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Corresponding Author:	Kirk J Wangensteen University of Pennsylvania Philadelphia, PA UNITED STATES
Corresponding Author's Institution:	University of Pennsylvania
Corresponding Author E-Mail:	wkirk@upenn.edu
Order of Authors:	Amber W Wang
	Adam M Zahm
	Kirk J Wangensteen
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TITLE:

Cell Type-Specific Gene Expression Profiling in the Mouse Liver

AUTHORS AND AFFILIATIONS:

Amber W. Wang[1], Adam M. Zahm[1], Kirk J. Wangensteen[1,2]

[1]Department of Genetics, University of Pennsylvania, Philadelphia, PA, USA
[2]Department of Medicine, University of Pennsylvania, Philadelphia, PA, USA

Email addresses of co-authors:
Amber W. Wang (wamber@pennmedicine.upenn.edu)
Adam M. Zahm (zahm@pennmedicine.upenn.edu)

Corresponding author:
Kirk J. Wangensteen (wkirk@pennmedicine.upenn.edu)

KEYWORDS:

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SUMMARY:

Translating ribosome affinity purification (TRAP) enables rapid and efficient isolation of cell type-specific translating mRNA. Here, we demonstrate a method that combines hydrodynamic tail-vein injection in a mouse model of liver repopulation and TRAP to examine the expression profile of repopulating hepatocytes.

ABSTRACT:

Liver repopulation after injury is a crucial feature of mammals which prevents immediate organ failure and death after exposure of environmental toxins. A deeper understanding of the changes in gene expression that occur during repopulation could help identify therapeutic targets to promote the restoration of liver function in the setting of injuries. Nonetheless, methods to isolate specifically the repopulating hepatocytes are inhibited by a lack of cell markers, limited cell numbers, and the fragility of these cells. The development of translating ribosome affinity purification (TRAP) technology in conjunction with the *Fah*$^{-/-}$ mouse model to recapitulate repopulation in the setting of liver injury allows gene expression profiling of the repopulating hepatocytes. With TRAP, cell type-specific translating mRNA is rapidly and efficiently isolated. We developed a method that utilizes TRAP with affinity-based isolation of translating mRNA from hepatocytes that selectively express the green fluorescent protein (GFP)-tagged ribosomal protein (RP), GFP:RPL10A. TRAP circumvents the long time period required for fluorescence-activated cell sorting that could change the gene expression profile. Furthermore, since only the repopulating hepatocytes express the GFP:RPL10A fusion protein, the isolated mRNA is devoid of contamination from the surrounding injured hepatocytes and other cell types in the liver. The affinity-purified mRNA is of high quality and allows downstream PCR- or high-throughput sequencing-based analysis of gene expression.

INTRODUCTION:

As the main metabolic organ in vertebrates, the liver is responsible for glucose homeostasis, serum protein synthesis, bile acid secretion, and xenobiotic metabolism and detoxification. The liver possesses an extraordinary capacity to regenerate the injured parenchyma upon exposure to toxins to prevent immediate liver failure[1]. However, failure of regeneration can occur in the setting of acetaminophen or alcohol overconsumption, which can lead to acute liver failure[2]. Furthermore, chronic liver injury caused by viral hepatitis infection, fatty liver disease, and steatohepatitis can cause liver fibrosis, cirrhosis, and hepatocellular carcinoma[3]. The only available curative treatment for end-stage liver disease is transplantation but is limited by organ shortage, preventing efficient treatment for all patients[4]. A better understanding of the recovery process after toxic liver injury is therefore crucial for the development of treatments to stimulate regeneration sufficient to rescue function in the diseased organ.

The most broadly applied model system for the study of liver regeneration is partial hepatectomy in rodents, in which a large proportion of the liver is resected to stimulate rapid hepatocyte expansion[5]. However, partial hepatectomy does not recapitulate hepatocyte expansion following toxic liver injury due to the lack of immune cell infiltration and hepatocyte cell necrosis often observed in the setting of acute liver injury in humans[6]. A more suitable system to model this form of organ renewal is the *Fah*$^{-/-}$ mouse, which lacks functional fumarylacetoacetate hydrolase (FAH) required for proper tyrosine metabolism and develops severe liver damage leading to death[7]. These mice can be maintained in a healthy state indefinitely by treatment with the drug nitisinone in the drinking water. Alternatively, FAH expression can be restored by transgene delivery to a subset of hepatocytes, which will expand to repopulate the liver upon nitisinone removal[8].

To profile the gene expression changes of repopulating hepatocytes, a tool to specifically isolate replicating hepatocytes in the *Fah*$^{-/-}$ mouse without contamination from the neighboring injured hepatocytes and other cell types is required. Unfortunately, fluorescence-assisted cell sorting (FACS) of hepatocytes is difficult since (1) the fragility of repopulating hepatocytes leads to poor recovery after liver perfusion, (2) replicating hepatocytes are highly variable in size, making isolation of a pure population by FACS difficult, and (3) the procedure time from liver perfusion to RNA isolation is greater than 2 hours, hence gene expression profiles may undergo substantial artificial changes before samples are acquired[9].

Alternatively, the expression of epitope-tagged ribosomes specifically in repopulating hepatocytes allows for the rapid isolation of actively translating mRNA bound by ribosomes using affinity purification immediately after organ harvest, with bulk liver tissue lysates. Here, we describe a protocol to perform translating-ribosome affinity purification (TRAP)[10] followed by high-throughput RNA-sequencing (TRAP-seq), to specifically isolate and profile mRNA in repopulating hepatocytes in the *Fah*$^{-/-}$ mouse[9]. Coexpression of green fluorescent protein-tagged ribosomal protein (GFP:RPL10A) with FAH allows affinity purification of translating mRNA bound by polysomes containing GFP:RPL10A. This method avoids any cell dissociation steps, such as liver perfusion to isolate fragile repopulating hepatocytes. Instead, it utilizes lysis of whole organ

tissue and antibodies to rapidly extract the RNA specifically from the target cells. Finally, isolation of abundant, high-quality mRNA via TRAP-seq enables downstream applications such as sequencing analysis to profile the dynamic change of gene expression during the repopulation process.

PROTOCOL:

All methods that involve the use of mice are consistent with the guidelines provided by the IACUC of the Penn Office of Animal Welfare at the University of Pennsylvania.

1. Reagent preparation

1.1. Cycloheximide

1.1.1. To make 500 µL of 0.1 g/mL cycloheximide, suspend 50 mg of cycloheximide in 500 µL of methanol.

CAUTION: Cycloheximide is extremely toxic to the environment and can cause congenital malformation. All wastes and buffers containing cycloheximide should be collected for proper disposal.

NOTE: Cycloheximide can be stored at 4 °C for up to 1 day. Cycloheximide inhibits translation.

1.2. Dithiothreitol (DTT)

1.2.1. To make 1 mL of 1 M DTT, suspend 0.15 g of DTT powder in RNase-free water.

CAUTION: DTT can cause irritation to the skin, eye, and respiratory tract.

NOTE: DTT is a detergent. DTT can be stored at -20 °C. It is recommended to store 1 M DTT in single-use aliquots of 50 µL.

1.3. Deoxycholate (DOC)

1.3.1. To make 10% DOC, suspend 1 g of DOC in a 50 mL conical tube and add RNase-free water up to 10 mL. Shake vigorously until the powder is dissolved.

NOTE: The 10% DOC solution is slightly yellow and can be stored at room temperature (RT) for up to 1 year. DOC is used for nuclear lysis.

1.4. GFP antibodies

1.4.1. Aliquot GFP antibodies when using for the first time. Snap freeze the aliquots and store at -80 °C.

NOTE: It is recommended to store 50 µg of GFP antibodies in single-use aliquots.

1.5. B biotinylated protein L

1.5.1. Resuspend biotinylated protein L in 1x phosphate-buffered saline (PBS) to make the final concentration 1 µg/µL.

NOTE: Resuspended solution can be stored at -20 °C for up to 6 months.

2. Buffer preparation

2.1. Bovine serum albumin (BSA) buffer

2.1.1. To make 50 mL of 3% BSA buffer, add 1.5 g of IgG- and protease-free BSA powder into 40 mL of PBS followed by vortex. After the BSA is dissolved, add PBS to a final volume of 50 mL.

NOTE: The BSA buffer can be stored at 4 °C for up to six months.

2.2. Dissection buffer

2.2.1. To make 50 mL of dissection buffer stock, combine 5 mL of 10x Hank's balanced salt solution (HBSS), 125 µL of 1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1,750 µL of 1 M glucose, and 200 µL of 1 M $NaHCO_3$. Add RNase-free water to a final volume of 50 mL.

NOTE: The dissection buffer stock can be stored at 4 °C for up to six months.

2.2.2. Immediately prior to use, add 100 µg/mL of 0.1 g/mL cycloheximide and keep on ice.

2.3. High-salt buffer

2.3.1. To make 50 mL of high-salt buffer stock, add 1 mL of 1 M HEPES, 8.75 mL of 2 M KCl, 500 µL of 1 M $MgCl_2$, and 500 µL of 100% nonylphenyl polyethylene glycol (**Table of Materials**) to RNase-free water.

NOTE: The high-salt buffer stock can be stored at 4 °C for up to six months.

2.3.2. Immediately prior to use, add 0.5 µL/mL of 1 M DTT and 1 µL/mL of 0.1 g/mL cycloheximide. Keep the fresh high-salt buffer on ice.

2.4. Low-salt buffer

2.4.1. To make 50 mL of low-salt buffer stock, add 1 mL of 1 M HEPES, 3.75 mL of 2 M KCl, 500 µL of 1 M $MgCl_2$, and 500 µL of 100% nonylphenyl polyethylene glycol to 44.25 Ml of RNase-free

water.

NOTE: The low-salt buffer stock can be stored at 4 °C for up to six months.

2.4.2. Add 0.5 µL/mL of 1 M DTT and 1 µL/mL of 0.1 g/mL cycloheximide prior to use. Keep the fresh low-salt buffer on ice.

2.5. Tissue lysis buffer

2.5.1. To make 50 mL of tissue lysis buffer stock, combine 1 mL of 1 M HEPES, 3.75 mL of 2 M KCl, and 500 µL of 1 M $MgCl_2$. Add RNase-free water to a final of 50 mL.

NOTE: The dissection buffer stock can be stored at 4 °C for up to six months.

2.5.2. Add 1 tablet/mL of EDTA-free protease inhibitor, 1 µL/mL of 0.1 g/mL cycloheximide, 10 µL/mL of RNase inhibitors each immediately prior to use. Keep the fresh tissue lysis buffer on ice.

3. Conjugation of antibodies to magnetic beads

3.1. Antibodies

3.1.1. Calculate the amount of GFP antibodies required for all samples and prepare for one extra sample.

NOTE: For each sample, 50 µg of each GFP antibody is required.

3.1.2. Thaw GFP antibodies on ice and spin at maximum speed (>13,000 x *g*) for 10 min at 4 °C and transfer supernatants to a new microcentrifuge tube.

NOTE: The antibody preparation step can be performed prior to bead preparation and the thawed antibodies can be kept on ice. Alternatively, this part can be performed during incubation of the magnetic bead and biotinylated protein L.

3.2. Resuspend magnetic beads

3.2.1. Resuspend magnetic beads (**Table of Materials**) by gentle pipetting.

3.2.2. For each sample, use 150 µL of magnetic bead. Calculate the volume of magnetic bead required for all samples and prepare one extra. Transfer the resuspended magnetic beads to a 1.5 mL or 2 mL microcentrifuge tube. If more than 1 mL is required for an experiment, split the total amount into equal volumes.

3.2.3. Collect beads on a magnetic stand for >1 min and remove the supernatant. Remove the microcentrifuge tube from the magnetic stand and add 1 mL of PBS followed by pipetting up and

down to wash the beads. Collect beads on a magnetic stand for >1 min and remove PBS.

3.3. Preparation of protein L-coated beads

3.3.1. For each sample, use 60 µL of biotinylated protein L. If protein L is previously resuspended and stored at -20 °C, thaw protein L on ice. If protein L is resuspended prior to use, take the amount required for all samples and prepare one extra.

3.3.2. Add the calculated volume of biotinylated protein L to the resuspended and washed magnetic beads. Add 1x PBS to make the final volume 1 mL if using a 1.5 mL microcentrifuge tube, or 1.5 mL if using a 2 mL microcentrifuge tube. Incubate magnetic beads with biotinylated protein L for 35 min at RT on a tube rotator.

NOTE: Antibodies can be prepared at this step while the beads are incubating with biotinylated protein L.

3.3.3. Collect protein L-coated beads on a magnetic stand for >1 min and remove the supernatant. Remove the microcentrifuge tube from the magnetic stand and add 1 mL of 3% BSA buffer followed by gentle pipetting for at least 5 times to wash the protein L-coated beads.

3.3.4. Collect coated beads on a magnetic stand for >1 min and remove the supernatant. Repeat the washing steps with 3% BSA for another 4 times (a total of 5 times).

3.4. Antibody binding

3.4.1. Add the calculated amount of GFP antibodies into the protein L-coated beads and incubate for 1 h at 4 °C on a tube rotator.

NOTE: After antibody incubation, take special care to not vortex the affinity matrix.

3.4.2. During incubation, prepare low-salt buffer by calculating the total volume required for all samples and add 0.5 µL/mL of 1 M DTT and 1 µL/mL of 0.1 g/mL cycloheximide to low-salt buffer stock prior to use.

NOTE: 3 mL of low-salt buffer for washing each tube of GFP-conjugated beads and 200 µL/sample for resuspension of the GFP-conjugated beads is required. Fresh low-salt buffer can be kept on ice for a couple of hours.

3.4.3. Collect the affinity matrix on a magnetic stand for >1 min and remove the supernatant. Add 1 mL of low-salt buffer and gently pipette up and down to wash the affinity matrix. Collect the affinity matrix on a magnetic stand for >1 min and remove low-salt buffer. Repeat the washing steps with low-salt buffer for another 2 times (a total of 3 times).

3.4.4. Resuspend the beads in low-salt buffer so that each sample has 200 µL of affinity matrix.

NOTE: The affinity matrix can be stored in 0.02% NaN_3 at 4 °C for up to 2 weeks. The affinity matrix should be quickly washed in low-salt buffer 3 times and resuspended gently on a tube rotator at 4 °C for at least 10 min if the affinity matrix is prepared within 1 week or overnight if the affinity matrix is stored for over 1 week. The protocol can be paused after this step.

CAUTION: Sodium azide is extremely toxic to the environment. Contact with acids produces toxic gas. All wastes should be collected for proper disposal.

4. Liver tissue lysis

4.1. Buffer preparation and equipment setup

4.1.1. Calculate the number of microcentrifuge tubes required, label and chill on ice.

NOTE: Usually, seven 1.5 mL microcentrifuge tubes are required for each sample: 1 for the remaining dissected liver, 4 for 4 mL of homogenized liver lysate, and 2 for transferring supernatants.

4.1.2. Prepare fresh dissection buffer by calculating the total volume required for all samples and add 1 µL/mL of 0.1 g/mL cycloheximide. Place the fresh dissection buffer on ice to keep cold throughout the experiment.

NOTE: For each sample, 10 mL of dissection buffer is required.

4.1.3. Prepare fresh lysis buffer by calculating the total volume required for all samples and add 1 tablet/mL of EDTA-free protease inhibitor, 1 µL/mL of 0.1 g/mL cycloheximide, and 10 µL/mL of RNase inhibitors each. Keep the lysis buffer on ice throughout the experiment.

NOTE: For each sample, 4 mL of lysis buffer is required.

4.1.4. Set up the tissue grinder (**Table of Materials**) so that the polytetrafluoroethylene (PTFE)-glass tubes can be placed on ice during homogenization of liver pieces. Put 4 mL of cold lysis buffer in the PTFE-glass tubes.

4.2. Repopulating liver homogenization

4.2.1. Euthanize an 8−12-week-old *Fah*$^{-/-}$ mouse injected with the TRAP vector and repopulated for 1−4 weeks with anesthesia and cervical dislocation according to approved animal experimental guidelines.

4.2.2. Place the mouse on a dissection board and spray the abdomen with 70% ethanol. Tent the skin and peritoneum low in the abdomen using forceps and use scissors to make a transverse incision. Continue to cut with the scissors to make a wide U-shaped peritoneal flap, with care to

not cut the viscera. Flip the peritoneal flap over the sternum to expose the liver.

4.2.3. Carefully remove the liver using fine scissors and forceps and quickly place the tissue in cold dissection buffer to rinse. To homogenize frozen tissues, quickly move the desired amount of liver tissue into PTFE-glass tubes with cold lysis buffer without thawing the tissue.

NOTE: The dissected tissue can be flash-frozen and stored at -80 °C after it is washed with dissection buffer. The protocol can be paused after this step.

4.2.4. Weigh the liver on a Petri dish and Isolate 200−500 mg of liver pieces and move into the PTFE-glass tubes. Place the remaining liver tissue into a pre-chilled microcentrifuge tube and flash freeze.

4.2.5. Homogenize the samples in a motor-driven homogenizer starting at 300 rpm to dissociate hepatocytes from the liver structure for at least 5 strokes. Lower the glass tube each time but take care to not let the pestle rise above the solution to prevent aeration that could cause protein denaturation.

4.2.6. Raise the speed to 900 rpm to fully homogenize the liver tissues for at least 12 full strokes.

4.2.7. Transfer the lysate into the labeled and pre-chilled tubes, with no more than 1 mL of lysate per 1.5 mL microcentrifuge tubes. If 4 mL of lysis buffer is used, keep 1 tube and flash freeze the remaining 3 tubes.

NOTE: The lysates can be kept on ice for up to 1 h while dissecting the next animal and preparing fresh lysates. The homogenized liver can be flash frozen after the lysis step and stored at -80 °C. There could be a 50% decrease in isolated RNA if frozen lysates are used. The protocol can be paused after this step.

4.3. Nuclear lysis

4.3.1. Centrifuge the liver lysate at 2,000 x *g* at 4 °C for 10 min and transfer the supernatant to a new, prechilled microcentrifuge tube on ice.

4.3.2. Add 1/9 of the supernatant volume of 10% nonylphenyl polyethylene glycol to make the final concentration 1% nonylphenyl polyethylene glycol and mix by gently inverting the microcentrifuge tubes.

4.3.3. Quickly spin down the microcentrifuge tubes and add 1/9 of the sample volume of 10% DOC to make the final concentration 1% DOC and mix by gently inverting the microcentrifuge tubes. Quickly spin down the microcentrifuge tubes and incubate on ice for 5 min.

4.3.4. Centrifuge the nuclear lysate at 20,000 x *g* at 4 °C for 10 min and transfer the supernatant to a new, prechilled microcentrifuge tube on ice.

NOTE: The mitochondria-depleted supernatant can be placed on ice for a couple of hours while the remaining samples are being collected.

5. Immunoprecipitation

5.1. For each tube, take out 1% of the total volume of the supernatant as a pre-immunoprecipitation control to compare target enrichment after incubation with the affinity matrix. Place pre-immunoprecipitation controls on a tube rotator at 4 °C overnight, the same way as the immunoprecipitated samples are processed.

5.2. Add 200 µL of affinity matrix to each sample. Take extra care to resuspend the beads by gentle pipetting prior to adding the affinity matrix to each sample. Incubate the lysates with affinity matrix at 4 °C overnight with gentle mixing on a tube rotator.

NOTE: The protocol can be paused for up to a day after this step.

6. RNA isolation

6.1. Buffer preparation and equipment setup

6.1.1. Place the magnetic rack at 4 °C for at least 30 min to pre-chill and keep the rack on ice throughout the experiment.

6.1.2. Calculate the number of microcentrifuge tubes required and pre-chill on ice or at 4 °C.

NOTE: Usually, each sample requires 1 microcentrifuge tube for the final purified RNA.

6.1.3. Quickly spin down the tubes from step 5.2 and collect the beads by placing on the magnetic rack for at least 1 min. Collect or discard the supernatant in additional microcentrifuge tubes.

NOTE: The collected supernatant that contains unbound fraction can be flash-frozen and stored at -80 °C to compare with the bound fraction for transcript enrichment after purification. The protocol can be paused after this step.

6.1.4. Prepare high-salt buffer by adding 0.5 µL/mL of 1 M DTT and 1 µL/mL of 0.1 g/mL cycloheximide to high-salt buffer stock.

NOTE: For each sample, 5 mL of high-salt buffer is required.

6.2. RNA isolation

6.2.1. Add 1 mL of fresh high-salt buffer to each tube followed by gentle pipetting for at least 5 times without introducing bubbles.

NOTE: Insufficient washing could introduce backgrounds of unbound transcripts while the introduction of bubbles could accelerate RNA degradation.

6.2.2. Collect beads on a magnetic stand for >1 min and remove the supernatant. Repeat the washing steps with high-salt buffer for another 4 times (a total of 5 times).

6.2.3. Remove remaining high-salt buffer and remove microcentrifuge tubes from the magnetic stand and place at RT for 5 min to warm up.

6.2.4. Resuspend the beads in 100 µL of lysis buffer (provided in the RNA isolation kit) with β-mercaptoethanol.

NOTE: Any RNA isolation and purification kit that contains the denaturant guanidine thiocyanate can be used as a lysis buffer to release bound RNA from the affinity matrix. RNA extraction should be processed at RT since guanidine thiocyanate can crystallize at low temperatures.

6.2.5. Vortex the beads and buffer for at least 5 s at the highest speed, quickly spin down to collect the buffer on the side of the microcentrifuge tube and incubate the beads with the buffer at RT for 10 min to release the bead-bound RNA into the lysis buffer.

6.2.6. Collect beads on a magnetic stand for >1 min and collect the supernatant to proceed immediately to RNA cleanup according to the RNA purification protocol as specified in the kit (**Table of Materials**).

NOTE: The supernatant containing the eluted RNA in lysis buffer can also be stored at -80 °C for up to 1 month before cleanup by warming up to RT upon thawing.

6.2.7. To achieve maximum quality of the isolated RNA, perform all optional steps including DNase digestion and all RNA elution steps. Heat up the elution buffer provided by the RNA isolation kit or RNase-free water to 60 °C for maximum RNA recovery.

NOTE: The isolated RNA can be stored at -20 °C for up to 1 month or -80 °C for several years. The protocol can be paused after this step.

7. Optional RNA quality analysis (**recommended**)

7.1. Assess RNA quality using a bioanalyzer[11] and quantity with a spectrophotometer[12] to determine if repeating the immunoprecipitation process is required to obtain ample and high-quality RNA.

NOTE: The optimal RNA quality for high-throughput sequencing should follow protocols specified by library preparation kits and sequencing platforms.

8. Downstream applications

NOTE: Total RNA isolated by the TRAP protocol can be used in a number of standard downstream applications, including RNA-seq (TRAP-seq). Standard reverse transcription and quantitative PCR protocols can also be used following TRAP.

8.1. For TRAP-seq, perform RNA-seq library preparation according to standard methods[13].

8.2. For RNA-seq, prepare cDNA sequencing libraries using commercial RNA-seq kits with oligo d(T)-based enrichment of polyadenylated poly(A) transcripts. Alternatively, if the total RNA quality is lower than recommended for poly(A) enrichment, use rRNA depletion modules. However, expect to see more rRNA alignment after sequencing.

REPRESENTATIVE RESULTS:

To profile gene expression in repopulating hepatocytes of the *Fah*$^{-/-}$ mouse, *Gfp:Rpl10a* fusion and *Fah* transgenes are co-delivered within a transposon-containing plasmid[8] (TRAP vector) to livers by hydrodynamic injection (**Figure 1A**). The removal of nitisinone induces a toxic liver injury that creates a selection pressure for hepatocytes stably expressing FAH to repopulate the injured parenchyma[9]. Immunofluorescence staining confirms the co-expression of FAH and the GFP:RPL10A fusion protein in repopulating hepatocytes after two weeks of liver repopulation (**Figure 1B**).

In the following representative experiment, TRAP-seq was performed using quiescent and repopulating mouse hepatocytes. First, to obtain GFP-tagged ribosomes from quiescent hepatocytes, transgenic *Rosa*$^{LSL\text{-}GFP\text{-}L10A}$ mice were injected with AAV8-TBG-Cre 7 days prior to sacrifice to induce GFP:RPL10A expression in all hepatocytes[14]. We also processed a liver sample collected from a wild type mouse as a negative control to ensure isolation of translating mRNA was specific, meaning RNA could only be extracted from mice expressing GFP:RPL10A. The concentration of isolated RNA correlated with the number of cells expressing the fusion protein, with the quiescent sample producing the highest yield since all hepatocytes express GFP:RPL10A after AAV8-TBG-Cre injection (**Figure 2A**). Conversely, barely any RNA was detectable in wild type controls that did not have the GFP:RPL10A transgene, indicating the TRAP procedure is highly specific and has a low background. When TRAP was used on liver tissue undergoing repopulation with GFP:RPL10A-transduced hepatocytes, abundant, high-quality RNA was obtained (**Figure 2B**). In contrast, no RNA trace was detected via bioanalyzer for the negative control sample.

Downstream gene expression analysis can be carried out via reverse transcription and quantitative PCR or RNA-seq on TRAP-isolated RNA. *Gsta1* encodes glutathione S-transferase that plays an important role for the metabolism of glutathione, the main detoxifying peptide to protect the liver against oxidative stress damage[15]. *Gsta1* expression is induced by over 10-fold in repopulating hepatocytes as compared to quiescent hepatocytes, while no threshold cycle (Ct) value was detected with TRAP-isolated RNA from the wild type mouse due to the lack of input RNA (**Figure 3A**). Note that RNA quality can greatly impact the gene expression analysis. In the case of RNA-seq experiments, assessment of RNA quality should be performed according the

recommendations of the library preparation kit and the sequencing platform (**Figure 3B**). A bioanalyzer is often used to determine the RNA integrity number (RIN), with a high RIN correlating with a higher rate of mRNA alignments to the genome (**Figure 3B, left**), whereas a lower RIN leading to a higher rate of ribosomal reads, indicating mRNA degradation (**Figure 3B, right**). **Figure 3C,D** demonstrates that TRAP-seq can identify differential gene expression in quiescent and repopulating hepatocytes. For instance, *Alb* expression is inhibited and *Afp* expression is upregulated during liver repopulation, reflecting that the replicating hepatocytes assume a less differentiated state to inhibit liver metabolic functions during repopulation[9,16].

FIGURE LEGENDS:

Figure 1: Implementation of TRAP with *Fah*$^{-/-}$ to profile gene expression change of repopulating hepatocytes. (**A**) Schematic of expressing the GFP:RPL10A fusion protein with FAH in the *Sleeping Beauty* transposon system followed by injection into the *Fah*$^{-/-}$ mouse. Green hexagons indicate repopulating hepatocytes with stable expression of FAH and GFP:RPL10A, whereas black hexagons represent injured, dying hepatocytes. (**B**) Representative immunofluorescence staining demonstrates coexpression of GFP-tagged ribosomal protein L10A (green) and FAH (red) in the repopulating hepatocytes. Scale bar = 50 μm.

Figure 2: TRAP allows cell type-specific isolation of high-quality RNA. (**A**) The yield of RNA is positively correlated with the number of hepatocytes expressing GFP:RPL10A. The low yield of RNA from a wild type mouse demonstrates the specificity of TRAP from sources without the expression of GFP:RPL10A. (**B**) Bioanalyzer traces of total RNA isolated from repopulating livers expressing GFP:RPL10A and from wild type livers demonstrate the specificity of TRAP. Total RNA isolated from wild type liver tissue devoid of the GFP:RPL10A transgene shows that minimal RNA has been collected, whereas transgene-expressing tissue provides ample high-quality RNA. Note that ribosomal RNA peaks are present following successful TRAP[10]. FU, fluorescence unit. RIN, RNA integrity number.

Figure 3: TRAP-isolated RNA can be used for downstream gene expression analysis. (**A**) Representative reverse transcription and quantitative PCR results of *Gsta1* in quiescent and repopulating hepatocytes. No Ct value was detected with RNA isolated from wild type animals. (**B**) Alignment analysis of isolated RNA after high-throughput sequencing, demonstrating the importance of determining RNA integrity after isolation. High-quality RNA results in a higher percentage of mRNA reads (green), while low-quality RNA leads to a much higher percentage of ribosome reads (red), as most mRNA is degraded. RIN, RNA integrity number. (**C**,**D**) Integrative Genomics Viewer (IGV) tracks of RNA-seq reads of mRNA affinity-purified from quiescent and repopulating hepatocytes at the (**C**) *Alb* and (**D**) *Afp* loci. Note that the 3' read bias is typical of a poly(A) selection pipeline.

DISCUSSION:

TRAP-seq is a technique for cell type-specific isolation of translating mRNA via epitope-tagged ribosomes and presents an alternative to FACS approaches, as it circumvents limitations such as time requirements of FACS[9]. Instead, TRAP allows rapid and efficient isolation of RNA directly

from bulk tissues, helping to avoid any alterations in gene expression. TRAP-seq is especially well-suited for use in the repopulating *Fah*$^{-/-}$ mouse liver, as hepatocyte expansion following removal of nitisinone is cell autonomous and enables gene expression profiling of the subset of hepatocytes with integrated transgenes. The TRAP vector can also be coexpressed with gene-activating or -silencing molecules[17], including cDNA, short-hairpin RNA, and guide RNA, to study the effects on global gene expression of activation or inhibition of a specific gene. Alternatively, the *Rosa*$^{LSL\text{-}GFP\text{-}L10A}$ transgenic mouse provides the ability to profile gene expression in any cell with Cre recombinase activity. Since GFP:RPL10A can be specifically expressed in any cells that express Cre, the role of other cell types in the liver during liver injury and repopulation could be studied. For instance, crossing CK19-Cre mouse with the TRAP transgenic mouse could be used to express GFP:RPL10A in cholangiocytes followed by TRAP-seq to study the change of gene expression in the biliary epithelium during the repopulation process.

To ensure accurate profiling of gene expression, it is critical to prepare all buffers and the affinity matrix prior to tissue dissection. All steps should be performed on ice with cold buffers unless otherwise specified to ensure polysome stabilization[10] and prevent RNA degradation. All buffers should be prepared with RNase-free reagents and the TRAP-seq protocol should be carried out in an RNase-free environment to prevent RNA degradation and low yield of immunoprecipitated RNA. The affinity matrix can be prepared up to 2 weeks prior to use with gentle resuspension on a tube rotator overnight. Special care should be taken to not vigorously shake the matrix to prevent disruption of the antibody-conjugated, protein L-coated magnetic beads. The methods to prepare the affinity matrix includes conjugation of magnetic beads to biotinylated protein L followed by incubation with anti-GFP antibodies. However, commercially available protein A/G magnetic beads can be substituted; if used, skip the initial conjugation step and proceed directly to antibody binding. Furthermore, alternative epitope tags are presumably feasible with the above protocol with appropriate modification.

There are various points in which the RNA isolation and purification step can be paused (see protocol above). However, once liver samples have been harvested, continuing to the immunoprecipitation is recommended, as the yield of isolated RNA could drop by ~50% with freezing at this step[10]. Tissues should be quickly rinsed with dissection buffer that contains cycloheximide to inhibit mRNA translation. Insufficient tissue lysis could also contribute to low RNA yield. It is critical to homogenize tissues on ice until no tissue chunks are visible with the motor homogenizer while ensuring minimal aeration[10]. Additionally, sufficient washing with high-salt buffer is crucial to ensure removal of nonspecific binding of ribosomal proteins to the affinity matrix. Including a wild type negative control helps to assess the specificity of the immunoprecipitation and the efficiency of the wash steps. Additionally, using a commercial RNA purification kit that includes RNase-free DNase treatment will increase RNA purity.

Moreover, it is recommended to verify the expression and abundance of the GFP:RPL10A fusion protein and assess the amount of tissue required to obtain ample RNA for downstream analysis. Tissue sections or lysates could be used for immuno-based detection methods to validate the expression of GFP:RPL10A. The amount of RNA isolated can vary by: (1) the number of cells expressing GFP:RPL10A, (2) the expression level of the transgene, and (3) the size and ploidy of

the cells expressing the transgene. A pilot experiment using half and double the amount of the recommended amount of tissue could be useful in determining the optimal input lysate for TRAP-seq. In our hands, we could obtain ~150 ng of RNA with as little as 1-2% of hepatocytes expressing GFP:RPL10A from 200 mg of the repopulating *Fah*$^{-/-}$ liver, representing ~2x10^5 polyploid hepatocytes with transgene expression[9].

The TRAP-seq methodology isolates ribosome-bound mRNA to profile a cell's translating mRNA pool. The resulting sequencing reads therefore correspond to the 'translatome' rather than the transcriptome. Note that translating ribosome footprints will not be collected, as TRAP is performed on native rather than crosslinked complexes. If footprinting analyses are desired, the above protocol should be modified with relevant cross-linking followed by immunoprecipitation (CLIP) methodologies[18]. Another limitation of TRAP is the requirement of a sufficient number of cells expressing the GFP:RPL10A fusion protein. For experiments in which the cell type of interest is small, combining multiple biological samples may be required to isolate sufficient RNA to enable RNA-seq[19]. Furthermore, TRAP-seq requires the presence of GFP:RPL10A in the cell type of interest. This could pose a challenge if there is no specific delivery system to the cells or if a cell-type specific promoter to drive Cre expression is not available.

The recent development of single-cell RNA-seq (scRNA-seq) technology has allowed direct sequencing followed by *in silico* identification of various cell types, enabling sequencing without sorting for specific cell types of interests[20-22]. However, scRNA-seq still requires dissociation of cells from the organ. In the case of the *Fah*$^{-/-}$ repopulation model, liver perfusion and hepatocyte isolation is extremely difficult and inefficient due to the fragility of both the injured and replicating hepatocytes. In fact, we have not yet been able to isolate sufficient hepatocytes from *Fah*$^{-/-}$ mice undergoing repopulation after hydrodynamic injection of *Fah* plasmids. Additionally, in the time it takes to process tissues, gene expression levels could change. Protocols for liver perfusion take up to 30 minutes of warm ischemia time. Future methodologies to optimize liver perfusion to decrease processing time and increase isolation efficiency could allow scRNA-seq integration to the *Fah*$^{-/-}$ mouse model system and possibly to other injury and repopulation models. This would also enable the study of all liver cell types.

In conclusion, the integration of TRAP-seq with the *Fah*$^{-/-}$ mouse allows specific isolation and gene expression profiling of replicating hepatocytes to identify therapeutic targets that could promote liver repopulation. This method can be implemented to study other cell types in the liver and other organ systems for disease-specific identification of gene expression changes to identify potential drug targets or biomarkers. An analogous technique can be used to collect nuclei from repopulating hepatocytes using affinity purification, and then to perform an epigenetic analysis of these specific cells[16].

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

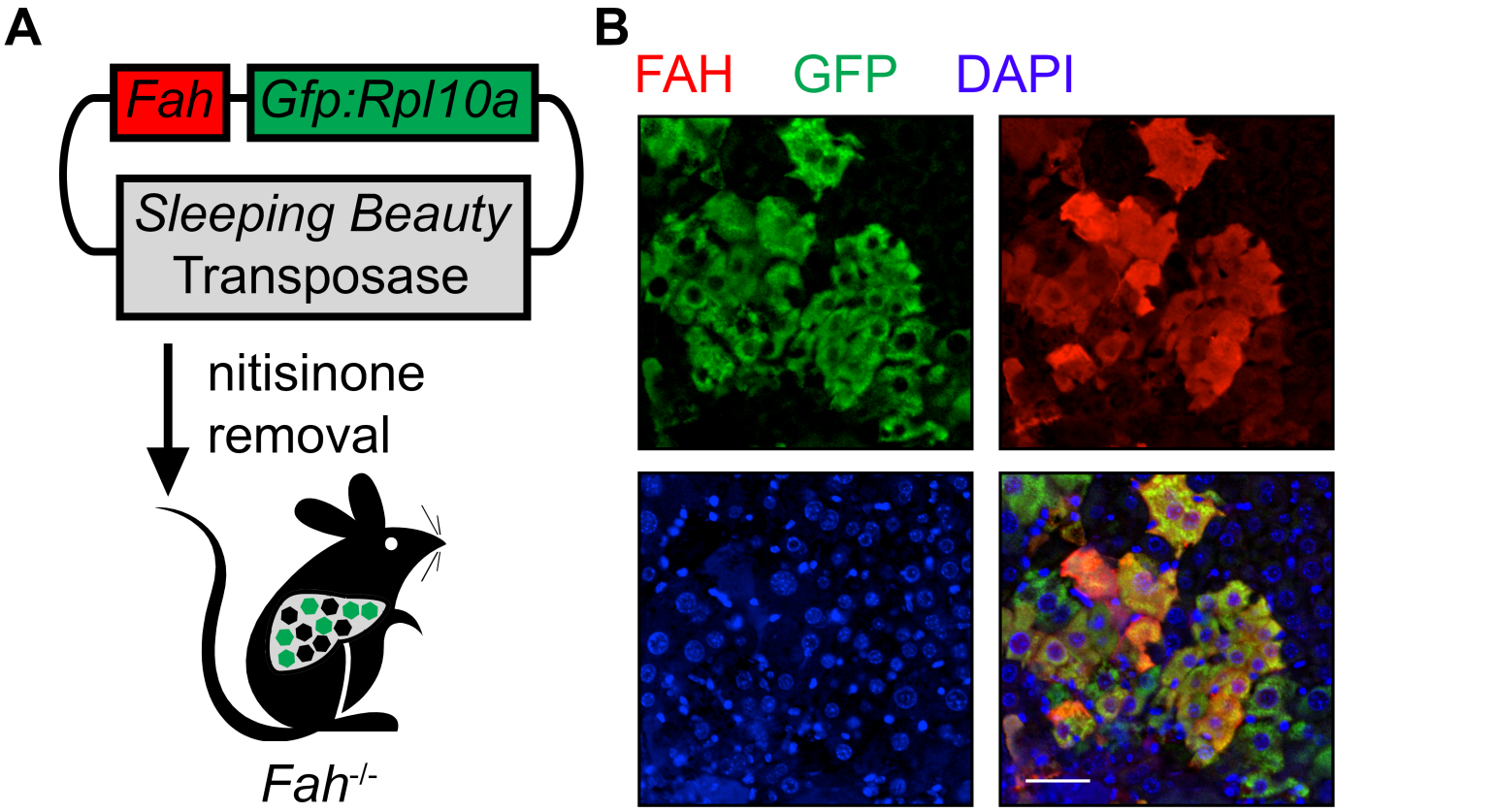


Figure 2

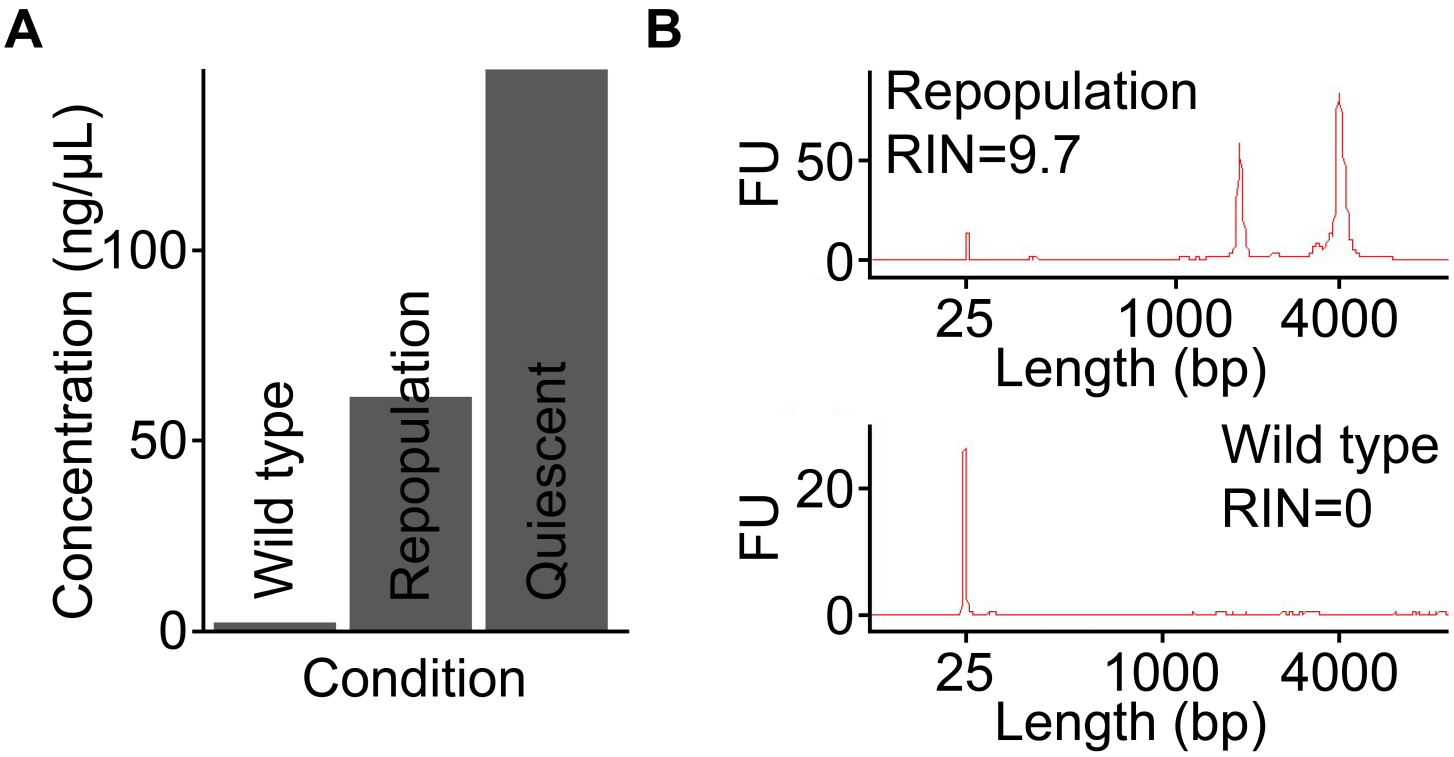
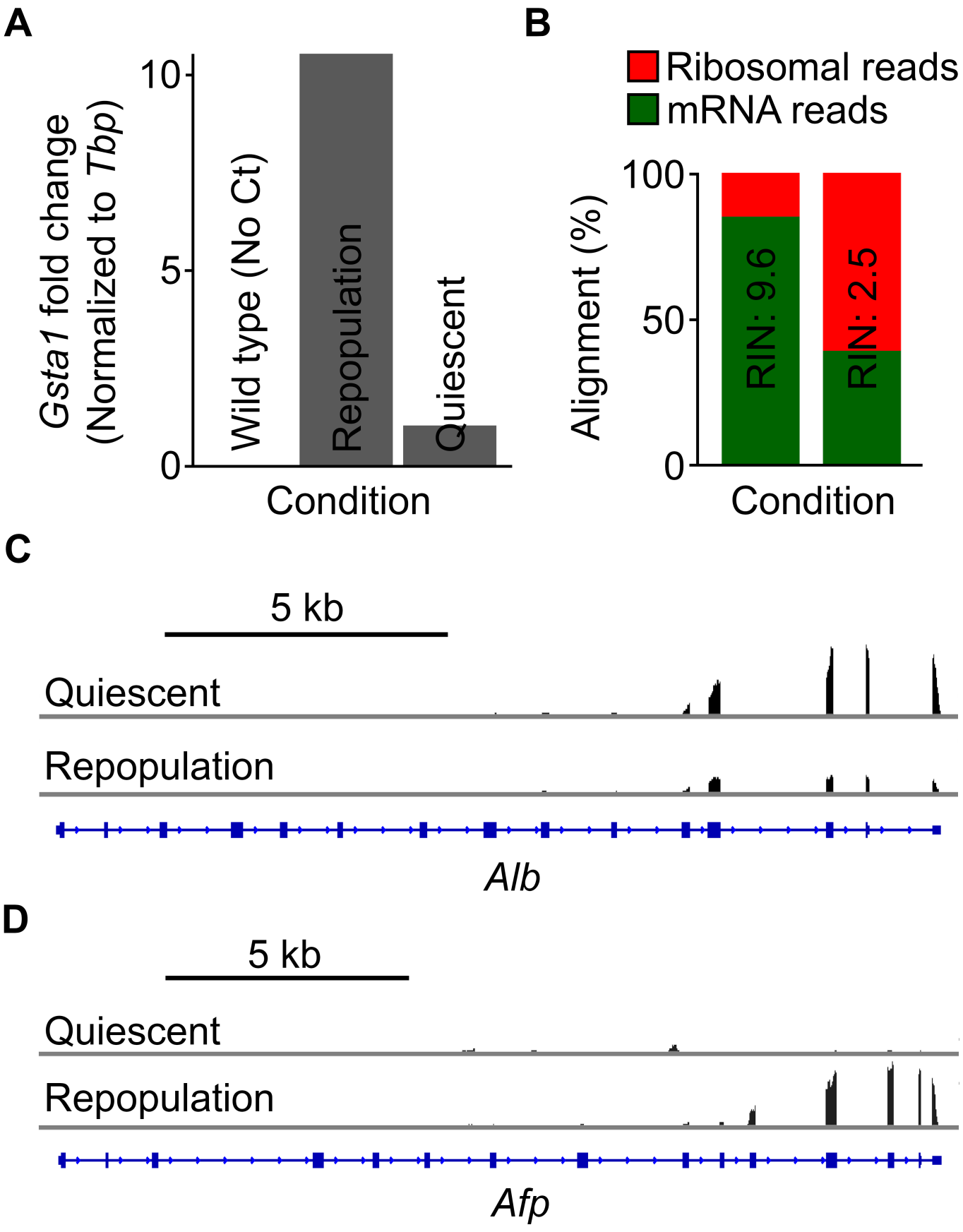


Figure 3



Name of Material/ Equipment

10 mL Tissue Grinder, Potter-Elv, Coated
Absolutely RNA Miniprep Kit
Anti-GFP antibodies
Bovine Serum Albumin, IgG-Free, Protease-Free
cOmplete, Mini, EDTA-free Protease Inhibitor Cocktail
Cycloheximide
Deoxycholic acid, DOC
D-Glucose, Dextrose
DL-Dithiothreitol
Dynabeads MyOne Streptavidin T1
Fisherbrand Petri Dishes with Clear Lid
HBSS (10x), calcium, magnesium, no phenol red
HEPES, 1M Solution, pH 7.3, Molecular Biology Grade, Ultrapure, Thermo Scientific
Magnesium chloride, $MgCl_2$
Methanol
NanoDrop 2000/2000c Spectrophotometer
NEBNext Poly(A) mRNA Magnetic Isolation Module
NEBNext Ultra RNA Library Prep Kit for Illumina
Nonylphenyl polyethylene glycol, NP-40. IGEPAL CA-630
Nuclease-Free Water, not DEPC-Treated
Overhead Stirrer
PBS Buffer (10x), pH 7.4
Pierce Recombinant Protein L, Biotinylated
Potassium chloride, KCl
RNA 6000 Pico Kit & Reagents
RNaseZap RNase Decontamination Solution
RNasin Ribonuclease Inhibitors
Sodium azide, NaN_3
Sodium bicarbonate, $NaHCO_3$
SUPERase·In RNase Inhibitor

Company	Catalog Number	Comments/Description
DWK Life Sciences (Wheaton)	358007	
Agilent	400800	
Memorial Sloan-Kettering Antibody & Bioresource Cor	GFP Ab #19C8 and GFP Ab #19F7	
Jackson ImmunoResearch	001-000-162	
Roche	11836170001	
Millipore Sigma	C7698	
Millipore Sigma	D2510	
Fisher Scientific	D16	
Millipore Sigma	D9779	
Thermo Fisher Scientific	65602	
Fisher Scientific	FB0875712	
Thermo Fisher Scientific	14065-056	
Thermo Fisher Scientific	AAJ16924AE	
Millipore Sigma	M8266	
Fisher Scientific	A452	
Thermo Fisher Scientific	VV-83061-00	
New England BioLabs	E7490S	
New England BioLabs	E7530S	
Millipore Sigma	I8896	
Ambion	AM9932	
DWK Life Sciences (Wheaton)	903475	
Ambion	AM9625	
Thermo Fisher Scientific	29997	
Millipore Sigma	P4504	
Agilent	5067-1513	
Invitrogen	AM9780	
Promega	N2515	
Millipore Sigma	S2002	
Millipore Sigma	S6297	
Invitrogen	AM2694	

This piece of the submission is being sent via mail.

Perelman School of Medicine
University of Pennsylvania

Kirk J. Wangensteen, M.D., Ph.D.
Assistant Professor of Medicine
Department of Medicine
Division of Gastroenterology

June 29, 2019

Ronald Myers, PhD.
Senior Science Editor
JoVE
1 Alewife Center, Suite 200
Cambridge, MA 02140

Dear Dr. Myers,

We would like to thank you and the reviewers for their favorable revision of our work and for the constructive feedback. We feel this has helped improve the manuscript substantially. Additionally, we are thankful for the opportunity to share this study with the scientific community.

We have made all of the changes suggested by the editor. We have also incorporated all the suggestions in the manuscript and we address the reviewers' comments point-by-point as follows:

Reviewer #1:

1) In the introduction, the authors make the statement "while gene expression analysis of mixed cell populations has been improved with single-cell RNA-seq technologies, the fragility and variability in size of repopulating hepatocytes leads to dropout of lowly-abundant transcripts and inefficient capture of the target cells." Certainly, the amplification used in sc-RNA seq can result in the dropout of abundant transcripts, but I do not feel that there is inefficient capture of target cells. Multiple papers have been published on single cell sequencing of hepatocytes with capture of their target population of cells. Nature. (2017) 542: 352-356. Nature (2017) 546: 533-538. Even very rare populations of cells have been resolved with sc-RNA seq such as the pulmonary ionocyte. It would be helpful for the authors to explain more how this method will fit into this landscape and what advantages/disadvantages it may have.

We thank reviewer 1 for pointing out the discrepancies between the text and others' studies and made several changes to the manuscript. In the revised manuscript, we mention our difficulties in capturing live cells in the *Fah*$^{-/-}$ injury and repopulation model. We found that in this model, capturing live cells after liver perfusion and hepatocyte dissociation was highly inefficient due to cell fragility. We discuss scRNA-seq and explore its potential in future studies of liver repopulation. Should novel methods to efficiently isolate hepatocytes be established, scRNA-seq would certainly aid in understanding the role of all cell types during liver repopulation and potentially identify novel transitional cell types during the regenerative process. All discussions regarding the limitation and potential of scRNA-seq can be found in the second-to-last paragraph of the discussion.

421 Curie Boulevard, 910 BRB ■ Philadelphia, PA 19104 ■ Phone: 215-573-7314 ■ Fax: 215-573-2024
wkirk@upenn.edu ■ https://www.med.upenn.edu/wangensteenlab

2) Figure 3b: This is not really a control experiment. Comparing high RIN and Low RIN sequencing data seems unnecessary as it is commonly known that low RIN RNA is degraded and should not be sequenced.

We thank reviewer 1 for this comment and have modified the text and figure legend to emphasize the necessity to assess RNA degradation, especially for downstream RNA-seq analysis. While using a Bioanalyzer to assess RIN is a recommended step in our protocol, we feel that a demonstration of how a low-quality RNA sample could lead to poor sequencing results would be useful for the readers. We have also added a representative results section that low RIN should not be sequenced at all since it indicates RNA degradation.

3) No technique is perfect, the authors should comment briefly about the limitations of TRAP so that others can decide which approach to take.

We thank reviewer 1 for the constructive suggestion and have included the limitations of TRAP in the third-to-last paragraph in the discussion. Mainly, (1) TRAP-seq is a measurement of the 'translatome' and not 'transcriptome', (2) TRAP-seq is not an assessment of ribosome footprinting, (3) TRAP isolation still requires a certain number of cells (at least 1:100 in our experience) to express GFP:RPL10A, and (4) genetic elements are required to ectopically express the GFP:RPL10A transgene in the cell-type-of-interest and could therefore limit its use in cells types that do not yet have an efficient expression or delivery system.

Reviewer #2:

1) Can the authors provide some criteria for evaluating RNA quality? How are the assignments of "high-quality" and "low-quality" RNA made in figure 3B?

We thank reviewer 2 for the constructive feedback and have expanded the description regarding the evaluation of RNA quality for RNA-seq purposes in the final paragraph of the representative results section. We suggest analysis of RNA quality with a Bioanalyzer assay of RNA quality. If there is evidence of RNA degradation, the samples should be excluded. However, as different RNA-seq library preparation kits use different chemistries, it is difficult to make a clear cut-off for RIN. For instance, poly(A) selection-based kits generally require RIN values above 7, while ribosomal RNA removal kits sometimes allow RIN to be as low as 5. We have also edited the figure legend and removed the usage of 'high-quality' and 'low-quality', and instead suggest referring to the kit to decide whether or not to include an RNA sample.

2) What is the minimum amount of tissue or cell number needed for TRAP-seq to work?

We have included an estimation of the lowest amount of cells for a successful TRAP-seq experiment in the manuscript; this can be found in the 4th paragraph of the discussion. We have used 200 mg of the repopulating liver in which only 1-2% of hepatocytes were expressing the GFP:RPL10A transgene. We calculate that ~150 ng of RNA can be purified from roughly 2x105 hepatocytes using TRAP. In the original TRAP paper, the authors were able to isolate as

421 Curie Boulevard, 910 BRB ■ Philadelphia, PA 19104 ■ Phone: 215-573-7314 ■ Fax: 215-573-2024
wkirk@upenn.edu ■ https://www.med.upenn.edu/wangensteenlab

little as 50 ng of RNA from 30 mg of mouse striatum that includes ~7x105 neurons expressing the transgene.

Regards,

Kirk Wangensteen

421 Curie Boulevard, 910 BRB ■ Philadelphia, PA 19104 ■ Phone: 215-573-7314 ■ Fax: 215-573-2024
wkirk@upenn.edu ■ https://www.med.upenn.edu/wangensteenlab