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TITLE:

Isolate Cell-Type-Specific RNAs from Snap-Frozen Heterogeneous Tissue Samples without Cell Sorting

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KEYWORDS:

cell-type-specific RNA; translating ribosome affinity purification; testis; microarray; EGFP; Gli1

SUMMARY:

This protocol aims to isolate cell-type-specific translating ribosomal mRNAs using the NuTRAP mouse model.

ABSTRACT:

Cellular heterogeneity poses challenges to understanding the function of complex tissues at a transcriptome level. Using cell-type-specific RNAs avoids potential pitfalls caused by the heterogeneity of tissues and unleashes the powerful transcriptome analysis. The protocol described here demonstrates how to use the Translating Ribosome Affinity Purification (TRAP) method to isolate ribosome-bound RNAs from a small amount of EGFP-expressing cells in a complex tissue without doing cell sorting. This protocol is suitable for isolating cell-type-specific RNAs using the recently available *NuTRAP* mouse model and could also be used to isolate RNAs from any EGFP-expressing cells.

INTRODUCTION:

High-throughput approaches, including RNA sequencing (RNA-seq) and microarray, have made it possible to interrogate gene expression profiles at the genome-wide level. For complex tissues such as the heart, brain, and testis, the cell-type-specific data will provide more details comparing the use of RNAs from the whole tissue[1-3]. To overcome the impact of cellular heterogeneity, the Translating Ribosome Affinity Purification (TRAP) method has been developed since early 2010s[4]. TRAP is able to isolate ribosome-bound RNAs from specific cell types without tissue dissociation. This method has been used for translatome (mRNAs that are being recruited to the ribosome for translation) analysis in different organisms, including targeting an extremely rare population of muscle cells in Drosophila embryos[5], studying different root cells in the model plant *Arabidopsis*

thaliana[6], and performing transcriptome analysis of endothelial cells in mammals[7].

TRAP requires a genetic modification to tag the ribosome of a model organism. Evan Rosen and colleagues recently developed a mouse model called Nuclear tagging and Translating Ribosome Affinity Purification (*NuTRAP*) mouse[8], which has been available through the Jackson Laboratory since 2017. By crossing with a Cre mouse line, researchers can use this *NuTRAP* mouse model to isolate ribosome-bound RNAs and cell nuclei from Cre-expressing cells without cell sorting. In Cre-expressing cells that also carry the *NuTRAP* allele, the EGFP/L10a tagged ribosome allows the isolation of translating mRNAs using affinity pulldown assays. At the same cell, the biotin ligase recognition peptide (BLRP)-tagged nuclear membrane, which is also mCherry positive, allows the nuclear isolation by using affinity- or fluorescence-based purification. The same research team also generated a similar mouse line in which the nuclear membrane is labeled only with mCherry without biotin[8]. These two genetically modified mouse lines give access to characterize paired epigenomic and transcriptomic profiles of specific types of cells in interest.

The hedgehog (Hh) signaling pathway plays a critical role in tissue development[9]. GLI1, a member of the GLI family, acts as a transcriptional activator and mediates the Hh signaling. *Gli1*$^{+}$ cells can be found in many hormone-secreting organs, including the adrenal gland and the testis. To isolate cell-type-specific DNAs and RNAs from *Gli1*$^{+}$ cells using the *NuTRAP* mouse model, *Gli1-CreER*^{T2} mice were crossed with the *NuTRAP* mice. *Shh-CreER*^{T2} mice were also crossed with the *NuTRAP* mice aim to isolate sonic hedgehog (Shh) expressing cells. The following protocol shows how to use *Gli1-CreER*^{T2}*;NuTRAP* mice to isolate ribosome-bound RNAs from *Gli1*$^{+}$ cells in adult mouse testes.

PROTOCOL:

All performed animal experiments followed the protocols approved by the Institutional Animal Care and Use Committees (IACUC) at Auburn University.

NOTE: The following protocol uses one testis (about 100 mg) at P28 from *Gli1-CreER*^{T2}*; NuTRAP* mice (*Mus musculus*). Volumes of reagents may need to be adjusted based on the types of samples and the number of tissues.

1. Tissue collection

1.1. Euthanize the mice using a CO_2 chamber, sanitize the abdomen surface with 70% ethanol.

1.2. Open the lower abdomen with scissors and remove the testes—Snap-freeze freshly dissected testes using liquid nitrogen (LN_2) immediately upon collection.

1.3. Store the samples in the vapor phase of LN_2 until use.

2. Reagents and beads preparation

2.1. Prepare the homogenization stock solution: Add 50 mM Tris (pH7.4), 12 mM $MgCl_2$, 100 mM KCl, 1% NP-40, and 1 mg/mL heparin. Store the solution at 4 °C until use (up to 1 month).

2.2. Prepare the homogenization working buffer from the stock solution (step 2.1) freshly before use: Add DTT (final concentration: 1 mM), cycloheximide (final concentration: 100 µg/mL), recombinant ribonuclease (final concentration: 200 units/mL), and protease inhibitor cocktail (final concentration: 1x) to the homogenization stock solution to make the required amount of the homogenization working buffer. Store the freshly prepared working buffer on ice until use.

2.3. Prepare the low-salt and the high-salt wash buffers:

2.3.1. To prepare low-salt wash buffer mix 50 mM Tris (pH 7.4), 12 mM $MgCl_2$, 100 mM KCl, and 1% NP-40. Add DTT (final concentration: 1 mM) and cycloheximide (final concentration: 100 µg/mL) before use.

2.3.2. To prepare high-salt wash buffer mix 50 mM Tris (pH7.4), 12 mM $MgCl_2$, 300 mM KCl, 1% NP-40. Add DTT (final concentration: 2 mM) and Cycloheximide (final concentration: 100 µg/mL) before use.

2.4. Prepare protein G beads (**Table of Materials**):

2.4.1. Each sample will need 50 µL of protein G beads. Place the required amount of beads in a 1.5 mL centrifuge tube and separate the beads from the solution using a magnetic rack by leaving the tube on the rack for 30–60 s.

2.4.2. Remove the supernatant by pipetting. Wash the beads three times with 1 mL of ice-cold low-salt wash buffer.

3. Tissue lysis and homogenization

3.1. Add 2 mL of ice-cold homogenization working buffer (freshly prepared from step 2.2) to a glass tissue grinder set. Quickly place the frozen sample into the grinder and homogenize the tissue with 30 strokes on ice using a loose pestle.

3.2. Transfer the homogenate to a 2 mL round-bottom tube and centrifuge at 12,000 x *g* for 10 min at 4 °C.

3.3. Transfer the supernatant to a new 2 mL tube. Save 100 µL to a 1.5 mL tube as the “input”.

3.4. Incubate the supernatant in the 2 mL tube with the anti-GFP antibody (5 µg/mL; 1:400) at 4 °C on an end-over-end rotator overnight.

4. Immunoprecipitation

4.1. Transfer the homogenate/antibody mixture to a new 2 mL round-bottom tube containing the washed protein G beads from step 2.4. Incubate at 4 °C on an end-over-end rotator (24 rpm) for 2 h.

4.2. Separate the magnetic beads from the supernatant using a magnet rack. Save the supernatant as the "negative fraction". The negative fraction contains (1) RNAs in EGFP-negative cells and (2) RNAs in EGFP-positive cells that are not bound to ribosomes.

4.3. Add 1 mL of high-salt wash buffer to the beads and briefly vortex the tube to wash the beads. Place the tube in a magnet rack.

4.4. Remove the wash buffer. Repeat the washing step two more times. The beads now contain the beads-ribosome-RNA complex from EGFP-positive cells.

5. RNA extraction

NOTE: The following steps are adapted from the RNA isolation kit (**Table of Materials**). Treat each fraction (i.e., input, positive, and negative) as an independent sample and isolate RNAs independently.

5.1. Incubate the beads from step 4.4 with 50 µL of Extraction Buffer (from the RNA isolation kit) in a thermomixer (42 °C, 500 rpm) for 30 min to release RNAs from beads.

5.2. Separate the beads with a magnet rack, transfer the supernatant which contains the beads-ribosome-RNA complex to a 1.5 mL tube.

5.3. Centrifuge the tube at 3000 x *g* for 2 min, then pipette the supernatant to a new 1.5 mL tube. This tube contains the "positive fraction" of the TRAP step.

NOTE: For the input and the negative fractions, extract RNA from 25 µL of samples using 1 mL of Extraction Buffer. Incubate in a thermomixer (42 °C, 500 rpm) for 30 min.

5.4. Pre-condition the RNA purification column: Pipette 250 µL of Conditioning Buffer onto the purification column. Incubate for 5 min at room temperature (RT). Centrifuge the column at 16,000 x *g* for 1 min.

5.5. Pipette equal volume (around 50 µL for the positive fraction and 1 mL for the input and the negative fractions) of 70% EtOH into the cell extract from step 5.3. Mix well by pipetting up and down.

5.6. Pipette the mixture into the column from step 5.4.

5.7. Centrifuge the column at 100 x *g* for 2 min to allow RNA binding to the membrane in the column, then continue centrifuge at 16,000 x *g* for 30 s immediately. Discard the flow-through.

NOTE: For the input and the negative fractions, add 250 µL of the mixture to the column each time. Repeat steps 5.6 and 5.7 until all mixtures are used.

5.8. Pipette 100 µL of Wash Buffer 1 (W1) into the column and centrifuge at 8,000 x *g* for 1 min. Discard the flow-through.

5.9. Pipette 75 µL of DNase solution mix directly into the purification column membrane. Incubate at RT for 15 min.

5.10. Pipette 40 µL of W1 into the column and centrifuge at 8,000 x *g* for 30 s. Discard the flow-through.

5.11. Pipette 100 µL of Wash Buffer 2 (W2) into the column and centrifuge at 8,000 x *g* for 1 min. Discard the flow-through.

5.12. Pipette 100 µL of W2 into the column and centrifuge at 16,000 x *g* for 2 min. Discard the flow-through. Re-centrifuge the same column at 16,000 x *g* for 1 min to remove all traces of wash buffer prior to the elution step.

5.13. Transfer the column to a new 1.5 mL microcentrifuge tube.

5.14. Pipette 12 µL of RNase-free water directly onto the membrane of the purification column. The pipet tip should not touch the membrane. Incubate at RT for 1 min and centrifuge at 1000 x *g* for 1 min. Then continue centrifugation at 16,000 x *g* for 1 min to elute the RNA.

6. RNA concentration and quality

6.1. Use a bioanalyzer to assess the quality and quantity of the extracted RNA[10].

7. Storage and further analysis

7.1. Store the RNA at -80 °C (up to 1 year) until further analysis (e.g., microarray, quantitative PCR (qPCR), and RNA-seq, etc.).

NOTE: For details of the qPCR analysis, including cDNA synthesis, refer to Lyu et al.[11]. Primers for qPCR are listed in the **Table of Materials**.

REPRESENTATIVE RESULTS:

Gli1-CreER^{T2} mouse (Jackson Lab Stock Number: 007913) were first crossed with the *NuTRAP* reporter mouse (Jackson Lab Stock Number: 029899) to generate double-mutant mice. Mice carrying both genetically engineered gene alleles (i.e., *Gli1-CreER* ^{T2} and *NuTRAP*) were injected with tamoxifen once a day, every other day, for three injections. Tissue samples were collected on the 7th day after the 1st day of the injection. Immunofluorescence analysis showed that the

EGFP was expressed in interstitial cells in testes (**Figure 1**). The adrenal gland capsule has been known to be another cell population positive of *Gli1*[12,13]. EGFP was also found in adrenal capsular cells in *Gli1-CreER*^{T2}*;NuTRAP* mice (**Figure 1**). Our lab also carries *Shh-CreER*^{T2}*;NuTRAP* mice. Note that in *Shh-CreER*^{T2}*;NuTRAP* mice, the *EGFP*$^{+}$ cell population resides in the outer cortex of the adrenal gland underneath the capsule (**Figure 1**), the same expression site of EGFP^{+} cells in *Shh*$^{+}$ area in the adrenal gland[13] confirming the expression of EGFP in Cre-expressing cells.

After the extraction of the cell-type-specific RNAs, the quantity and quality of isolated RNAs in each fraction from two independent extractions (one testis used in each extraction) were assessed using a bioanalyzer (**Figure 2**). The bioanalyzer result indicates that this protocol is able to obtain high-quality RNAs from all three fractions. All fractions have a similar RNA Integrity Number (RIN).

To further test whether the extracted RNA is cell-type-specific, extracted RNA was sent for microarray analysis using a commercial microarray service (see **Table of Materials**). The microarray result showed that about 3,000 genes were enriched in the positive fraction comparing to genes in the negative fraction, whereas about 4,000 genes were enriched in the negative fraction (**Figure 3**). Among these differentially expressed genes, Leydig-cell-associated genes *Hsd11b1* and *Hsd3b6*[14,15] were enriched in the positive fraction. In the negative fraction, the Sertoli-cell-associated genes *Dhh* and *Gstm6*[16,17] were enriched. Only a few differentially expressed genes were identified when comparing the negative fraction with the input.

Real-time quantitative RT-PCR (qPCR) was also used to confirm the expression of key genes in the positive fraction and the negative fraction. Similar to what was found in the microarray assay, steroidogenic enzymes 3β-Hydroxysteroid dehydrogenase (encoded by *Hsd3b*) and cholesterol side-chain cleavage enzyme (encoded by *Cyp11a1*) were enriched in the positive fraction. Whereas the Sertoli cell marker *Sox9* (SRY-box Transcription Factor 9), and the germ cell marker *Sycp3* (Synaptonemal Complex Protein 3), were enriched in the negative fraction (**Figure 4**). Together these data demonstrated that the transcriptomes in *Gli1*$^{+}$ cells were successfully pulled down and enriched by the above protocol.

FIGURE AND TABLE LEGENDS:

Figure 1: Immunofluorescence images of the EGFP expression in *NuTRAP* reporter mouse models. The *Gli1-CreER*^{T2}*;NuTRAP* or the *Shh-CreER*^{T2}*;NuTRAP* mice were treated with tamoxifen to activate the EGFP expression. In the testis, *Gli1*$^{+}$ cells were in the interstitium, whereas in the adrenal gland, *Gli1*$^{+}$ cells were in the adrenal capsule. In the adrenal gland, cells underneath the capsule were positive of SHH (EGFP^{+} cells in *Shh-CreER*^{T2}*;NuTRAP* mice). SHH which is the ligand of the SHH signaling pathway eliciting its function in *Gli1*$^{+}$ capsular cells[13]. Scale bars: 50 µm.

Figure 2: RNA quality and quantity from the TRAP extraction. RNAs of the positive fraction, the negative fraction, and the input were evaluated using a bioanalyzer. The positive fraction contains RNAs extracted from protein G beads after the incubation with the GFP antibody (step 4.1). The negative fraction contains RNAs that remain in the supernatant at step 4.2. The input contains RNAs from the homogenate (step 3.3). In the electropherogram, because the

concentrations of the lower marker (displayed as the first peak at 20–25 s of the migration time of samples shown on the X-axis) and the ladder (not shown in these electropherograms) are known, the concentration of each sample can be calculated. The two major peaks at 40–50 s represent the 18S and 28S rRNA. The ribosomal ratio (based on the fluorescence intensity shown on the Y-axis) is used to determine the integrity of the RNA sample. The RNA Integrity Number (RIN) of each sample is shown on the top right corner of each plot. The dot plot shows the amount of RNA that was extracted from one single testis. The amount of RNA of each sample in the negative fraction and the input was extracted from 25 µL of samples.

Figure 3: Microarray analysis for TRAP samples. Results of two extractions (one testis each) are shown. The microarray analysis identified a similar number of differentially expressed genes from two independent extractions (Testis A and Testis B). Around 4,000 genes were enriched (red dots) in the positive fraction, whereas ~3,000 genes were enriched (green dots) in the negative fraction. *Hsd11b1* and *Hsd3b6* were enriched in positive fractions. *Dhh* and *Gstm6* were enriched in negative fractions. Only a few genes were identified as differentially expressed genes between the negative fraction and the input, suggesting the testis only contains a very small number of *Gli1*$^{+}$ cells.

Figure 4: qPCR analysis for TRAP samples. qPCR analysis showed the relative expression of cell-type-specific genes in the positive fraction and the negative fraction. The expression of each gene was first normalized with *Actb.* The relative expressions of genes within the positive fraction were then calculated based on the expression of *Sox9* (set as 10). For the positive fraction, *Cyp11a1* (set as 10) was used to normalize the relative expression. Note that the relative expression of target genes can only be compared within each fraction. Genes that encode the steroidogenic enzymes (*Hsd3b* and *Cyp11a1*) were enriched in the positive fraction. Whereas the marker gene for germ cells (*Scyp3*) and Sertoli cells (*Sox9*), were enriched in the negative fraction. Three biological replicates (three independent extractions, one testis each) were shown.

DISCUSSION:

The usefulness of the whole-tissue transcriptome analysis could be dampened, especially when studying complex heterogeneous tissues. How to obtain cell-type-specific RNAs becomes an urgent need to unleash the powerful RNA-seq technique. The isolation of cell-type-specific RNAs usually relies on the collection of a specific type of cells using micromanipulation, fluorescent-activated cell sorting (FACS), or laser capture microdissection (LCM)[18]. Other modern high-throughput single-cell collection methods and instruments have also been developed[19]. These methods usually employ the microfluidics techniques to barcode single cells followed by single-cell RNA-seq. Cell dissociation is the required step to obtain the suspended cell solution, which then will go through either a cell sorter or a microfluidic device to barcode each cell. The cell dissociation step introduces challenges to these methods for cell-type-specific studies because the enzymatic treatment not only breaks down tissues but also affects cell viability and transcriptional profiles[20]. Moreover, the expense for single-cell RNA-seq is usually high and requires specialized equipment on site.

Recently, two studies successfully isolated RNA/DNA of specific cell types from whole tissues

using the *NuTRAP* mice[8,21]. Without using specific equipment and tools, the *NuTRAP* mouse model allows obtaining RNAs and DNAs from specific types of cells. The *NuTRAP* allele could target Cre-expressing cells without the cell dissociation step, allowing to avoid changing cell's viability and transcriptional profiles. Rol et al. used the *NuTRAP* mouse model to isolate nuclei and translate mRNA simultaneously from adipose tissue. The other study also demonstrated that the *NuTRAP* mouse model could work for glial cells in the central nervous system[8].

In our lab, we are interested in studying the stem cell populations in steroidogenic tissues such as *Gli1*$^+$ interstitial cells in the testis[22] and *Gli1*$^+$ capsular cells in the adrenal gland[13]. The challenge of studying *Gli1*$^+$ cells in these two organs is that the number of *Gli1*$^+$ cells in the testis and the adrenal gland is small. For example, the proportion of Leydig cells, which are the major population of *Gli1*$^+$ cells in the testis, only occupy about 3.8% of the total testis volume in adult mice[23]. Because the TRAP technique aims to specifically pull down translating ribosome-bound RNAs in complex tissue, the *NuTRAP* mouse model could be a powerful tool suitable for studying a rare cell population in a complex tissue. The previously published protocols using *NuTRAP* mice target adipocytes and glial cells that are more abundant in the brain and adipose tissue compared to *Gli1*$^+$ cells in the testis and in the adrenal gland. To ensure obtaining required RNAs from a small number of cells in a complex tissue, we revised the existing protocols by (1) increasing the incubation time with the GFP antibody from 1 h to overnight; (2) using another type of RNA extraction kit which aims to isolate a small amount of RNA at a picogram level.

We demonstrated that this protocol could obtain high-quality cell-type-specific RNAs from a small number of cells in a complex tissue. The quality and quantity of extracted RNAs are capable for qPCR and a commercial microarray service. Results from microarray and qPCR confirmed that Leydig-cells-associated genes are enriched in the positive fraction coming from one testis in an adult mouse in which the targeted cell population only occupies 3.8% of the testis volume. The protocol here provides a detailed approach to isolate cell-type-specific translating ribosome mRNAs using the *NuTRAP* mouse model. This protocol may also be used to isolate RNAs from any EGFP-expressing cells.

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DISCLOSURES

The authors declare no conflict of interest.

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Figure 1

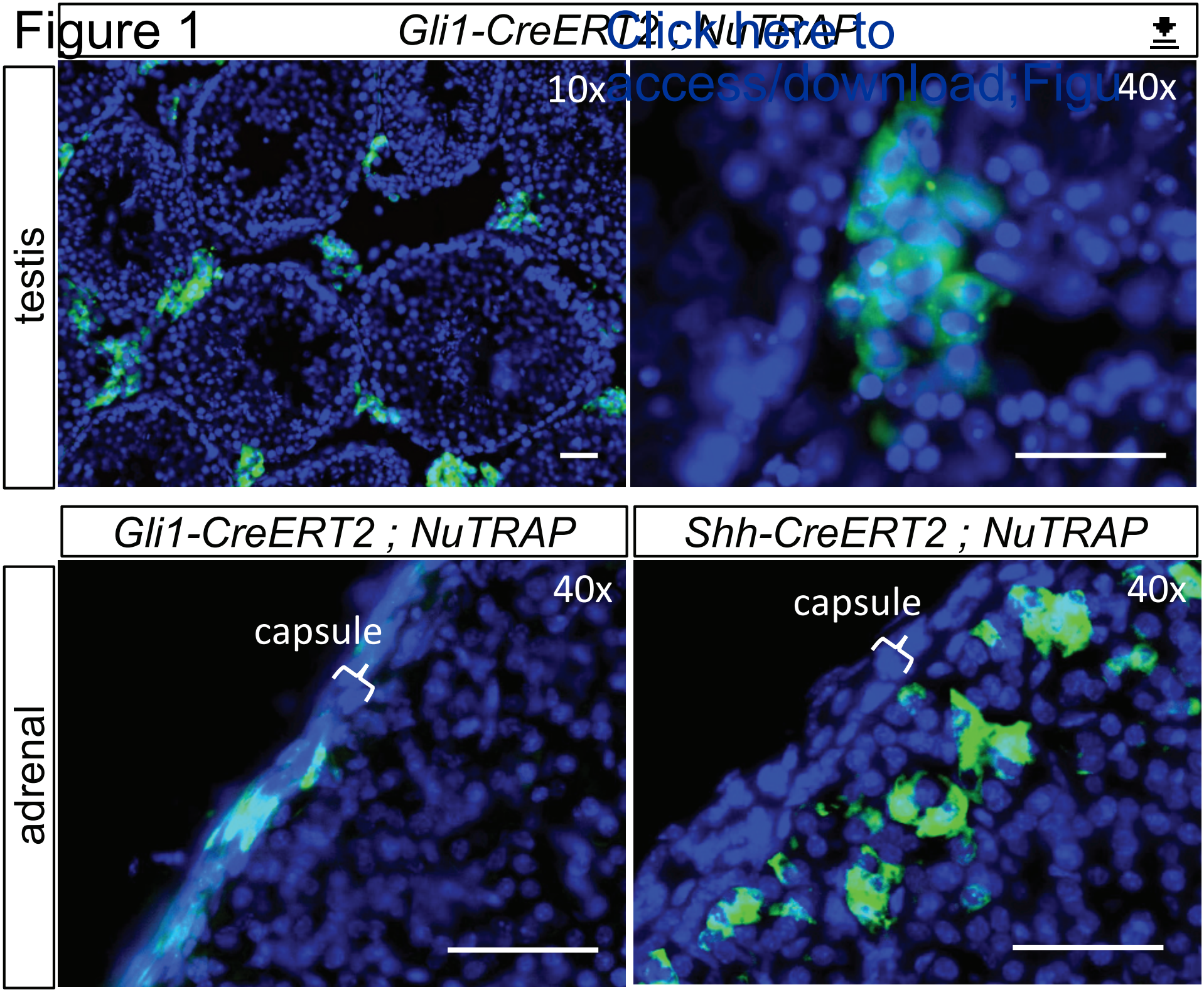
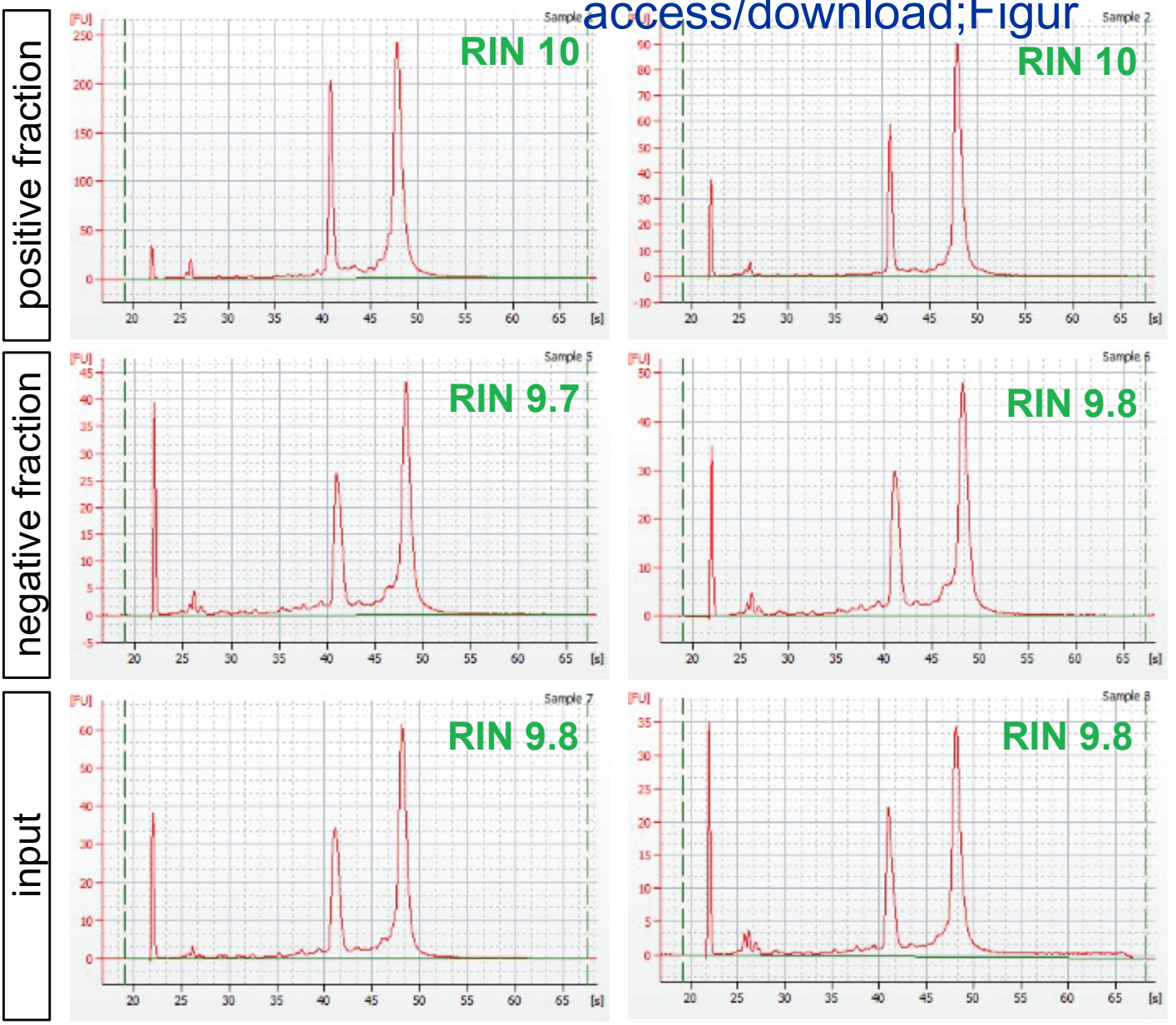


Figure 2



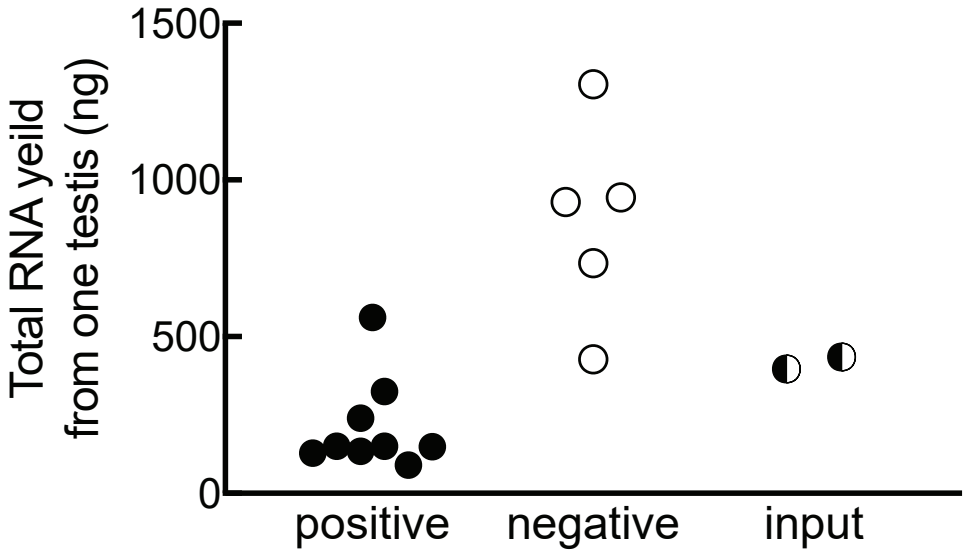


Figure 3

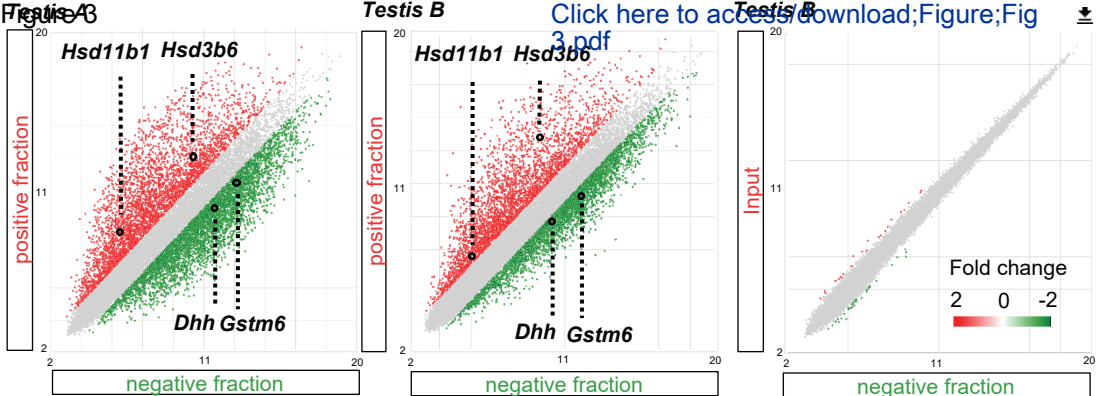
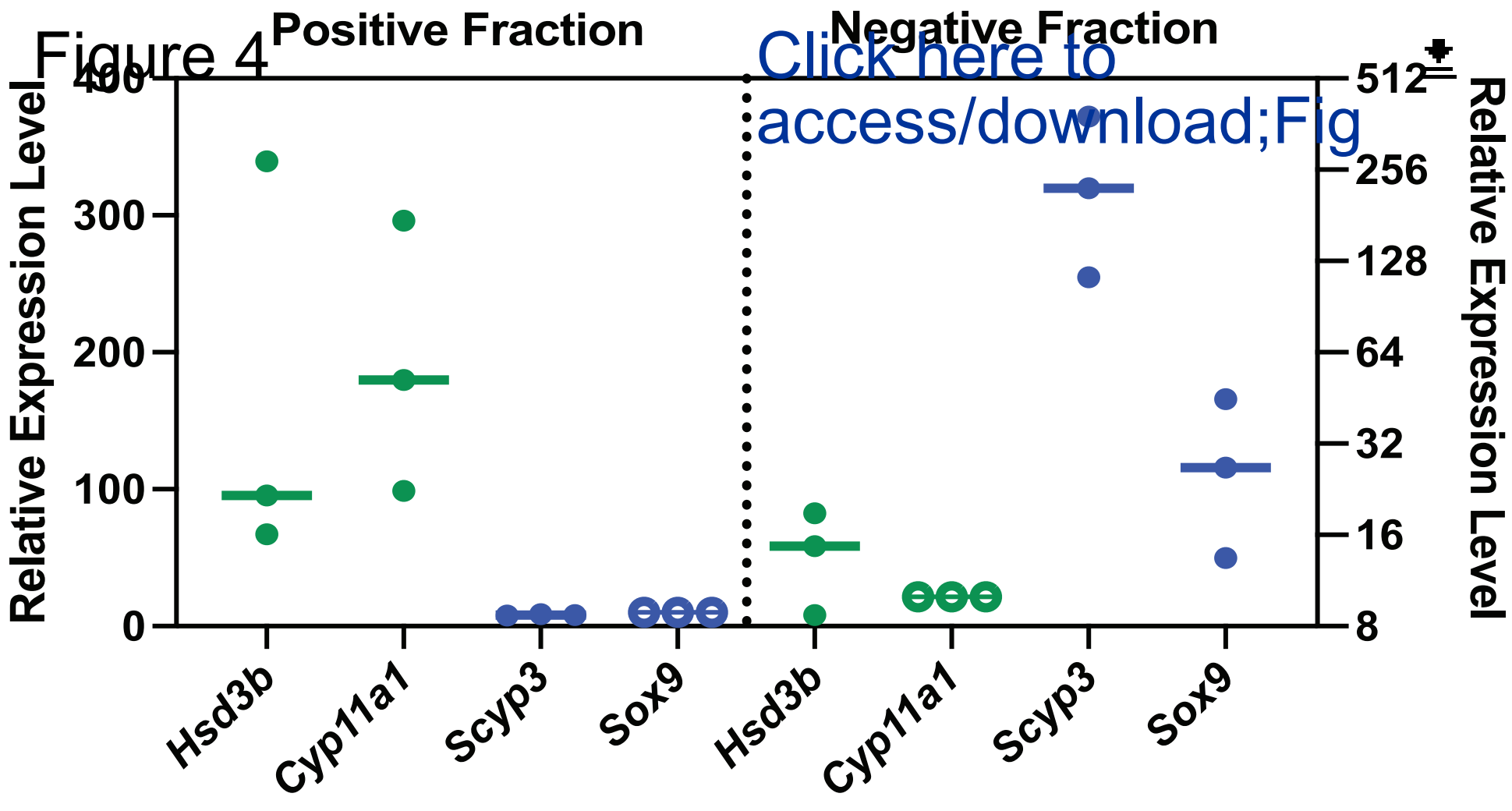


Figure 4



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We thank the constructive comments from the editor and reviewers. Changes have been made as suggested. Major changes are highlighted in yellow in the revision.

Editorial comments:

Editorial Changes

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Spelling and grammar issues have been fixed.

2. Please revise the following lines to avoid previously published work: 106-107, 109-110, 142, 144-145, 163, 166-167. Please refer to the iThenticate report attached.

These sentences have been revised.

3. Please provide an institutional email address for each author.

Institutional email addresses now have been provided.

4. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Personal pronouns are now only used in the Discussion section and Acknowledgments.

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For example: Dynabead, Abcam, PicoPure, e BioAnalyzer, Agilent, Clariom S Assay microarrays, ThermoFisher Scientific, etc.

Commercial language has been removed from the text and is only shown in the Table of Materials.

6. Please ensure that the Introduction includes all of the following:
a) A clear statement of the overall goal of this method
b) The rationale behind the development and/or use of this technique
c) The advantages over alternative techniques with applicable references to previous studies
d) A description of the context of the technique in the wider body of literature
e) Information to help readers to determine whether the method is appropriate for their application.

The introduction has been revised as requested.

7. Line 65-68: Please specify the strain and the age of the mice used.

The strain and the age of the mice has been added to Line 66.

8. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

The manuscript has been revised as requested.

9. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step.

The manuscript has been revised as requested.

10. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g., button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

11. Line 72: Please specify how the testes are dissected. If the animals were euthanized to remove testes, please specify the method of euthanasia.

The manuscript has been revised as requested.

12. Line 109-110: Please specify the dilution of the antibody used.

The dilution of the antibody has been added.

13. Line 115: Is any specific rpm maintained in the rotator.

The rotator rpm has been added.

14. Line 121: Please elaborate on the washing step.

The manuscript has been revised as requested.

15. Line 171: Please add more details to the qualification and quantification step. If not, please cite relevant references.

The reference has been cited.

16. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Essential steps have been highlighted in green.

17. The representative results and figure legends describe microarray analysis and qPCR. Please ensure that the relevant references for performing these assays are included in section 6 of the protocol. You can also consider including the steps to perform these assays.

Step 7 has been added.

18. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

Changes have been made as suggested.

19. Please do not use the &-sign or the word "and" when listing authors in the references.

Changes have been made as suggested.

20. Please submit each figure individually as a vector image file to ensure high resolution throughout production: (.psd, ai, .eps.).

Each figure has been converted to the ai format.

21. Figure 1: Please ensure that the scale bar mentioned in the figure legend represents the scale bars included in all the images of the panel (e.g., the sale bar in the first testis image is small compared to the other panels. Ensure that the scale bar is 50 µm).

The size of the scale bar has been double checked.

22. Figure 2: Please specify the x and y-axis of the plots shown.

Details have been added to the figure legend.

23. Figure 4: Please include error bars in the figure and define them in the figure legends.

Fig. 4 uses qPCR as a quick test to see whether a similar trend of results can be observed using different approaches. Two biological replicates had been examined using qPCR and a similar trend was observed. Only data from one testis (extraction) is shown.

24. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please sort the Materials Table alphabetically by the name of the material.

The table has been revised as requested.

Reviewers' comments:

Reviewer #1:

Manuscript summary:

This manuscript presents a protocol for a cell type-specific gene expression analysis using GFP-tagged ribosome pulldown, based on the NuTRAP reporter mouse. While there are several different methods that allow for RNA isolation from specific cell types, TRAP ribosome pulldown is one of the simplest and most robust approaches with reasonable purity and yields. The authors present a clear protocol with the details using readily available reagents and include sufficient induction and discussion. While there are already several protocol papers published on TRAP methods, this paper added some modifications such as longer incubation for immunoprecipitation and a different RNA extraction method. This would be additional useful information to readers. Given the growing interest in cellular heterogeneity, this TRAP protocol would be of interest to many scientists in biomedical research.

We thank Reviewer 1 for the comments/suggestions.

Minor concerns:

There are several minor comments:

1. Cycloheximide and DTT are usually used freshly. The authors mentioned that the buffer added with these chemicals can be stored at 4 degree for up to 1 month. Was this actually tested? Is fresh addition recommended?

We agree that cycloheximide and DTT should be added before use. The text has been revised and this information has been added to the revision.

2. The authors isolated RNA from the input and negative fraction, but this part does not contain the details of how. It would be useful to know how much volume/fraction was used and how these are processed for RNA extraction.

The details have been included in step 5.

3. The authors mentioned that they used extended overnight immunoprecipitation to increase RNA yield. Although it highly depends on target cell types, some information on the expected RNA amounts from TRAP pulldown would be very useful for readers to determine the success of experiments. At least, the authors can show RNA yields from their experiments after 1hr or overnight IP.

We agree that the incubation time could be case/tissue dependent. Our result indicated that an overnight incubation does not compromise the quality of the extracted RNA. End users of this protocol may adjust the incubation time based on their needs. We have revised the text and included the total RNA yield data in Figure 2.

4. There is no error bar in Figure 4. Is it n=1? If so, it would be impossible to make any strong conclusions from them. Please add more replicates for statistical analysis. Alternatively, indicate the limitations of their data due to the lack of replicates.

We used qPCR as a quick test here to see whether a similar trend of results can be observed using different approaches. Two biological replicates had been examined using qPCR and a similar trend was observed. Only data from one testis (extraction) is shown. We have included this information in the figure legend.

5. Typo line 232. Figure 3 -> Figure 4.

Corrected.

6. Please clarify the difference between the first two plots in Figure 3.

These two figures represent two biological replicates. Legends have been added to the figure.

Reviewer #2:

Manuscript Summary:

The authors present a protocol for TRAP isolation of ribosome bound RNAs from the NuTRAP model.

Major Concerns:

This is a fairly basic protocol but could be of use to those new to the area with the video instructions.

We agree with Reviewer 2 that this is a fairly basic protocol. Our step-by-step protocol demonstrated how to extract cell-type-specific RNA from a rare cell population in a complex organ using the NuTRAP mouse model.

Minor Concerns:

Error bars for Fig 4

We used qPCR as a quick test here to see whether a similar trend of results can be observed using different approaches. Two biological replicates had been examined using qPCR and a similar trend was observed. Only data from one testis (extraction) is shown. We have included this information in the figure legend.

Reviewer #3:

Manuscript Summary:

This protocol from Zheng and Huang describes how to use the NuTRAP system to isolate ribosome-bound RNAs from the EGFP-expressing Gli1+ cells in adult mouse testis. The protocol is of interest to a broad community since the approach can be adapted to other cell types.

Thank you.

Major Concerns:

No major concerns

Thank you.

Minor Concerns:

It would be helpful to readers/users to know the expected RNA yield from X amount of cells. This will vary with cell type but a range should be denoted.

We thank Reviewer 3 for this suggestion. Leydig cells (the major cell population in the testis interstitium) only occupy 3.8% of the testis volume. Our data indicates that this protocol is able to isolate high-quality RNAs from a rare cell population from a complex tissue. Data of the total amount of RNA can yield from one testis now have been included in Fig. 2.

Figure 1

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Fig 1.ai

Figure 2

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Figure 3

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Figure 4

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Fig 4 211022.ai