Fig. S1

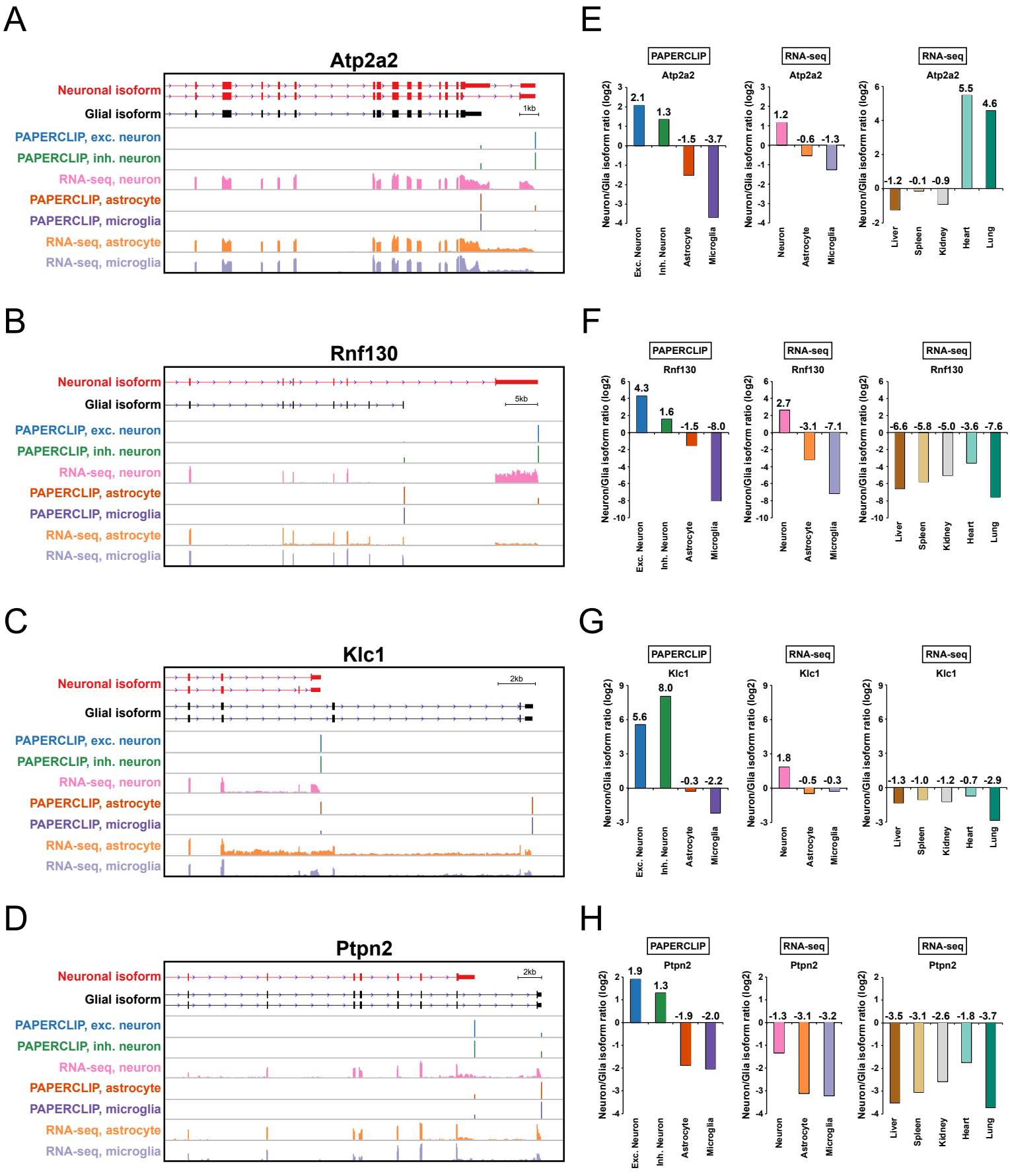
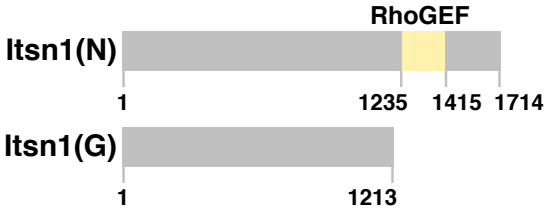

A
Atp2a2
Neuronal isoform
Glial isoform
1kb
PAPERCLIP, exc. neuron
PAPERCLIP, inh. neuron
RNA-seq, neuron
PAPERCLIP, astrocyte
PAPERCLIP, microglia
RNA-seq, astrocyte
RNA-seq, microglia
B
Rnf130
5kb
C
Klc1
2kb
D
Ptpn2
E
PAPERCLIP
RNA-seq
Neuron/Glia isoform ratio (log2)
2.1
1.3
-1.5
-3.7
1.2
-0.6
-1.3
-1.2
-0.1
-0.9
5.5
4.6
Exc. Neuron
Inh. Neuron
Astrocyte
Microglia
Neuron
Liver
Spleen
Kidney
Heart
Lung
F
4.3
1.6
-1.5
-8.0
2.7
-3.1
-7.1
-6.6
-5.8
-5.0
-3.6
-7.6
G
5.6
8.0
-0.3
-2.2
1.8
-0.5
-0.3
-1.3
-1.0
-1.2
-0.7
-2.9
H
1.9
1.3
-1.9
-2.0
-1.3
-3.1
-3.2
-3.5
-3.1
-2.6
-1.8
-3.7

Figure S1. cTag-PAPERCLIP identifies distinct APA preferences in neurons, related to Figure 3. (**A-D**) Diagrams demonstrating differential APA patterns between neurons and glia at *Atp2a2* (A), *Rnf130* (B), *Klc1* (C) and *Ptpn2* (D) loci. X-axis: position of PAPERCLIP or RNA-seq reads across RNA transcripts, as indicated in the top two tracks. Exc.: excitatory. Inh.: inhibitory. Y-axis: normalized read depth (scaled in individual tracks). RNA-seq data is from (Y. Zhang et al., 2014). (**E-H**) Bar graphs showing the relative ratio of neuronal and glial APA isoforms of *Atp2a2* (E), *Rnf130* (F), *Klc1* (G) and *Ptpn2* (H) as determined by PAPERCLIP (left), RNA-seq in mouse brain (middle), or RNA-seq in five different mouse tissues (right). Exc.: excitatory. Inh.: inhibitory.

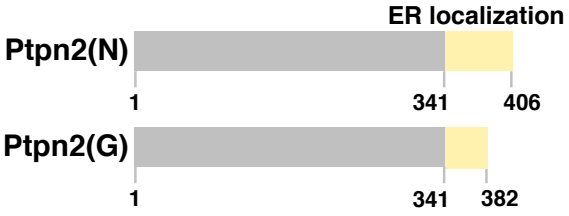
Fig. S2

A

B



C



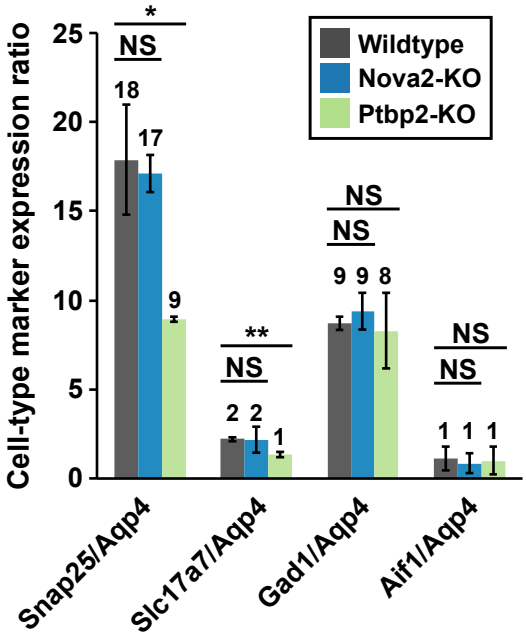
D

E

F

G

H



I

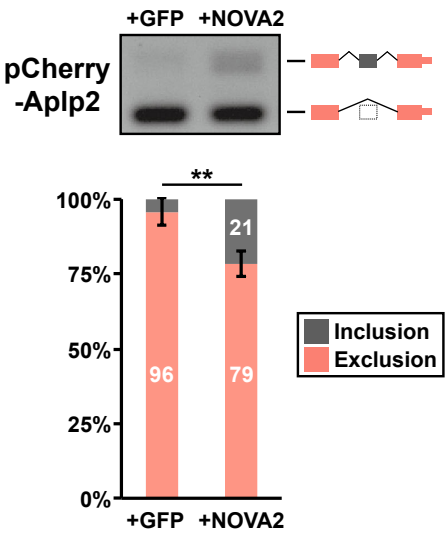
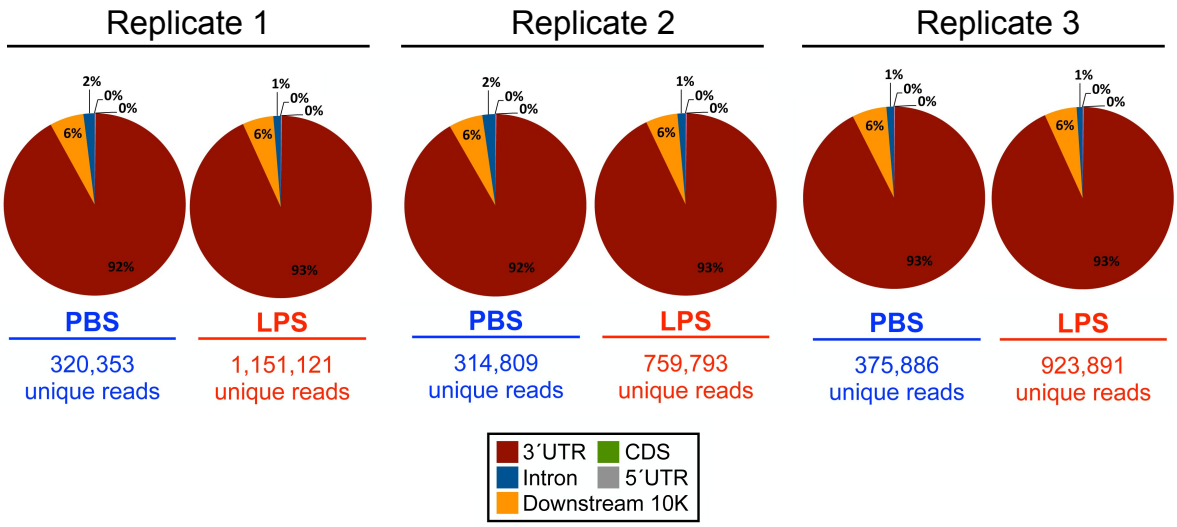


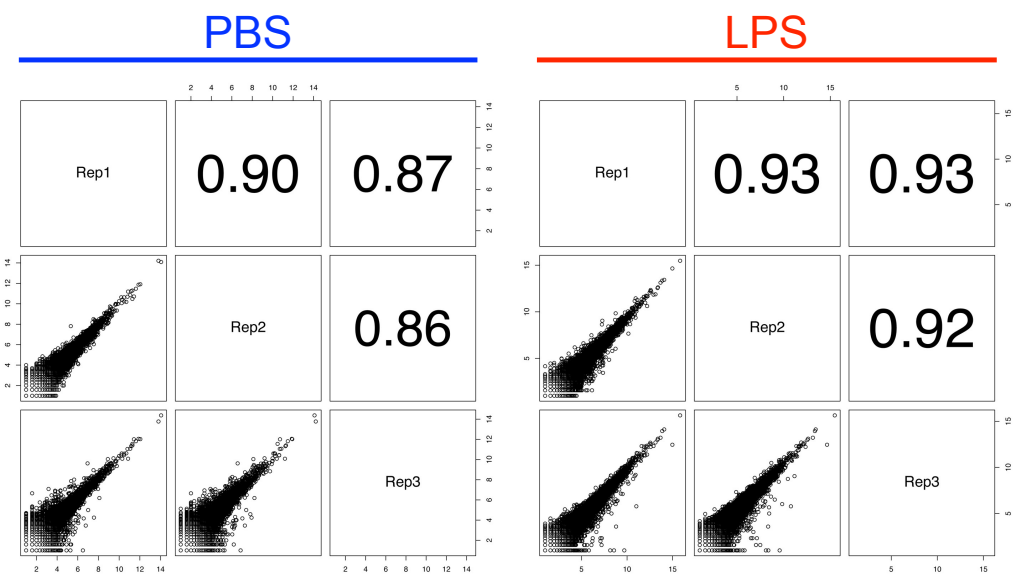
Figure S2. APA results in distinct protein isoform production in neurons and glia, related to Figure 3. (**A-C**) Diagrams showing that the APA isoforms are predicted to generate protein isoforms that lose known domains partially or entirely in neurons (N) or glia (G); shown are Map4 (A), Itns1 (B), Ptpn2 (C). Yellow: known protein domain. (**D-G**) Diagrams showing that the APA isoforms are predicted to generate protein isoforms that have distinct C-termini in Klc1 (D), Rnf130 (E), Atp2a2 (F) and Cdc42 (G). Green/Magenta: indicate different amino acid sequences. Numbers indicate corresponding amino acid residues. (**H**) Bar graphs comparing the cell-type-specific marker expression ratio in E18.5 brain cortex of wildtype, Nova2-KO and Ptbp2-KO mice as determined by RNA-seq. Aqp4, an astrocyte marker, serves as the common denominator. Snap25: pan-neuronal marker. Slc17a7: excitatory neuron marker. Gad1: inhibitory neuron marker. Aif1: microglia marker. Error bars: standard deviation. *: $p<0.05$. **: $p<0.01$. NS: not significant. (**I**) Results from a pCherry mini-gene assay demonstrating an increase in *Apip* cassette exon inclusion when co-transfected with a NOVA2-expressing plasmid. *Apip* is a direct target of *Nova2*. *Top*: A representative agarose gel image from an RT-PCR experiment showing exon-inclusion products (top band) and exon-exclusion products (bottom band). *Bottom*: Bar graphs summarizing the results from three RT-PCR experiments. Error bars: standard deviation. **: $p<0.01$.

Fig. S3

A


Replicate 1
Replicate 2
Replicate 3
2%
0%
0%
6%
92%
1%
0%
0%
6%
93%
PBS
LPS
320,353
unique reads
1,151,121
unique reads
314,809
unique reads
759,793
unique reads
375,886
unique reads
923,891
unique reads
3´UTR
CDS
Intron
5´UTR
Downstream 10K

B


PBS
LPS
Rep1
Rep2
Rep3
0.90
0.87
0.86
0.93
0.93
0.92

C

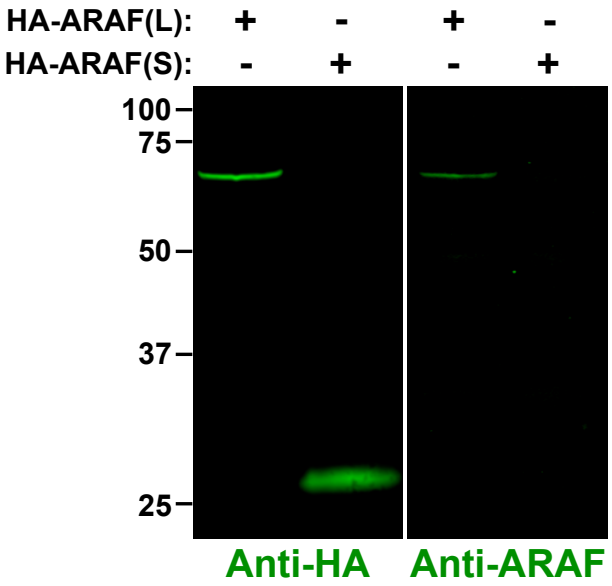
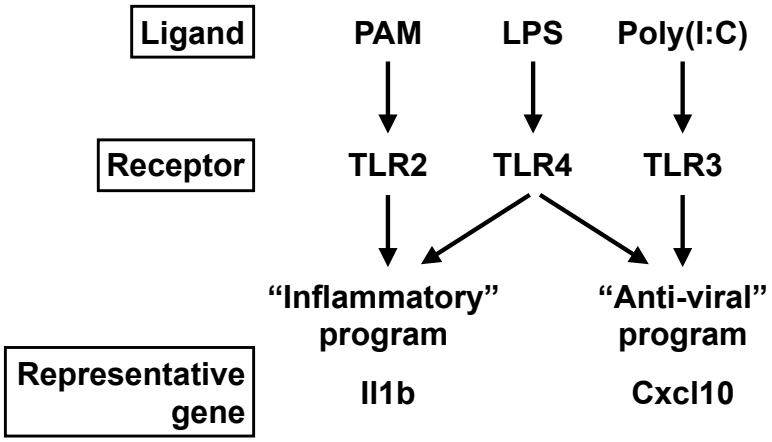

HA-ARAF(L): + - + -
HA-ARAF(S): - + - +
100
75
50
37
25
Anti-HA
Anti-ARAF

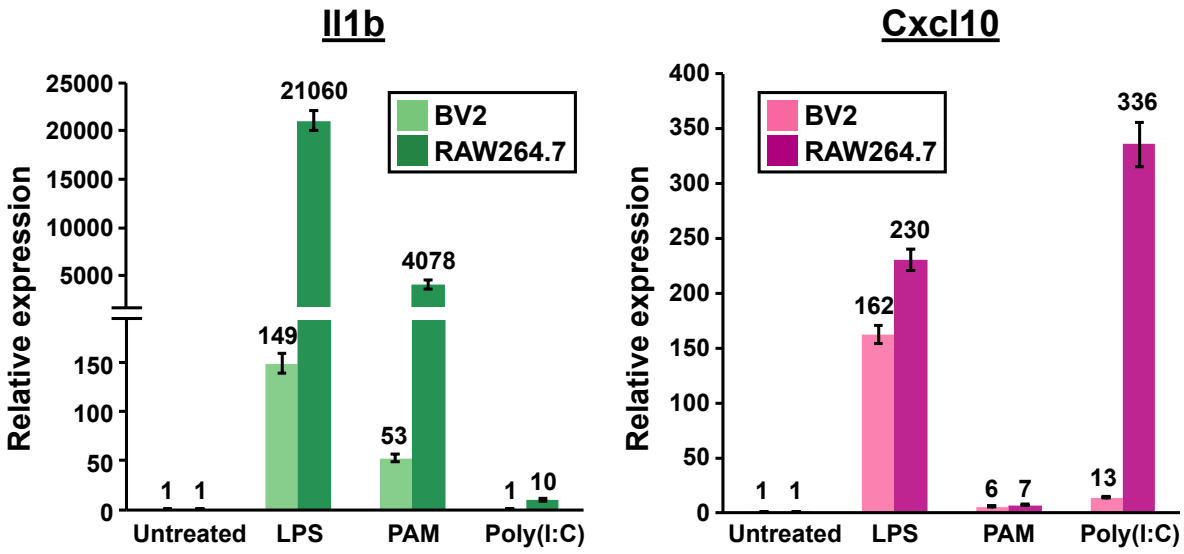
Figure S3. A summary of the three biological replicate cTag-PAPERCLIP experiments for microglia activation, related to Figure 6. (**A**) Pie charts showing the genomic distribution of unique reads in each replicate experiments. (**B**) Diagrams showing the correlation between replicate experiments. (**C**) Fluorescence immunoblot results showing the ARAF antibody used in the study recognizes the full-length *Araf* protein isoform [ARAF(L)] but not the short *Araf* protein isoform [ARAF(S)].

Fig. S4

A


Ligand
PAM
LPS
Poly(I:C)
Receptor
TLR2
TLR4
TLR3
"Inflammatory" program
"Anti-viral" program
Representative gene
Il1b
Cxcl10

B


Il1b
Cxcl10
Relative expression
BV2
RAW264.7
25000
20000
15000
10000
5000
150
100
50
0
21060
149
4078
53
1
1
1
10
400
350
300
250
200
150
100
50
0
230
162
336
6
7
13
Untreated
LPS
PAM
Poly(I:C)

C

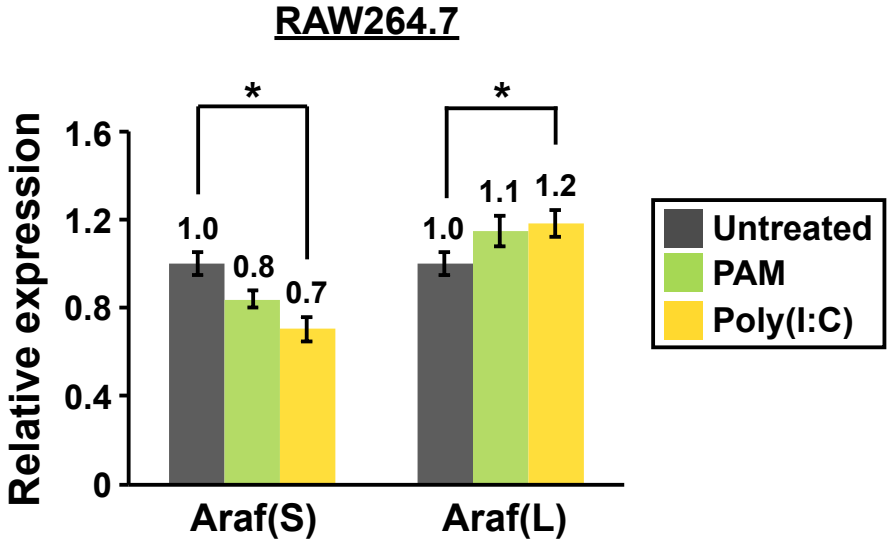
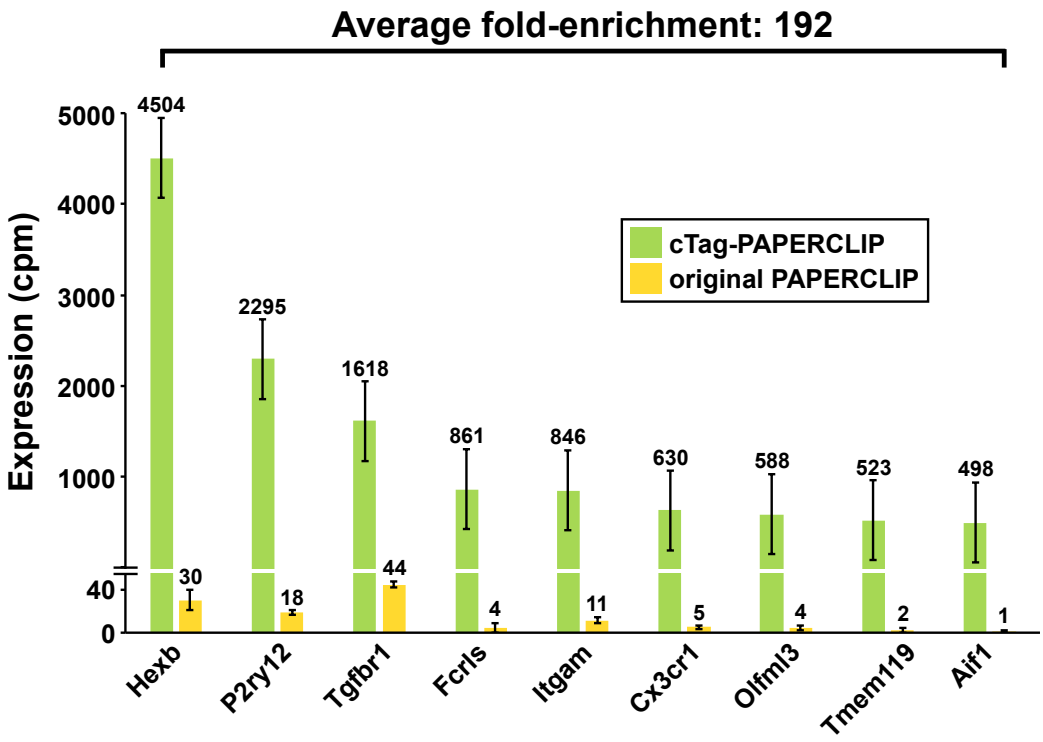

RAW264.7
Relative expression
1.6
1.2
0.8
0.4
0
*
*
1.0
0.8
0.7
1.0
1.1
1.2
Araf(S)
Araf(L)
Untreated
PAM
Poly(I:C)

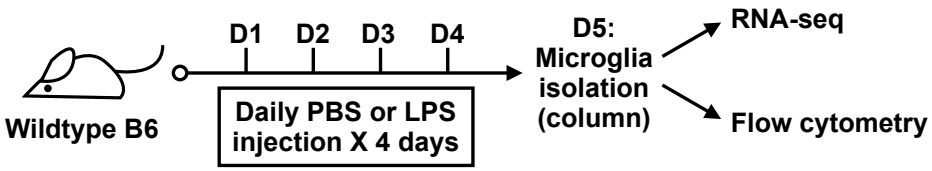
Figure S4. The *Araf* APA switch is also induced by a TLR3 ligand, related to Figure 7. (**A**) A diagram showing the TLR ligands, receptors and representative response genes. (**B**) Bar graphs showing qRT-PCR results measuring the response of BV2 microglia and RAW264.7 macrophages to different TLR ligands. Error bars: standard error. (**C**) Bar graphs showing qRT-PCR results measuring the abundance of *Araf* mRNA isoforms in RAW264.7 macrophages treated by TLR2 and TLR3 ligands. Error bars: standard error. *: $p<0.05$.

Fig. S5

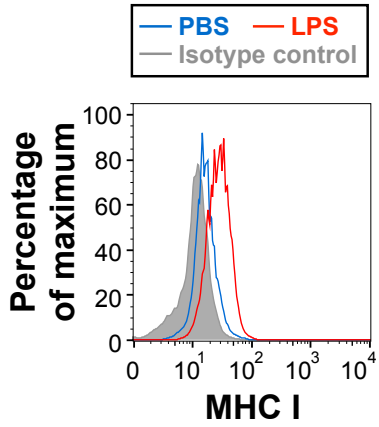
A


Average fold-enrichment: 192
Expression (cpm)
5000
4000
3000
2000
1000
40
0
cTag-PAPERCLIP
original PAPERCLIP
4504
30
2295
18
1618
44
861
4
846
11
630
5
588
4
523
2
498
1
Hexb
P2ry12
Tgfbr1
Fcrls
Itgam
Cx3cr1
Olfml3
Tmem119
Aif1

B


Wildtype B6
D1
D2
D3
D4
Daily PBS or LPS injection X 4 days
D5: Microglia isolation (column)
RNA-seq
Flow cytometry

C


PBS
LPS
Isotype control
Percentage of maximum
100
80
60
40
20
0
0
10^1
10^2
10^3
10^4
MHC I

D

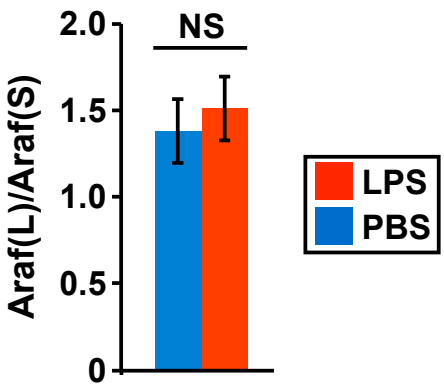
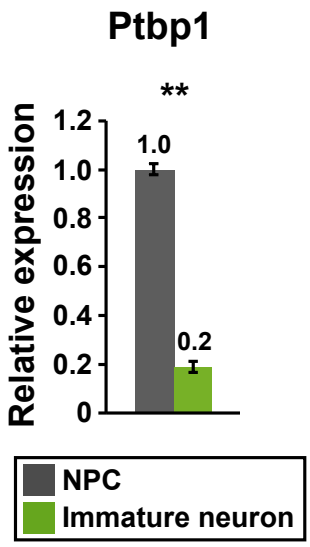

NS
Araf(L)/Araf(S)
2.0
1.5
1.0
0.5
0
LPS
PBS

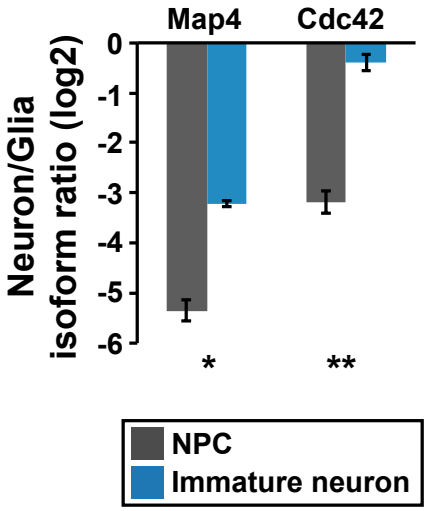
Figure S5. RNA-seq on *ex vivo* isolated microglia failed to identify the *Araf* APA switch, related to Figure 5. (**A**) Bar graphs comparing the expression of 9 microglia cell-type markers between cTag-PAPERCLIP data from *Cx3cr1-Cre; Pabpc1*^{cTag} mice (current study) and the whole cortex PAPERCLIP data from our original PAPERCLIP study (Hwang et al., 2016). Error bars: standard error. cpm: counts per million. (**B**) A schema showing the experimental design. RNA-seq data from the PBS group was also evaluated for microglia activation status (see Fig. 5B). (**C**) Flow cytometry results showing a full activation of brain microglia by the LPS injection protocol. (**D**) Bar graphs showing the ratio of Araf(L)/Araf(S) as measured by RNA-seq on microglia isolated from 3 biological replicates of PBS- or LPS-injected wildtype B6 mice. Error bars: standard error. NS: not significant.

Fig. S6

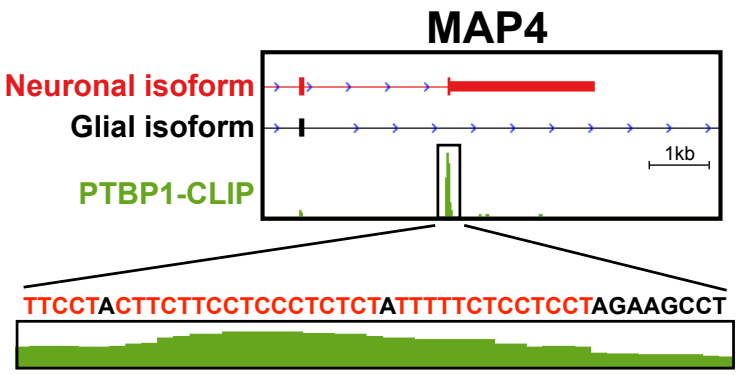
A


Ptbp1
**
Relative expression
1.2
1.0
0.8
0.6
0.4
0.2
0
1.0
0.2
NPC
Immature neuron

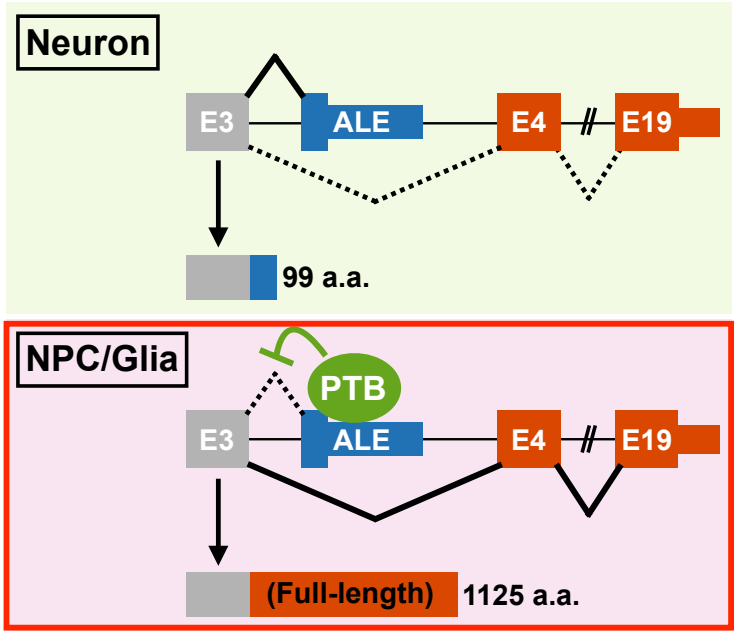
B


Map4
Cdc42
Neuron/Glia
isoform ratio (log2)
0
-1
-2
-3
-4
-5
-6
*
**
NPC
Immature neuron

C


MAP4
Neuronal isoform
Glial isoform
PTBP1-CLIP
1kb
TTCCTACTTCTTCCTCCCTCTCTATTTTTCTCCTCCTAGAAGCCT

D


Map4
Neuron
E3
ALE
E4
E19
99 a.a.
NPC/Glia
PTB
E3
ALE
E4
E19
(Full-length)
1125 a.a.

E

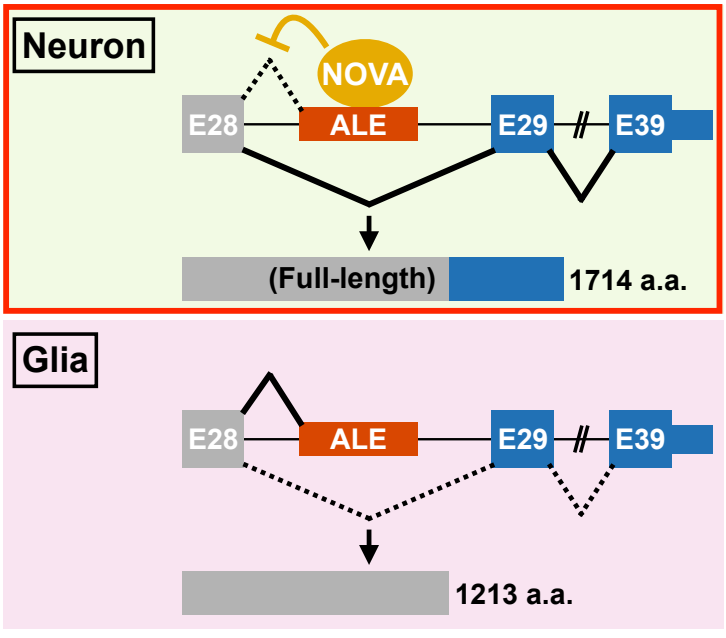
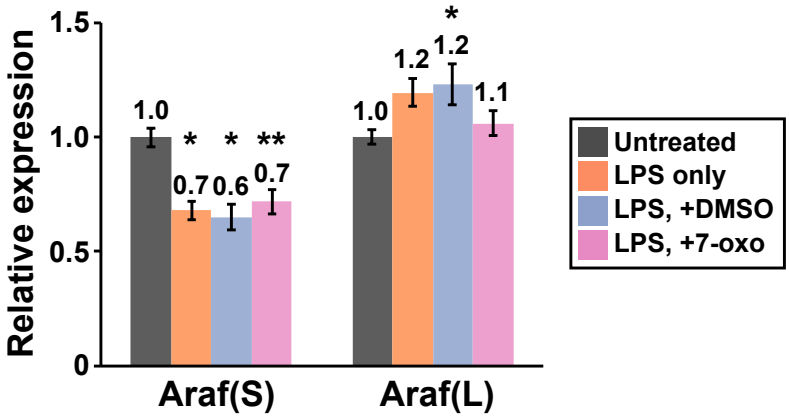

Itsn1
Neuron
NOVA
E28
ALE
E29
E39
(Full-length)
1714 a.a.
Glia
E28
ALE
E29
E39
1213 a.a.

Figure S6. NOVA and PTB restrict the APA isoform expression of *Itsn1*, *Map4* and *Cdc42* in different cell-types in adult brain or during brain development, related to Figure 4. (**A**) A bar graph showing a decrease in Ptbp1 abundance during neuron differentiation as determined by RNA-seq isoform analysis from purified neural progenitor cells and neurons from E14.5 mouse cortex. RNA-seq data is from (X. Zhang et al., 2016). NPC: neural progenitor cells. **: $p<0.01$. (**B**) Bar graphs showing an increase in abundance of the neuronal isoform of *Map4* and *Cdc42* during neuronal differentiation from the same RNA-seq dataset shown in (A). NPC: neural progenitor cells. **: $p<0.01$. *: $p<0.05$. (**C**) Diagrams showing PTBP1-CLIP footprints in the alternative last exons of *MAP4* in HepG2 cells. The PTBP1-CLIP data was obtained from the ENCODE database. The genomic sequence is shown in the 5′-3′ direction. PTB binding motifs (poly-pyrimidines) are highlighted in red. Due to space limitation, additional PTB binding motifs are not shown. (**D**) Schematics illustrating how PTB regulate the differential *Map4* APA isoform expression between adult neurons and glia or between developing neurons and NPC. Red rectangle denotes the cell-type in which the full-length protein is expressed. (**E**) Schematics illustrating how NOVA regulate the differential *Itsn1* APA isoform expression in adult neurons and glia. Red rectangle denotes the cell-type in which the full-length protein is expressed.

Fig. S7

A


Relative expression
1.5
1.0
0.5
0
1.0
*
*
**
0.7
0.6
0.7
1.0
1.2
*
1.2
1.1
Araf(S)
Araf(L)
Untreated
LPS only
LPS, +DMSO
LPS, +7-oxo

B

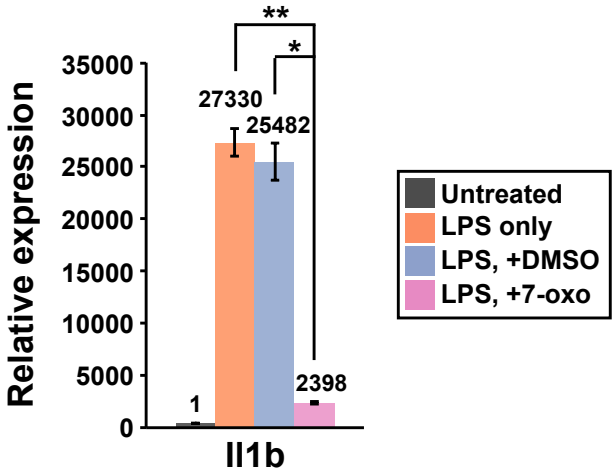

Relative expression
35000
30000
25000
20000
15000
10000
5000
0
**
*
27330
25482
1
2398
Il1b
Untreated
LPS only
LPS, +DMSO
LPS, +7-oxo

Figure S7. TAK1 inhibition does not affect the *Araf* APA switch induced by LPS treatment in RAW264.7 macrophages, related to Figure 7. (**A and B**) Bar graphs showing the qRT-PCR results measuring the abundance of *Araf* mRNA isoforms (A) or Il1b (B) in RAW264.7 macrophages with various treatments. 7-oxo: (5Z)-7-Oxozeaenol. Error bars: standard error. *: $p<0.05$; **< $p<0.01$.

Method S1. The complete cTag-PAPERCLIP protocol, related to the STAR Methods.

cTag-PAPERCLIP [Conditionally-Tagged *P*oly(*A*) binding *P*rotein-mediated mRNA 3´*E*nd *R*etrieval by *C*ross*L*inking *I*mmuno*P*recipitation]

***Modifications from the original PAPERCLIP protocol are highlighted in red.**

Day 1

I. General method for UV cross-linking of tissue/cell lines

a. UV cross-linking

For mouse tissue:

Harvest tissue and let tissue sit in ice cold 1x PBS (or 1x HBSS) until harvest is complete.

[The optimal amount of tissue depends on the cell-type of interest and needs to be tested. The usual input for excitatory cortical neurons is the entire cortex tissue from TWO adult mice.]

Add ~10 tissue volumes of 1x PBS (or 1x HBSS) and triturate tissue with 18-gauge needle and syringe a few times.

[Because UV light can penetrate a few cell layers, stringent trituration to the single-cell is not necessary.]

Spread the tissue suspension in 10-cm petri dishes and put the dishes on ice in a tray with the lid off. Put the tray in Stratalinker 2400 (Stratagene) and irradiate three times for 400mJ/cm^2. Rotate the tray 90° between each irradiation.

[The length of crosslinking may need to optimized for other types of tissue or a different UV-crosslinker is used. For a preliminary experiment, try 100, 200 and 400mJ/cm^2, and then use the shortest condition that gives >70% of the maximum signal.]

b. Post-crosslinking processing

Transfer the tissue suspension to 1.5mL or 2mL eppendorf tubes, pellet at 6000 rpm for 3 min at 4°C, remove supernatant and freeze pellets at -80°C until use.

[To accommodate the volume of lysis buffer (~5X tissue volume), we usually put the entire cortex tissue from ONE adult mouse (approximately 100~150mg) in one 1.5mL eppendorf tube.]

To prepare **1x HBSS**:

50ml 10x Hank's Balanced salt solution, Ca-Mg-free (Invitrogen, #14185-012)
5ml 1M HEPES, pH 7.3
445ml ddH_2O

Day 2

II. Immunoprecipitation

a. Solutions

Antibody Binding Buffer
1x PBS (tissue culture grade; no Mg++, no Ca++)
0.02% Tween-20

1x PXL Buffer
1x PBS (tissue culture grade; no Mg++, no Ca++)
0.1% SDS
0.5% NP-40
0.5% Sodium deoxycholate

5x PXL Buffer
5x PBS (tissue culture grade; no Mg++, no Ca++)
0.1% SDS
0.5% NP-40
0.5% Sodium deoxycholate

1x PNK Wash Buffer
50mM Tris-Cl pH 7.4
10mM $MgCl_2$
0.5% NP-40

1x PNK+EGTA Wash Buffer
50mM Tris-Cl pH 7.4
0.5% NP-40
20mM EGTA

b. Bead preparation

For each group (e.g., the entire cortex tissue from TWO adult mice), use 300µl of Dynabeads Protein G (Invitrogen, 10004D).

[Prepare enough beads for all reactions. The minimum number of reactions is two (one high RNase and one low RNase).]

Wash beads 3x with Antibody Binding Buffer.

Re-suspend beads in 300µl Antibody Binding Buffer and add 40µg of anti-GFP mixture (20µg of 19F7 and 20µg of 19C8).

Rotate beads at room temperature for at least 30 minutes.

Wash beads three times with 1x PXL Buffer; if you are not yet ready to add crosslinked lysate, leave beads in last

wash step on ice.

c. Prepare crosslinked lysate

Resuspend each tube of crosslinked tissue using 500µl~1mL 1x PXL Buffer (>5x tissue volume); sit on ice for 5-10 min to lyse. Pool the lysates if using more than 1 tube of tissues for one reaction.

Add 30~50 µl of RQ1 DNase (Promega, M6101) to each tube; incubate at 37° for 5 min, 1000 rpm.

[We perform all incubation/shaking steps in Eppendorf Thermomixer. A thorough and even shaking is critical.]

Make a dilution of RNase A (USB, 70194Y) at 1:100 in 1x PXL Buffer (High-RNase).

Make a dilution of RNase A (USB, 70194Y) at 1:100,000 in 1x PXL Buffer (Low-RNase).

[Each experiment should be done in duplicate with two RNAse concentrations – the dilution depends also on the batch of RNAse, so in the first experiment, several dilutions should be tested. The High-RNase group is used as a control to confirm that the size of the radioactive band on SDS-PAGE gel changes in response to different RNAse concentrations (which confirms that the band corresponds to a protein-RNA complex).]

Add 30~50µl of 1:100 diluted RNase A per 1mL lysate to the High-RNase tube.

Add 15µl of 1:100,000 diluted RNase A per 1mL lysate to the Low-RNase tube(s).

Incubate at 37°C for 5 minutes, 1000 rpm.

[Optimal amount of RNase A needs to be experimentally determined.]

Spin lysates in pre-chilled microcentrifuge at 14,000 rpm (Max, ~20,800g) for 10 minutes at 4°C.

Remove the supernatant from the precipitates.

d. Immunoprecipitation

Divide washed beads; add supernatant to one prepared tube of beads.

Rotate beads/lysate mix for 2 hours at 4°C.

e. Post-IP washes

Wash beads in the following order (all buffers are ice-cold and kept on ice during washes):
-Two times with 1x PXL Buffer
-Two times with 5x PXL Buffer
-Two times with 1x PNK Wash Buffer

III. CIP Treatment, (On-Bead)

CIP mix:

6 μl 10x Fast AP Buffer
2.3 μl Fast AP (Fermentas, EF0654)
0.6 μl 10% Tween-20
51.1 μl water
60 μl total

[The reaction volume is based on 300µl protein-G beads and can be scaled down if fewer amounts of beads are used.]

Add 60 μl of PNK mix to each tube and incubate in Thermomixer R (Eppendorf) at 37° for 20 minutes (1200 rpm every 1.5 minutes for 15 seconds).

Wash:
-Once with 1x PNK Wash Buffer
-Once with 1x PNK+EGTA Wash Buffer
-Two times with 1x PNK Wash Buffer

IV. 5′ labeling, (On-Bead)

PNK mix:

6 μl 10x PNK Buffer (NEB)
3 μl T4 PNK (NEB, M0201L)
1.5 μl ^{32}P-γ-ATP
49.5 μl water
60 μl total

[The reaction volume is based on 300µl protein-G beads and can be scaled down if fewer amounts of beads are used.]

Add 60 μl of PNK mix to each tube and incubate in Thermomixer R (Eppendorf) at 37° for 20 minutes (1200 rpm every 1.5 minutes for 15 seconds).

Wash:
-Three times with 1x PNK Wash Buffer

Leave beads in the last wash and store at 4°C overnight.

[It is possible to complete all Day 2 and Day 3 procedures in a very long day. In this case, one can proceed to the next part without storing the reactions.]

Day 3

V. SDS-PAGE & nitrocellulose transfer

a. Elution

Elution mix:

27 μl 1x PNK Buffer
3 μl 1M DTT
30 μl NuPAGE 4x LDS Sample Buffer (Invitrogen, NP0007)
60 μl total

Remove the last 1x PNK Wash Buffer; add 60 μl elution mix to each tube.

Elute Protein/RNA complexes from beads by incubating at 70°C for 10 minutes (1200rpm).

Load 1 tube per 2 wells (30 μl/well) of a 20 well Novex NuPAGE 10% Bis-Tris Midi gel.

Run the gel at 200V in the cold room. (Run time: ~1.5hrs with 1x MOPS buffer)

[Some pre-stained molecular weight markers may run differently on Novex NuPAGE gels. We use rainbow marker (GE Healthcare, RPN800E), which runs at the expected molecular weights.]

After gel run, transfer gel to nitrocellulose membrane using the Novex wet transfer apparatus.

[This pure nitrocellulose is a little fragile, but it works better for the RNA/protein extraction step.]

Transfer at 30V in NuPAGE Transfer Buffer with 10% methanol. (Transfer time: 75~90 minutes)

After transfer, rinse the nitrocellulose membrane in 1x PBS, and gently blot on Kimwipes; wrap membrane in plastic wrap and expose to film in a cassette at -80°C.

[Use a luminescent sticker, so that you can later align the membrane back to the autoradiogram. A band a little bit above the 102kDa marker should be readily observed for the High-RNase group. (The molecular weight of PABP-GFP is ~100kDa)]

Day 4

VI. RNA Isolation and Purification

1x PK Buffer:

100 mM	Tris-Cl pH 7.5
50 mM	NaCl
10 mM	EDTA

1x PK Buffer/7M urea (this buffer must be fresh):

100 mM	Tris-Cl pH 7.5
50 mM	NaCl
10 mM	EDTA
7 M	Urea

Cut nitrocellulose membrane (bottom margin: slightly above the top of the High-RNase band; top margin: ~225kDa marker) using a clean scalpel blade, and cut into small pieces (the smaller the better). Put the nitrocellulose pieces into 1 eppendorf tube for each group.

Make a 4mg/ml proteinase K (Roche, 03115828001) solution in 1x PK Buffer; pre-incubate this stock at 37°C for 10-20 minutes to kill any RNases.

Add 200 μl of proteinase K solution to each tube of isolated nitrocellulose pieces; incubate 20 min at 37°C at 1200 rpm.

Add 200 μl 1x PK/7M urea solution; incubate another 20 min at 37°C at 1200 rpm.

Add 400 µl RNA phenol (Sigma, P4682-100ML) and 130 µl of $CHCl_3$ (Sigma, 25668-100ML) to solution; vortex at high for 20 seconds; incubate 20 min at 37°C at 1200 rpm.

[RNA phenol can also be prepared by equilibrating pure phenol with 0.15 M NaOAc pH 5.2; $CHCl_3$ is chloroform 49:1 with isoamyl alcohol.]

Spin tubes at full speed in microcentrifuge; transfer aqueous phase to new eppendorf tubes.

Add the following to each tube:

50 μl	3M NaOAc, pH 5.2
1 μl	Ultra pure glycogen (Invitrogen, 10814-010)
1 ml	1:1 mix of ethanol and isopropanol

Precipitate overnight at -20°C.

Day 5

VII. cDNA synthesis and purification

a. Bead Preparation: Blocking with Denhardt's Solution

Ab Binding Buffer:
1X PBS, pH 7.4
0.02% Tween-20

50µl Protein-G Dynabeads per sample (25µl per cDNA purification step), include -RT and/or -Template

Wash 3 times with Ab binding buffer

Add 225µl Ab binding buffer, 25µl 50X Denhardt's Solution (Sigma, D2532 or Invitrogen, 750018); total volume is 5X original bead volume

Rotate at RT for at least 45 minutes ~ 1 hour

b. Reverse Transcription

Spin down the RNA at max speed (14,000 rpm, ~20,800g) for 20 minutes at 4°C. Wash 2 times with 75% ethanol, and dry the pellet.

Add 8µl nuclease-free water to RNA pellet (tap to resuspend, quick spin down). Denature at 65°C for 5 minutes (in microfuge tube), place tube on ice (to avoid loss of RNA, do not over-dry pellet and do not pipette until after denaturing step)

Transfer to PCR tube (on ice)

Mix I:
4µl 5X RT Buffer
1µl dATP
1µl dCTP } 8.2mM (Invitrogen, 10297-018)
1µl dGTP
1µl Br-dUTP (8.2mM; Sigma, B0631)
8µl total

Add 8µl of Mix I, 1µl of 25µM indexed RT primer to each tube.

Denature 3 minutes at 75°C, ramp down to 48°C and hold.

Mix II:
1µl 0.1M DTT
1µl RNasin Plus (Promega, N2611)
1µl SuperScript III (Invitrogen)
3µl total

Add 3µl of Mix II (pre-warm to 48°C in PCR block before adding).

Reverse transcription: 45 minutes at 48°C, 15 minutes at 55°C, 5 minutes at 85°C, 4°C hold.

c. Bead Preparation: Antibody binding

1x IP Buffer:
0.3X SSPE
1mM EDTA
0.05% Tween-20

Wash blocked beads 3 times with Ab binding buffer.

Add 5µl 50X Denhardt's Solution, 5~7.5µl (5~7.5µg) anti-BrdU antibody (Millipore, MAB3222), and 37.5~40µl Ab binding buffer to bring the total reaction volume to 50µl.

Rotate at RT for at least 45 minutes.

Wash 3 times with 1x IP Buffer.

d. Post-RT clean-up:

Add 1µl (at 2U/µl) RNase H (Invitrogen 18021-071 or NEB M0297L) to each RT tubes.

Incubate for 20 minutes at 37°C, hold at 4°C.

Add 10µl nuclease-free water (to bring volume above 25µl needed for G-25 column).

Spin the RNase H-digested RT products through Illustra Microspin G-25 column (GE Healthcare, 27-5325-01) to remove free BrdUTP (discard G-25 column as solid radioactive waste).

e. cDNA Purification: Immunoprecipitation I

2x IP Buffer:
0.6X SSPE
2mM EDTA
0.1% Tween-20

Nelson Low Salt Buffer:
15mM Tris pH 7.5
5mM EDTA

Nelson Stringent Buffer:
15mM Tris-HCl pH7.5
5mM EDTA
2.5mM EGTA
1% Triton X-100
1% Sodium deoxycholate
0.1% SDS
120mM NaCl
25mM KCl

Measure volume, add water up to 40µl and add 10µl 50X Denhardt's Solution and 50µl 2x IP Buffer for a total volume of 100µl (Denhardt's and 2X IP Buffer can be added to the G-25 column collection tube prior to spinning samples through, volume can then be adjusted up to 100µl).

Denature 5 minutes at 70°C, equilibrate to room temperature.

Add to prepared tube of beads (25µl original slurry volume, store remaining beads for second purification at 4°C overnight), rotate at RT for 45 minutes.

(all washes 3-5 minutes rotating at RT, 1ml)

Wash 1 time with 1x IP Buffer (5x Denhardt's)
2 times with Nelson Low Salt Buffer (1x Denhardt's)
2 times with Nelson Stringent Buffer (1x Denhardt's)
2 times with 1x IP Buffer

f. cDNA Purification: Heat Elution

Elution Buffer:
50µl 2x IP Buffer
40µl Water
90µl

Add 90µl elution buffer to each tube of beads.

Incubate at 98°C for 1 minute in thermomixer (1200 rpm) to elute cDNA from beads and place on magnet

Collect eluate and add 10µl 50X Denhardt's to each tube for a total of 100µl

Store overnight at 4°C

Day 6

VIII. cDNA purification and library construction

a. cDNA Purification: Immunoprecipitation II

CircLigase Wash Buffer:
33mM Tris-Acetate
66mM KCl
(pH 7.8)

Denature 5 minutes at 70°C, equilibrate to room temperature.

Add to prepared tube of beads, rotate at RT for 45 minutes.

(all washes 3-5 minutes rotating at RT, 1ml)

Wash 1 time with 1x IP Buffer (5x Denhardt's)
2 times with Nelson Low Salt Buffer (1x Denhardt's)
2 times with Nelson Stringent Buffer (1x Denhardt's)
2 times with CircLigase Wash Buffer

b. cDNA Circularization with CircligaseII

Phusion Wash Buffer:
50mM Tris
(pH 8.0)

Circligase Reaction Mix:
2µl CircLigase 10X Reaction Buffer
4µl Betaine (5M)
1µl $MnCl_2$ (50mM)
1µl CircLigase ssDNA Ligase II (100U) (Epicentre, CL9021K)
12µl Water
20µl total

Incubate 1 hour at 60°C in thermomixer (interval: shake at 1300rpm every 30" for 15").

(all washes 3-5 minutes rotating at RT, 1ml)

Wash 2 times with Nelson Low Salt Buffer
2 times with Nelson Stringent Buffer
2 times with Phusion wash buffer

c. PCR: Phusion Polymerase, SYBR Green

Mix I:
10µl 5X Phusion HF Buffer
1µl 10mM dNTPs
37µl Water

48µl total

Mix II:
0.5µl DP5-PE (20µM)
0.5µl DP3-PAT (20µM)
0.5µl Phusion DNA Polymerase (NEB, M0530)
1.5µl total

Add 48µl Mix I to beads.

Incubate at 98°C for 1 minute in thermomixer (1200 rpm) to elute cDNA from beads and place on magnet.

Collect eluate and place in PCR tube with optically clear cap.

Add 1.5µl Mix II, 0.5µl 50X SYBR Green I (dilute 10,000X stock to 50X in Phusion Wash Buffer) to mix and place in real-time PCR machine (Bio-Rad, CFX96).

(PCR cycle parameters)

Initial denaturation:	98°C	30”
Cycle:	98°C	10”
	60°C	15”
	72°C	20”

Remove reaction tube when RFU signal reaches ~250-500 (usually results in 2.5-5nM).

e. Post-PCR processing: library purification and quantitation

Purify PCR product using Agencourt AMPure XP beads (Beckman Coulter) according to manufacturer’s instructions.

Quantitate using TapeStation (Agilent), pool samples according to TapeStation results.

MiSeq:
Dilute to 2nM, sequence on MiSeq at 5pM final concentration using standard Read 1 primer already on cartridge.

HiSeq:
Dilute to 2-10nM Submit for HiSeq sequencing using standard Read 1 sequencing primer listed below.

IX. Primers

RT Primers:

RT-1 (CGAT):

/5Phos/DDDATCGNNNNNNNAGATCGGAAGAGCGTCGT/iSp18/CACTCA/iSp18/CAAG
CAGAAGACGGCATACGAGATTTTTTTTTTTTTTTTTTTVN

RT-2 (TAGC):

/5Phos/DDDGCTANNNNNNNAGATCGGAAGAGCGTCGT/iSp18/CACTCA/iSp18/CAAG
CAGAAGACGGCATACGAGATTTTTTTTTTTTTTTTTTTVN

RT-3 (CTAG):

/5Phos/DDDGACTNNNNNNNAGATCGGAAGAGCGTCGT/iSp18/CACTCA/iSp18/CAAG
CAGAAGACGGCATACGAGATTTTTTTTTTTTTTTTTTTVN

RT-4 (GATC):

/5Phos/DDDAGTCNNNNNNNAGATCGGAAGAGCGTCGT/iSp18/CACTCA/iSp18/CAAG
CAGAAGACGGCATACGAGATTTTTTTTTTTTTTTTTTTVN

PCR Primers:

DP5-PE: (Allowing standard Read1 sequencing primer)

5′-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT

DP3-PAT:

5′-CAAGCAGAAGACGGCATA

Sequencing Primers:

Illumina Standard Read1 Sequencing Primer:

5′-ACACTCTTTCCCTACACGACGCTCTTCCGATCT