads to retrograde flow of the injected solution into the liver, creating high colloidal pressure that facilitates the transfer of plasmids through the fenestrated endothelium of the hepatic capillary bed, and resulting in much higher transfection in the liver (10%–40%) than other tissues (< 0.1% of the liver transfection level in kidney, spleen, lung, and heart) ([Chen and Calvisi, 2014](#)). If even this minimal expression of transgene is causing undesired phenotypes in non-liver tissues, then the experimenter may consider lowering plasmid concentration and injection volume to introduce less plasmids into cells (hepatocytes and non-hepatocytes) ([Liu et al., 1999](#)), or incorporate a hepatocyte-specific promoter in the cloning steps to further restrict expression of the transgene to hepatocytes.

### Problem

High mouse mortality following hydrodynamic tail vein injection in step 9.

### Potential solution

For hydrodynamic tail vein injection, it is important to make sure there are absolutely no air bubbles inside the syringe before injecting mice because bubbles will get trapped in the mouse circulation and may lead to embolism and death. Only push the injection mix through the needle if the needle is inside of the tail vein, otherwise an edema might form at site of injection and dramatically decreases chance of success. If the injection fails, another try can be made in the other tail vein. The age of the mice may affect the success rate of the tail vein injection, with younger mice having smaller veins and older mice with more coloration and fibrous tails, both making it harder to locate the tail vein. In our experience mice that are between 6–8 weeks old, which are used in this study, are the easiest to

inject and achieve higher transfection efficiency (Ruiz de Galarreta et al., 2019). Alternative to the heat lamp, one can use restrainers that simultaneously warm mouse tail such as the “Mouse Tail Illuminator Restrainer” (Cat.# MTI STD) from Braintree Scientific may improve injection success.

### Problem

Low hepatocyte viability following liver perfusion in step 11.

### Potential solution

Liver perfusion can be a tricky procedure as mice are small in size and difficult to cannulate. If cannulation of the portal veins fails, one can try to cannulate the inferior vena cava on the same mouse. A successful cannulation should lead to backflow of blood into the catheter, at which point it is possible to attach the tubing (buffer already flowing) without introducing air bubbles. In our experience, low hepatocyte viability often results from a bad perfusion – suboptimal cannulation resulting in incomplete draining of blood from the liver and perfusion with collagenase. It is also possible to over-digest the liver with collagenase (as the enzyme strength may vary with different batches), which will also lead to a decrease in hepatocyte viability. It is best to practice several times and check viability at the end to get a sense of the quality of the Collagenase. The stiffness of the liver is a good indicator for the degree of digestion, with the liver softening overtime as the collagenase solution pass over it.

### Problem

High mouse mortality following PH in step 16.

### Potential solution

Major mouse surgery such as 2/3 PH is difficult to master and thus requires a lot of practice to become proficient before the actual experiment. The dosage of anesthesia (isoflurane) needs to be monitored very carefully, using the minimum dosage that maintains unconsciousness, otherwise the mouse will not recover and will die within a few hours post-surgery. If the mouse experiences internal bleeding during the surgery or is under anesthesia for more than 35 min, it is strongly advised that the mouse be sacrificed while unconscious rather than allowed to wake up because survival will be unlikely.

### Problem

Do not detect an effect of the transgene overexpression on hepatocyte behavior during liver regeneration at the end of step 19.

### Potential solution

It is possible that the transgene simply has no effect on liver regeneration. In our example we found that E2F1 overexpression only induced cell cycle gene and proliferation in *Uhrf1<sup>hepKO</sup>* mouse livers but not wild-type mouse livers (Wang et al., 2019). However, before completely ruling out the contribution of this transgene to liver regeneration one could carry out a more detailed time-course study to check for potential effects of the transgene. For example, in our study we only examined 72 h following hydrodynamic injection for cell cycle gene expression in transfected hepatocytes, but one can test earlier (e.g., 24 and 48 h) and later timepoints (96 h to 7 days) to see if the overexpressed transgene has a more acute or more chronic effect on cell cycle gene expression. Similarly, we checked a single timepoint of 30 h following PH for a potential effect of E2F1 overexpression, this timepoint was selected based on our previous data showing maximum difference between wild-type and *Uhrf1<sup>hepKO</sup>* mice at this post-surgery time point (see Figure 4D from Wang et al., 2019); however, the experimenter might want to also test 40 and 48 h following PH for an effect of the transgene, which is when the strongest cell cycle gene expression is expected in this model.



## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by Shuang Wang ([shuang.wang@mssm.edu](mailto:shuang.wang@mssm.edu)).

### Materials availability

This study did not generate any unique materials or reagents.

### Data and code availability

This study did not generate any unique datasets or code.

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## AUTHOR CONTRIBUTIONS

S.W. and K.C.S. conceived of and designed the experiments. S.W., M.R.G., and A.L. carried out the experiments. K.C.S. and A.L. supervised the studies. S.W. wrote the manuscript with input from M.R.G., K.C.S., and A.L.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

## REFERENCES

- Carlson, C.M., Frandsen, J.L., Kirchoff, N., Mcivor, R.S., and Largaespada, D.A. (2005). Somatic integration of an oncogene-harboring Sleeping Beauty transposon models liver tumor development in the mouse. *Proc. Natl. Acad. Sci. U S A* *102*, 17059.
- Chen, X., and Calvisi, D.F. (2014). Hydrodynamic transfection for generation of novel mouse models for liver cancer research. *Am. J. Pathol.* *184*, 912–923.
- Li, W.-C., Ralphs, K.L., and Tosh, D. (2010). Isolation and culture of adult mouse hepatocytes. In *Mouse Cell Culture: Methods and Protocols*, A. Ward and D. Tosh, eds. (Humana Press), pp. 185–196.
- Liu, F., Song, Y.K., and Liu, D. (1999). Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. *Gene Ther.* *6*, 1258–1266.
- Mitchell, C., and Willenbring, H. (2008). A reproducible and well-tolerated method for 2/3 partial hepatectomy in mice. *Nat. Protoc.* *3*, 1167–1170.
- Ruiz de Galarreta, M., Bresnahan, E., Molina-Sánchez, P., Lindblad, K.E., Maier, B., Sia, D., Puigvehi, M., Miguela, V., Casanova-Acebes, M., Dhainaut, M., et al. (2019 Aug).  $\beta$ -Catenin Activation Promotes Immune Escape and Resistance to Anti-PD-1 Therapy in Hepatocellular Carcinoma. *Cancer Discov.* *9*, 1124–1141.
- Wang, S., Zhang, C., Hasson, D., Desai, A., Senbanerjee, S., Magnani, E., Ukomadu, C., Lujambio, A., Bernstein, E., and Sadler, K.C. (2019). Epigenetic Compensation Promotes Liver Regeneration. *Dev. Cell* *50*, 43–56.e6.