Free circular introns with an unusual branchpoint in neuronal projections

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Abstract
The polarized structure of axons and dendrites in neuronal cells depends in part on RNA localization. Previous studies have looked at which polyadenylated RNAs are enriched in neuronal projections or at synapses, but less is known about the distribution of non-adenylated RNAs. By physically dissecting projections from cell bodies of primary rat hippocampal neurons and sequencing total RNA, we found an unexpected set of free circular introns with a non-canonical branchpoint enriched in neuronal projections. These introns appear to be tailless lariats that escape debranching. They lack ribosome occupancy, sequence conservation, and known localization signals, and their function, if any, is not known. Nonetheless, their enrichment in projections has important implications for our understanding of the mechanisms by which RNAs reach distal compartments of asymmetric cells.

Introduction
In polarized cells, such as neurons and oocytes, RNA localization to distinct subcellular compartments is important for spatial control of protein expression (Holt and Bullock, 2009). Known mechanisms for asymmetric distribution of RNA include active transport (e.g., Actb, Ross et al., 1997), spatially restricted capture by an anchor (e.g., Nanos, Forrest and Gavis, 2003), and control of RNA degradation (e.g., Hspa3, Bashirullah et al., 2001). In some cases, RNA localization depends on splicing. For example, a detained intron (i.e., an intron with regulated post-transcriptional splicing, as opposed to constitutive co-transcriptional splicing) restricts Sr35 mRNA export from the nucleus (Boutz et al., 2015), and a retained intron (i.e., an alternative unspliced isoform) promotes dendritic localization of Calm3 (Sharangdhar et al., 2017). The Robo3 gene, which is important for commissural axon development in mice, expresses both a fully spliced mRNA and another retaining intron 26, and these isoforms encode different proteins that have opposing functions in axon guidance (Chen et al., 2008). Spatial and temporal control of protein expression from the intron-retaining Robo3 isoform depends on its susceptibility to nonsense-mediated decay due to the presence of a premature termination codon in the retained intron (Colak et al., 2013).

Provocatively, some retained introns have been proposed to undergo splicing in dendrites (Glanzer et al., 2005). For example, an intron in the calcium-activated potassium channel Kcna1 was reported to undergo splicing in dendrites of rat hippocampal neurons (Bell et al., 2010), and this was suggested to be a mechanism for locally tailoring calcium-activated potassium currents. Because pre-mRNA splicing by the spliceosome is generally thought to be restricted to the nucleus (Steitz et al., 2008), this proposal has been controversial, and it has not yet been independently confirmed.
The interplay between intron retention and neuronal RNA localization has been studied in several individual cases (Chen et al., 2008; Bell et al., 2010; Buckley et al., 2011; Khaladkar et al., 2013; Ortiz et al., 2017; Sharangdhar et al., 2017). In this work, our aim was to systematically identify localized RNAs in primary rat hippocampal neurons by sequencing total RNA (rRNA depleted) as opposed to polyadenylated (polyA+) RNA, with a particular focus on the repertoire of projection-localized introns (both retained and excised). Our analyses identify hundreds of transcripts with retained introns. Unexpectedly, we also found a set of free circular introns localized to distal neuronal projections.

Results

Experimental Design and Validation

To physically separate cellular projections from cell bodies, we cultured dissociated primary rat hippocampal cells on membranes with 1 μm diameter pores (Poon et al., 2006). These cultures are a mixture of neuronal and glial cells; we add a DNA replication inhibitor to block cell division and prevent dividing glia from overgrowing post-mitotic neurons. We refer to the projections as “neuro-glial” projections because both neuronal (Map2-immunopositive) and non-neuronal (Gfap/Vimentin-immunopositive) projections extend through the pores and continue growing on the underside of the membrane, whereas cell bodies and nuclei are restricted to the top surface (Figure 1A and Figure 1—Figure Supplement 1). Lysates prepared by scraping the underside are highly enriched for projections (“projection” samples), while lysates prepared from the top surface comprise whole cells with nuclei and projections (“whole cell” samples).

To capture both adenyated (polyA+) and non-adenylated (polyA-) long RNAs in our lysates, we prepared rRNA-depleted total RNAseq libraries (mean insert size ~200 nt) from five biological replicates (10 samples total). The RNAseq libraries were subjected to paired-end sequencing (100-125 nt reads) on the Illumina platform to obtain 30-80 million mate pairs per sample.

We generated additional datasets to help interpret projection and whole cell RNAseq data. To distinguish retained introns from intronic polyadenylation (polyA) sites, we prepared polyA-site sequencing (PASseq) libraries (Ashar-Patel et al., 2017) from a subset of samples (three biological replicates each, 6 samples total). To identify coding exons, we generated ribosome profiling (Ricci et al., 2014) and polyA+ selected RNAseq libraries (Heyer et al., 2015) from the purified cytoplasmic (nuclei-depleted) fraction of primary rat hippocampal neurons cultured on plates (three biological replicates) (Figure 1A, B) (Table S1).

For preliminary characterization of these data, we aligned RNAseq reads to the rat genome (Ensembl release 81, Rnor_6.0) (Zerbino et al., 2018) (Figure 1—Figure Supplement 3). Ribosomal RNAs and small (<150 nt) noncoding RNAs (e.g., snRNAs, tRNAs) were depleted effectively. Larger abundant nonpolyadenylated noncoding RNAs were observed as expected, such as SRP (or 7SL, the RNA component of the signal recognition particle) and RMRP (the RNA component of mitochondrial RNase P); both were more prevalent in projection than in whole cell libraries. Reads mapping to the mitochondrial genome were also highly abundant and enriched in projection libraries, consistent with the abundance of mitochondria in neurons and neuronal projections (Palay, 1956) (Figure 1—Figure Supplement 1). To minimize spurious genome alignments due to expected abundant species, we filtered out reads mapping entirely to rRNA, SRP, mitochondrial genome, and repeat elements cataloged by RepeatMasker (Jurka, 2000) before moving on to other analyses described below.

To qualitatively assess our success in separating projections from whole cells, we aligned filtered reads to the rat genome with TopHat2 (Kim et al., 2013) and visualized read density tracks of known nuclear and projection localized RNAs on a rat genome browser (Integrative Genomics Viewer, Robinson et al., 2011). Known nuclear-localized RNAs such as the noncoding RNA Xist (Brown et al., 1992) and Srsf5 mRNA (which contains a detained intron) (Boutz et al., 2015) were depleted from projections relative to whole cells (Figure 1C and Figure 1—Figure Supplement 1). Conversely, the known projection-localized Pabpc1 mRNA (Poon et al., 2006) was enriched in projection data.
### Figure 1

Experimental design and data validation. (A) Imaging of MAP2 protein immunostaining (neuronal marker, green) and DAPI fluorescence (nuclear marker, blue) confirms that the bottom surface of neuronal cultures on a semipermeable membrane (cartoon, top) consists only of neuro-glial projections. Total RNAseq and polyA site RNAseq (PASseq) datasets were generated from the top surface (“whole cell”) and bottom surface (“projection”) lysates. (B) Standard plate cultures, fractionated to remove nuclei, were used to prepare ribosome profiling and cytoplasmic polyA+ RNAseq datasets. (C) Genome browser plots of read densities (sum of three replicates) and sequence conservation (PhyloP scores on 20 aligned vertebrate genomes, Pollard et al., 2010) for a projection-localized mRNA and a nuclear noncoding RNA (RPM = Reads per million mapped).

**Figure 1—Figure supplement 1.** Separation of neuro-glial projections from cell bodies.
**Figure 1—Figure supplement 2.** Microcapillary electrophoresis of total RNA from whole cells and projections.
**Figure 1—Figure supplement 3.** Data analysis workflow.
Figure 2. Quantitative analysis of RNA localization. (A) Scatter plot comparing log mean read counts and log fold difference in projections versus whole cells, for 19,815 RNA transcripts with non-zero read counts. 1,440 (orange dots) are significantly enriched (q-value < 0.01 and fold-change > 1.5) in projections, 1,486 (blue dots) are enriched in whole cells, and 16,899 (gray dots) show no significant enrichment in either sample. Ribosomal protein encoding RNAs are shown as black dots; rhombi enclose labeled RNAs. (B-G) smFISH validation of examples of projection-localized (B) versus projection-depleted (C), and low (D), medium (E), high (F), and higher (G) abundance mRNAs. Upper left corner shows gene name, lower right corner shows projection:whole cell TPM. Scale bars = 20μm.

**Figure 2—Figure supplement 1.** Correlation between RNaseq from biological replicates.

**Figure 2—Figure supplement 2.** Gene ontology classes enriched in projections and whole cells.

**RNAs enriched in projections**

To comprehensively and quantitatively evaluate how well our datasets distinguish known localized RNAs, we employed Kallisto and Sleuth (Bray et al., 2016; Pimentel et al., 2017) for differential expression analysis of annotated RNA transcripts in projections versus whole cells. RNA abundances (in TPM, Transcripts Per Million) of biological replicates were well correlated (Spearman's correlation coefficient ≥ 0.83 in projection samples, ≥ 0.88 in whole cell) (Figure 2—Figure Supplement 1), but comparisons between projection and whole cell datasets showed substantial differences (Figure 2A; Table S2). As expected, known nuclear noncoding RNAs, including Xist, Malat1, Meg3, snoRNAs, and scaRNAs, were among the 1,486 genes significantly depleted (> 1.5 fold and q-value < 0.01) from projections. In contrast, 1,440 transcripts were significantly enriched in projections, including known projection-localized mRNAs such as Pabpc1, Map2, Dlg4 (neuronal), and Gfap (glial) (Poon et al., 2006; Garner et al., 1988; Cajigas et al., 2012; Sarthy et al., 1989). Gene ontology analysis showed that the set of projection-enriched mRNAs were significantly enriched for genes involved in mitochondrial functions (cytochrome-c oxidase activity) as well as nearly the entire set of ribosomal protein encoding mRNAs (RP mRNAs) (Figure 2—Figure Supplement 2). Indeed, seventy annotated...
Figure 3. A subset of introns localize to projections. (A) Cartoon illustrating how we define intron regions as the intersection of all annotated introns. For two mock alternatively spliced isoforms, black rectangles are exons, lines are introns; vertical dotted lines mark the intron region boundaries. (B) Scatter plot comparing mean mapped reads per intron region (+1 pseudocount) from five biological replicates of projections versus whole cell RNAseq data. Rhombi enclose labeled RNAs. Inset: Venn diagram showing the number of intron regions that we consider to show reliable read coverage (at least one read in each replicate and mean read density > 0.005 reads/nucleotide).

**Figure 3–Figure supplement 1.** Data analysis workflow to count reads on intron regions.

**Figure 3–Figure supplement 2.** Comparison with previously reported retained introns in dendrites.

RP mRNA isoforms were enriched more than two-fold in projections (black dots in Figure 2A). RP mRNA enrichment in projections is puzzling because ribosomal proteins are imported into the nucleus for ribosome assembly but their enrichment in distal cellular locations has been made consistently in other RNA localization studies (for e.g., most recently, Shigeoka et al. (2018) studied RP mRNA enrichment in Xenopus retinal cell axons), and we discuss it later.

To test the accuracy of our RNAseq abundance measurements, we performed single-molecule fluorescence in situ hybridization (smFISH) on example RNAs. Using exon-hybridizing RNAseq probe sets, we probed for six different mRNAs spanning a range of TPM values and projection:whole cell ratios (Polr2a, 2:4; Ppib, 43:50; Ubc, 162:146; Pabpc1, 40:12; Srsf5, 18:37; Rpl4, 297:96) (Figure 2B-G; Table S4). Consistent with their RNAseq projection:whole cell TPM ratios, Polr2a, Ppib, and Ubc mRNAs exhibited predominantly cytoplastic localization, with a strong gradient in spot density highest in cell bodies tapering off into the projections. Srsf5 mRNA was almost entirely confined to the cell bodies, and consistent with the presence of a detained intron (Boutz et al., 2015), Srsf5 mRNA spots were also visible in the nucleus. In contrast, Pabpc1 and Rpl4 mRNA spots exhibited a nearly uniform distribution throughout cell bodies and projections, with relatively few discernible spots in nuclei.

We concluded from these and other analyses that our RNAseq datasets reliably detect and quantify projection-localized RNAs in primary rat hippocampal cultures. We then turned to our main interest in localization of intron sequences.

**Intron regions enriched in projections**

Because of alternative splicing, transcribed genomic regions cannot be easily separated into introns and exons. To facilitate a comprehensive analysis, we define an “intron region” as a genomic interval that is annotated as intronic (and not exonic) in all annotated transcript isoforms that
span it (Ensembl release 81, Rnor_6.0, annotation downloaded on July 24, 2015) (Figure 3A and Figure 3—Figure Supplement 1). Out of a total of 190,180 such intron regions, we considered 57,432 to have reliable coverage (at least one read in each of the five biological replicates, with mean read density > 0.005 mapped reads/intron region length) in the whole cell libraries, but only 1,632 met these criteria in projections (Figure 3B - inset; Table S3). For the 33 intron regions that we considered reliably covered in projections but not in whole cells, individual examination showed that all had coverage in the whole cell libraries but had just missed the cut.

Introns are expected to be spliced and degraded in the nucleus and thus strongly depleted from projections, but a scatter plot of intron region abundance in projections versus whole cells (Figure 3B) shows a bimodal distribution. Intron regions that show coverage in projections define a subpopulation that has similar read coverage in projections and whole cells. This population is interesting because of the restrictive way that we define “intron regions”: no annotated transcript isoform of a given gene shows these regions as exonic, but their abundance in projections suggests that they are either unspliced, excised but stable, or independent transcripts. They include, for example, known (but unannotated) neuron-specific retained introns, such as in Calm2 (homolog of mouse Calm3, Sharangdhar et al., 2017).

As we looked at randomly selected examples of the 1,632 intron regions in the rat genome browser, we found, unsurprisingly, that many cases simply represented an unannotated alternative splicing event (i.e., alternative 5’ or 3’ splice site) or an unannotated transcription start site (TSS) or polyadenylation site (PAS) within an annotated intron. We found it useful to distinguish them into classes depending on the presence or absence of reads spanning the unspliced 5’ or 3’ splice sites (5’ exon-intron and intron-3’ exon reads, EI and IE) and spliced exon-exon junctions (EE) (Figure 4A, B and Figure 4—Figure Supplement 1).

Intron regions with high EI reads but few EI reads (Figure 4B-(i) upper left; n = 385 regions) correspond to unannotated alternative 5’ splice sites (e.g., Aplp1) or unannotated alternative polyadenylation sites (e.g., Cxadr). Conversely, regions with high IE but few EI reads (Figure 4B-(iii) lower right; n = 320 regions) correspond to unannotated alternative 3’ splice sites (e.g., Mts51) or unannotated alternative transcriptional start sites (e.g., Nell2).

High EI and IE reads correspond predominantly to retained introns (Figure 4B-(ii) upper right; n = 428 regions), the most abundant of which are a 3’ UTR intron in Calm2 and the last intron in Sept3 (intron 10). Previous work in mouse neurons showed that the dsRNA binding protein STAU2 interacts with the Calm3 intron to promote dendritic localization of the intron-retaining mRNA isoform (Sharangdhar et al., 2017). In our data, the intron-retaining isoform of Calm2 predominated in both projection and whole cell libraries, with no selective enrichment in either cell compartment (Figure 4—Figure Supplement 2). Retention of Sept3 intron 10 results in a protein isoform with a different C-terminal sequence than the canonical isoform. Conservation of this alternative coding sequence suggests it is likely functional (Figure 4C).

We looked specifically at Kcnma1 intron 23, which has previously been reported to be retained, localized to rat primary hippocampal neuron projections (Bell et al., 2008), and spliced locally in dendrites upon neuronal activation (Bell et al., 2010). In our data, no Kcnma1 introns appear to be retained or localized to projections (Figure 4—Figure Supplement 4). We detect only spliced Kcnma1 transcript isoforms in projections.

A fourth class of intron regions in projections had both low EI and IE reads (Figure 4B-(iv) lower left; n = 499 regions). Some of these regions proved to harbor a gene transcribed from the same strand and contained within the intron of a different gene, such as Cxol6a1 within the last intron of Gatc (Figure 4—Figure Supplement 1). Others corresponded to unannotated alternative terminal exons, as in Map4 (Figure 4—Figure Supplement 1). We used the presence of intronic polyadenylation sites from our PASseq data to identify these two cases (n = 96 combined). Another subset corresponded to unannotated alternative cassette exons, as in Abi2 (Figure 4—Figure Supplement 1), which we identified using evidence of ribosome occupancy (mean ribosome profiling reads ≥ 5 per replicate; n = 221). The remaining 278 regions showed no evidence of ribosome occupancy in our
**Figure 4.** Classification of 1,632 projection-localized intron regions. (A) Description of symbols used to show annotated gene structure and types of read alignments. Rectangles represent annotated (black) or inferred (or unannotated, red) coding (ORF, Open Reading Frame) and noncoding (UTR, UnTranslated Region) exons. Lines represent introns and arrows point in the 5’ to 3’ direction of the transcript. Uninterrupted read alignments are shown as lines whereas arcs depict split reads connecting spliced exon-exon junctions. (B) Scatter plot of mean read coverage (+0.1 pseudocount) of 50 nt exon-intron (EI) versus intron-exon (IE) boundary regions in projection RNAseq samples. Dashed lines indicate thresholds set to EI=1 and IE=1 that divide introns into four quadrants, i-iv. Representative sketches depicting the situations within each quadrant are shown. n shows the number of intron regions in each quadrant. (C) Genome browser views of read coverage (sum of three biological replicates) and phyloP conservation for a free (Creld1-intron 5) and retained intron (Sept3 - 3’ terminal intron). RPM = reads per million mapped.

**Figure 4—Figure supplement 1.** Genome browser views of introns labeled in Figure 4.

**Figure 4—Figure supplement 2.** Comparison of intron enrichment versus mRNA enrichment in projections.

**Figure 4—Figure supplement 3.** Subcellular location of regular, retained, detained, and free introns validated by smFISH.

**Figure 4—Figure supplement 4.** Genome browser view of *Kcnma1* shows only the spliced isoform in projections.
Mean read density per intron
\ln (projections/whole-cells read counts)

Zc3h18
Creld1
P4htm
Mov10
Adgrb2
Ppp2r5b
Otof
snoU85
scaRNA7
scaRNA21
snoU89
Cep170b
Rasal1
Sec61a2
Igf1rArvcf
Tent4a
Scaf1
Ddit4 Fmnl1
Arrdc1
Srsf5
Clk1
Free introns with circularly permuted reads

0.01 0.10 1.00
-2.5
0
2.5
-5.0
5.0

Figure 5. 278 free introns detected in projections. Scatter plot of log ratio of mean read density per intron region in projections versus whole cells, and read density averaged across all replicates of projections and whole cells. Examples of previously known detained introns and intronic snoRNAs/scaRNAs are labeled in black. Introns with circularly permuted reads and without known functional annotation are labeled in red.

Ribosome profiling data, nor of polyadenylation sites in our PASseq data, and thus they appeared to correspond to nonpolyadenylated noncoding RNAs – possibly free introns or other genes within an intron – and we looked into them further.

Circular introns with noncanonical branchpoints in projections

One explanation for detecting “free” intron regions in projection samples is that they could correspond to intron-encoded small RNAs such as small nucleolar RNAs (snoRNAs) or small Cajal body RNAs (scaRNAs) that are nuclear-localized yet abundant enough that we detect reads in both projection and whole cell data. We plotted relative enrichment (log ratio in projections/whole cells) versus average abundance of each of the 278 intron regions (Figure 5). This showed that most regions are indeed depleted in projection data, and we found that many do correspond to known snoRNAs and scaRNAs. Projection-depleted regions also included known detained (unspliced and nuclear-localized) introns of highly expressed genes, including Srsf5 and Clk1. We attribute their low EI and IE read counts in projection data to statistical fluctuation around our thresholds; in whole cell data, we see high EI and IE read counts for these abundant nuclear-localized introns.

However, many other intron regions were both abundant and enriched in projections (Figure 5). These have features indicative of a previously described class of RNAs called “stable intronic sequence RNAs” (sisRNAs; Gardner et al., 2012). SisRNAs are circular lariat products of splicing (i.e., free introns) that are inefficiently debranched in the nucleus and exported to the cytoplasm via an NFX1/NXT1-dependent mechanism (Talhouarne and Gall, 2018). The most prominent examples in our projection libraries, such as Creld1 (Figure 4C and Figure 5), Zc3h18, and Mov10, were devoid of exon-intron and intron-exon reads, with higher read density on the intron than on the flanking exons. Further, these species were not detected in polyA+ RNAseq and ribosome profiling data. We
also observed a lack of read coverage over a 20-30 nt region at the 3’ end of the intron.

Spliceosome-mediated intron excision from pre-mRNA releases a lariat molecule in which the branchpoint nucleotide, predominantly an adenosine (Taggart et al., 2017), is linked 2’-5’ to the 5’ end of the intron. Reverse transcriptase can occasionally traverse a 2’-5’ linkage, so lariat branchpoints result in circularly permuted reads in RNAseq data. To test whether projection-enriched free intron species had characteristics of an intron lariat, we searched for circularly permuted reads within them using find_circ.py (Memczak et al., 2013). For fourteen projection-enriched intron regions, we detected numerous circularly permuted reads (Figure 6A), enabling us to identify the branchpoints. In all 14 cases, the branchpoint was a cytosine, C (n = 12), or guanine, G (n = 2), instead of the canonical adenosine, A. The spliceosome can use C or G as a branchpoint nucleotide, but the lariat debranching enzyme is inefficient if hydrolyzing the 2’-5’ linkage at these residues (Jacquier and Rosbash, 1986). Thus lariats with C or G branchpoints might be expected to be more stable than other introns. We attribute the lack of read coverage at the 3’ end of these introns to exonucleolytic degradation of the lariat tail, leaving stable circular RNAs with a 2’-5’ linkage.

We asked if these 14 introns had any other unusual sequence features. We derived sequence logos centered at their aligned 5’ splice sites, branchpoints, and 3’ splice sites. Their 5’ and 3’ splice sites conform to the standard consensus sequences, but their branchpoint follows a CC consensus 17-49 nucleotides upstream of 3’ splice site (Figure 6B). Mismatches in read alignments at the branchpoint and the relative scarcity of circularly permuted reads versus linear reads were consistent with the existence of a 2’-5’ linkage that the reverse transcriptase traverses with lower efficiency and accuracy. Other than conservation of 5’ and 3’ splice site sequences, none of the 14 free introns showed notable phylogenetic sequence conservation (Figure 6C). Although we hypothesize that the C branch site is an essential feature in stabilizing these projection-localized free introns, C branchpoints were not notably conserved across multi-species alignments of these introns, indicating little evolutionary pressure to conserve this feature.

Discussion

In this study, we conducted a comprehensive analysis of transcripts localizing to neuro-glial projections of primary rat hippocampal cells. Our data add to the growing compendium of localized RNAs identified using high-throughput methods in diverse rat/mouse neuronal cell types (e.g., motor neurons, Briese et al. (2016); dorsal root ganglia, Gumy et al. (2011), Minis et al. (2014); retinal ganglion cells, Zivraj et al. (2010); neuropil, Cajigas et al. (2012); cortical cells, Taliaferro et al. (2016), Taylor et al. (2009), Poulopoulos et al. (2019); primary hippocampal cells, Poon et al. (2006), Miyashiro et al. (1994)). Because our main intent was to identify and characterize intron sequences in projections, we created rRNA-depleted total RNAseq libraries from rat hippocampal cells grown on membranes that provided physical separation between projections and cell bodies (Figure 1). As expected, 97% of the annotated intron regions that we could detect across all samples met our expression cutoffs in whole cells only (Figure 3). Of the 3% of intron regions that are also detectable in projections, the majority turned out to be attributable to incomplete annotation of alternative mRNA isoforms or to intron-encoded snoRNAs/scaRNAs (Figure 4). Integration of layers of information – polyA+ RNAseq, ribosome profiling, and PASseq libraries – from parallel hippocampal cultures proved essential for us to characterize these regions.

Full-length introns present at high abundance in projections fell into two classes, one expected (retained introns) and one unexpected (free introns). As a class, retained introns displayed no strong tendency toward preferential projection localization (Figure 4–Figure Supplement 2). Thus, while some transcripts may contain localization elements in an alternatively retained intron (Sharangdhar et al., 2017), this does not appear to be a general function of intron retention in neurons. Some retained introns clearly express alternative protein isoforms, as we observed for Sept3 intron 10 (Figure 4). Notably, Kcnn1 intron 23, previously proposed to undergo local splicing in primary rat hippocampal dendrites (Bell et al., 2010), had almost no coverage in our projection libraries in the
**Figure 6.** Free circular introns with a noncanonical branchpoint. (A) Projection RNAseq read density for linearly mapped (orange) and circularly permuted (gray) reads across Creld1 - intron5. Alignment mismatches at the junction of circularly permuted reads are colored. Individual circularly permuted read alignments are shown as horizontal gray bars, with each bar representing one read. (B) Schematic of circularly permuted read alignment and sequence composition at 5’ splice sites, branch sites (the branchpoint nucleotide is underlined), and 3’ splice sites of five example introns. (C) Cumulative distribution function plots for phyloP sequence conservation showing lack of conservation in projection-enriched free introns relative to exons, or even to retained introns. x-axis represents median phyloP score over a 50 nt sliding window with 10 nt step size. (D) Inferred molecular structure of projection-localized free circular introns.
same cell types (Figure 4–Figure Supplement 4). Further, the retained introns that we identified in neuro-glial projections exhibit almost no overlap with the set of “cytoplasmic intron sequence-retaining transcripts” (CIRTS) previously reported to localize to rat primary hippocampal dendrites (Figure 3–Figure Supplement 2) (Buckley et al., 2011). CIRTs and Kcna1 intron 23 were initially identified by sequencing RNA from 15-300 individually dissected dendrites. While the method was state-of-the-art at the time, the low amount of input RNA necessitates multiple rounds of RNA and cDNA amplification prior to sequencing. In contrast, we started with high RNA input, restricted cDNA amplification to ≤ 15 cycles, and sequenced deeply to capture even low abundance RNA species.

Our most surprising finding was the detection of free introns in projections (Figure 5), which we infer to be lariat species containing a cytosine or guanine branchpoint instead of the canonical adenosine (Figure 6). The most likely explanation for the persistence of such species is the inability of debranching enzyme to cleave the 2'-5' bond at a C or G branch. That stable circular introns (or stable intron sequences, sisRNAs) can escape the nucleus and accumulate in the cytoplasm has already been reported by Talhouarne and Gall (2018). In that study, the authors observed sisRNAs across diverse samples, including cell lines from multiple vertebrate species and mouse fibroblasts, red blood cells, liver, and brain. Despite this pervasiveness, these introns’ general lack of sequence conservation and absence of interspecies overlap with sisRNAs strongly disfavors any evolutionarily conserved function. Rather, it seems more likely that sisRNAs are gene expression byproducts, perhaps with little positive or negative influence on cellular function; they may simply be noise in the system. We hypothesize that substituting any intron branchpoint from a canonical A to G has little effect on splicing while making the excised intron resistant to lariat debranching, leading to a stable 2'-5' circular RNA without much phenotypic consequence, and that such substitutions are tolerated at low frequency in any organism with spliceosomal introns in its genes.

Our study extends previous work on sisRNAs by the Gall lab by finding that sisRNAs are enriched in neuro-glial projections. If these free circular introns lack evolutionary signatures of function, why would they appear to be enriched in projections? We observe that because RNAseq experiments measure relative rather than absolute abundances, relative enrichment does not necessarily imply active localization. We hypothesize that mRNAs are typically occupied by polyribosomes in the cell body, whereas an untethered stable noncoding RNA may more freely diffuse throughout the entire volume of the cell, including distant projections. Unlike most mRNAs, free circular introns showed no evidence of ribosome occupany. It has previously been observed for Actb mRNA in neurons that a lack of ribosomal engagement leads to faster mRNA diffusion kinetics than is observed for actively translating mRNAs (Katz et al., 2016). The intracellular environment of neurons and neuronal projections is full of highly motile proteins and organelles that are themselves actively transported (Stepekowski et al., 2017), as indeed we see reflected by the prevalence of mitochondrial RNA in our projection libraries (> half of all reads in our projection libraries were of mitochondrial origin; Figure 1–Figure Supplement 1). We imagine that the movement of large objects within the confines of narrow cellular projections mixes the surrounding cytoplasm and could well force unanchored molecules to move in both anterograde and retrograde directions. Thus, free stable circular introns may appear to be relatively enriched in projections simply because they are long-lived and more freely diffusing than other RNAs that are depleted in projections because they are less stable or tethered in the cell body (to polyribosomes, for example).

This hypothesis has implications for another long-standing puzzle in neuronal RNA localization. All transcriptome-wide studies of the mRNA content of dendrites, axons, or synapses, including ours, have observed an enrichment of the complete set of ribosomal protein (RP) mRNAs (Moccia et al., 2003; Poon et al., 2006; Zivraj et al., 2010; Cajigas et al., 2012; Puthanveettil et al., 2013; Ainsley et al., 2014; Briese et al., 2016; Taliaferro et al., 2016; Nakayama et al., 2017; Shigeoka et al., 2018, to cite a few). Ribosomes are assembled in the nucleus, so localization of RP mRNAs to distant projections is puzzling. Recently, ribosome protein synthesis and incorporation of some individual ribosomal proteins into assembled ribosomes in axons of Xenopus retinal cells has been
shown to occur and to be important for axon development (Shigeoka et al., 2018), however, only a subset of RP mRNAs in axons are translated. It has been previously shown that in quiescent or growth-arrested cells (such as neurons), 30-40% of RP mRNA molecules sediment in polysome profiles as free mRNPs, compared to only ~10% for non-RP mRNAs (Meyuhas et al., 1987). Thus, it appears that in neurons, a substantial fraction of RP mRNAs are not engaged with ribosomes, and like our free circular introns, unengaged RP mRNAs may be more free to diffuse about the cell. We propose that the relative enrichment of RP mRNAs in projections could simply be due to their low ribosome engagement, not to an active transport mechanism.

**Methods and Materials**

**Primary hippocampal neuron culture**

Animals were handled in accordance with protocols approved by the Institutional Animal Care and Use Committee at University of Massachusetts Medical School and Harvard University. All experiments were performed on primary hippocampal neurons of embryonic day 19 rat fetuses. Pregnant Sprague Dawley rats purchased from Charles River Laboratories at 19 days of gestation were euthanized by carbon dioxide asphyxiation immediately followed by diaphragm puncture to ensure death of the animal prior to surgical removal of fetuses. Fetuses were transferred to pre-cooled dishes and placed on ice. Fetal brains were gently extracted under sterile conditions and submerged in ice-cold sterile HBSS (Hank's Balanced Salt Solution, Gibco™ 14185052) for transport to the tissue-culture laboratory. The brains were transferred to ice-cold Hibernate E (BrainBits) for microdissection of the hippocampi in a sterile tissue culture hood. Isolated hippocampi were transferred to freshly prepared, pre-warmed pre-dissociation media (comprising Hibernate E, EBSS, Papain and DNase from Worthington LK003176) and incubated at 37°C for 30 minutes. Pre-dissociation solution was replaced by 2 ml MEM complete media (containing 50 ml 10X MEM (Invitrogen 11430-030), 15 ml 20% glucose, 15 ml 7.5% sodium bicarbonate, 2 ml 1N HCl, 400 ml water, 5 ml 200 mM glutamax (Gibco™ 35050061), 50 ml heat inactivated horse serum, 5 ml penicillin-streptomycin (Gibco™ 15140122)) and the hippocampi were dissociated by gentle trituration, first with a regular and then a fire-polished (with reduced tip diameter) glass pipette. Additional MEM complete media was added to the dissociated cells. Cells were counted using a hemocytometer and plated at optimized densities on poly-D-lysine hydrobromide (Sigma #P0899) coated plates/surfaces.

Two hours after plating, MEM complete media was replaced with Neurobasal media (500 ml Neurobasal (Invitrogen 21102-049), 1.3 mL 200 mM glutamax, 1X antibiotic/antimycotic (Gibco™ 15240062), 1x B27 (Gibco™ 17504001)). We obtained ~13 embryos per rat and ~1 million cells per embryo.

Mature neurons are post-mitotic but glial cells divide. To prevent glial cells from crowding out the neurons, we treated the culture with a DNA replication inhibitor, Cytosine β-D-arabinofuranoside (Sigma-Aldrich #C1768), on the third day in vitro (DIV). Half of the neurobasal media was replaced with fresh media on DIV = 6, and then on DIV = 12. Cells were harvested on DIV = 14.

**Physical separation of neuro-glial projections from cell bodies**

To separate neuro-glial projections from cell bodies, primary neurons were cultured on Falcon® Permeable Support with Polyethylene Terephthalate (PET) membranes (Corning Life Sciences #C353102) that have 1µm pores such that the cell bodies remain on top of the membrane, but cellular projections (axons, dendrites, and glial projections) can grow through the pores to the underside of the membrane (Poon et al., 2006). Lysate collected from the top surface of the membranes comprises whole cells (cell bodies and projections) whereas lysate from the underside of the membranes comprises neuro-glial projections and some cytoplasmic blebs.

Primary hippocampal neurons were cultured at a density of 0.2 x 10^6 per well on Falcon® Permeable Support designed to fit 6-well tissue culture plates (Corning Life Sciences #C353102). The plating density was optimized to ensure healthy neuronal cultures for harvesting on DIV = 14.
Before setting up the neuronal culture, membranes were immersed in poly-D-lysine hydrobromide solution (0.1 mg/ml in 0.05 M sodium borate pH 8.5) overnight at 37°C. Before plating neurons, membranes were rinsed three times with sterile water and incubated at 37°C for at least 2 hours while immersed in MEM complete media. Neuronal health was assessed for every biological replicate by immunofluorescence imaging using a dendritic marker, MAP2, to visually inspect cell morphology.

**RNA isolation from neurons cultured on semipermeable membranes**

To extract RNA from neuro-glial projections, we removed media from the Falcon® Permeable Support, turned it upside-down with the projections-side of the membrane facing upward, applied 200 µl of TRIzol® reagent (ThermoFisher #15596018) to the membrane, quickly scraped the surface with one stroke using a cell-scaper, tilted the membrane and gently collected the lysate from the edge of the membrane with a pipette. Lysate was similarly collected from the cell body side of the membrane by applying 500 µl of TRIzol® reagent.

RNA was extracted following the steps recommended by TRIzol® reagent manual with minor modifications. Briefly, after application of TRIzol® reagent, the lysate was transferred to 15 ml Falcon tubes and vortexed for 30 seconds, followed by a 5 minute incubation at room temperature to dissociate nucleoprotein complexes. 200 µl of chloroform per 1 ml of TRIzol® was then added for phase separation. The tubes were vigorously shaken by hand and centrifuged at 12,000 x g for 15 min at 4 °C. The aqueous phase was transferred to fresh tubes and the chloroform wash was repeated two more times. RNA was precipitated by mixing with 100% isopropanol, incubating at room temperature for 15 min, and centrifugation at 12,000 x g at 4°C for 15 min. The supernatant was removed without disturbing the precipitated RNA. The RNA precipitate was rinsed two times with 75% ethanol, air dried, and dissolved in RNase-free water by incubating for 10 min at 55-60°C. RNA was stored at -80°C until needed.

Typical RNA yield from neuronal cultures on 8 x 6-well plates with the semipermeable membrane inserts was ~5 µg from the projections lysate for every ~50 µg from the whole cell lysate. The quality and quantity of RNA was assessed by Nanodrop™ UV spectrophotometer (A260/A280 measurements) and Bioanalyzer Pico RNA microcapillary electrophoresis (Figure 1—Figure Supplement 2).

**rRNA-depleted total RNASeq library preparation**

RNASeq libraries were prepared following the protocol published in (Zhang et al., 2012). Briefly, 5 µg of RNA from each sample was treated with TURBO™ DNase (ThermoFisher AM2238) followed by clean-up and enrichment of RNA > ~150 nt using RNA clean and concentrator (Zymo Research #R1013). DNase activity was tested beforehand to ensure DNA digestion. Total RNA was depleted of rRNA using the Ribo-Zero rRNA removal kit (Illumina #MRZH11124), following manufacturer's protocol. RNA was hydrolyzed using 5X first strand buffer (provided with Superscript III reverse transcriptase, ThermoFisher #18080044) at 94°C for 4 minutes and 50 seconds and immediately moved to ice. The fragmented RNA was reverse transcribed using random hexamers (ThermoFisher #N8080127) and Superscript III to make single-stranded cDNA. To make strand specific libraries, the second strand complementary to the cDNA was transcribed with DNA polymerase I (New England Biolabs #M0209S) using dUTP instead of dTTP. Illumina sequencing adapters were ligated to the double stranded cDNA. The dUTP-containing strand was degraded using Uracll-DNA Glycosylase (New England Biolabs #M0280S). The resulting single-stranded cDNA was amplified with 13 or 15 PCR cycles followed by size-selection using Pippin Prep (Sage Science). Before subjecting to Illumina sequencing, the RNASeq library quality was assessed by running the samples on the Bioanalyzer and Sanger sequencing a subset of TOPO-TA cloned products. Only those libraries with sufficient final concentration (> 12 nM), a product of appropriate size range (mode 300 nt size), and comprising expected RNA sequences (for instance, exon regions of abundant mRNAs) were selected for Illumina sequencing.
A total of five biological replicates were sequenced over a span of 2 years, replicates 1-3 (paired-end 100) in 2014 and replicates 4,5 (paired-end 125) in 2016.

PolyA site sequencing

PolyA site sequencing (PASseq) libraries were prepared following the protocol in Ashar-Patel et al. (2017) and Heyer et al. (2015). 2-5 µg of total RNA from each sample (3 biological replicates of projection and whole-cell lysates) was treated with DNase and fragmented as described above. The RNA was reverse transcribed with Superscript III using an anchored oligo-dT primer containing Illumina sequencing adapters and a unique barcode for each sample. Single-stranded RNA was degraded with RNasel. The cDNA was denatured (65°C for 5 min) and resolved by electrophoresis on a 10% polyacrylamide gel to select 160-210 nt sized fragments (for a 50-100 nt expected insert size sans the adapter sequences). To extract cDNA from the gel, a piece of the gel containing the cDNA was cut at the appropriate location, crushed, and nuted overnight in a solution of 300 mM sodium chloride and 10 mM EDTA. The solution was recovered from gel pieces by centrifugation in Corning™ Costar™ Spin-X™ columns (#07200386) at 10,000 x g for 3 minutes. cDNA was precipitated using isopropanol, followed by washes in 70% ethanol. The cDNA was then circularized using CircLigase (EpiCentre BioTechnologies #CL4115K) and amplified with 12-14 PCR cycles. The amplified DNA library was further enriched for a product of size 180-280 nt to exclude insert-less product (150 nt) using Pippin Prep.

Ribosome profiling and polyA+ RNAseq from the cytoplasmic fraction

Ribosome profiling and corresponding RNAseq libraries were prepared from fractionated cytoplasmic lysate of primary hippocampal neurons following the protocol in Ricci et al. (2014) and Heyer et al. (2015).

Rat primary hippocampal neurons were cultured (as described above) on poly-D-lysine coated 6-cm plates (three plates per sample) at a density of 1 x 10^6. After 14 days in vitro, cyclohexamidine was added to the media at 100 µg/µl final concentration for 10 min to stall translation. The plates were placed on ice where the media was removed and the cells were washed two times with 2 ml ice-cold PBS containing 100 µg/ml cyclohexamidine. The cells were lysed in 200 µl lysis buffer (10 mM Tris-HCl pH 7.5, 5 mM MgCl₂, 100 mM KCl, 1% Triton™ X-100, 2 mM DTT, 100 µg/ml cycloheximide, protease inhibitor (Complete, EDTA-free, Roche)). The lysate was collected by scraping the plates, transferred to a clean microcentrifuge tube and incubated on ice for 5 min, followed by centrifugation at 1300 x g for 10 min to pellet the nuclei. The supernatant was recovered, flash frozen in liquid nitrogen, and stored at -80°C until needed. Half of the lysate was used for ribosome profiling and the other half for polyA+ RNAseq library preparation.

To purify ribosome occupied RNA sequences for ribosome profiling, the RNA was digested with 300 units of RNase T1 (Fermentas) and 500 ng of RNase A (Ambion) for 30 minutes at room temperature to break down polysomes into monosomes. The monosomes were purified by density gradient ultracentrifugation. Lysates were fractionated by centrifugation through a 10-50% (weight/volume) linear sucrose gradient (20 mM HEPES-KOH, pH 7.4, 5 mM MgCl₂, 100 mM KCl, 2 mM DTT, 100 µg/ml cycloheximide) at 35,000 r.p.m. for 2 hours and 40 minutes at 4°C. A gradient fractionator (Brandel) was used to identify and collect the monosome enriched fraction by measuring absorbance at 254 nm. RNA was extracted from the monosome fraction and resolved by electrophoresis on a denaturing polyacrylamide gel to select RNA fragments ranging from 26 - 32 nt in size.

To extract RNA for ribosome profiling and polyA+ RNAseq, SDS was added to 1% final volume, and proteinase K (Invitrogen) was added to a final concentration of 200 µg/ml. The samples were incubated at 42°C for 45 minutes. One volume of acid phenol/chlorofom (Ambion AM9720, pH 4.5) was added and the samples were vortexed for 30 seconds followed by centrifugation at 12,000 x g for 15 minutes. The supernatant was transferred to a clean microcentrifuge tube and 0.1 volume of sodium acetate (3 M, pH 5.2) and 10 mM final concentration of MgCl₂ were added. To precipitate
RNA, 1 volume of 100% isopropanol was added to the solution and centrifuged at 12,000 x g for 35 minutes. The precipitated RNA was rinsed with 70% ethanol, air dried, and reconstituted in 5 µl water.

For polyA+ RNAseq library preparation, the RNA was partially hydrolyzed using Fragmentation Reagent (Ambion) prior to CDNA library preparation.

To prepare cDNA libraries from ribosome occupied RNA fragments and from RNA fragments from the cytoplasmic lysate, the 3’ ends of RNA fragments were dephosphorylated with T4 polynucleotide kinase (New England BioLabs #M02015). A preadenylated DNA adaptor sequence was ligated to the 3’-hydroxyl ends of the RNA fragments using T4 RNA Ligase (T4 RNA Ligase 2, truncated K227Q, NEB #M0351S). The ligated RNA product was reverse transcribed using Superscript III and a barcoded primer with sequence complementarity to the adaptor. The reverse transcription primer also contained adaptors required by Illumina sequencers. The resulting cDNAs were enriched for desired product size, circularized, and amplified following the steps described for PASseq library preparation.

Ribosome profiling libraries were amplified with 8 PCR cycles whereas polyA+ RNAseq libraries were amplified with 13 PCR cycles and sequenced on Illumina HiSeq sequencers for 50 nt single reads.

**Immunofluorescence staining**

Cells were cultured either on semipermeable membrane inserts or Thermo Scientific™ Lab-Tek™ II chamber slides as described in previous sections. At DIV = 14, media was removed, the cells were rinsed two times with phosphate buffered saline (PBS, pH = 7.4) and subsequently treated with fixative (4% paraformaldehyde) for 10 min at room temperature. The fixative was removed and the cells were rinsed three times with PBS followed by permeabilization with 0.1% Triton™ X-100 for 5 minutes. Cells were then rinsed three times with PBS and quenched with 50 mM ammonium chloride (in double-distilled water) for 10-15 minutes, followed by three more rinses with PBS. The fixed and permeabilized cells were then incubated with 10% normal goat serum in PBS (blocking solution) for 30 minutes at room temperature. For cells grown on membranes, the membranes were cut out of their plastic support system using a sharp blade and transferred to small chambers for the next steps. The samples were kept moist at all times during the protocol.

The membranes or slides were incubated overnight at 4°C with primary antibody diluted in blocking solution then rinsed three times with PBS. Fluorescently-labeled secondary antibody was applied for 1 hour in the dark at room temperature and rinsed by three washes with PBS. The membranes were cut in half and placed on glass slides with either the whole cell or the projection side on top. ProLong™ Gold antifade media with DAPI was applied to the membranes before covering them with a 0.16-0.19 mm thick cover glass. The edges of cover glass were sealed with transparent nail-polish and allowed to set overnight in the dark at room temperature. The samples were imaged on DeltaVision or Zeiss Cell Discoverer microscopes.

**smFISH**

smFISH was performed following ACDBio protocol. Briefly, cells were cultured on Ibidi poly-D-lysine coated chambered coverslips. At DIV=14, media was removed, cells were rinsed two times with PBS, fixed for 30 minutes at room temperature, then rinsed three times with PBS. The cells were dehydrated by incubating them in sequentially higher concentrations of ethanol (50%, 70%, and 100%, respectively) for 5 minutes each and a final immersion in 100% ethanol for at least 10 min at room temperature. The dehydration was then reversed by incubation in 70% ethanol for 2 minutes, 50% ethanol for 10 minutes, and rinsing with PBS at room temperature. They were then treated with Protease III (ACD Bio) for 10 minutes at room temperature. smFISH probes were hybridized following manufacturer’s instructions. In the end, the cells were counterstained with DAPI and mounted with ProLong™ Glass Antifade Mountant (P36980). Probes were multiplexed to image up
to three different targets in the same sample. The samples with imaged on Zeiss Cell Discoverer at the Harvard Center for Biological Imaging. Regions targeted by smFISH probes are listed in Table S4.

**COMPUTATIONAL ANALYSIS**

An outline for the data analysis workflow is shown in Figure 1–Figure Supplement 3 and Figure 3–Figure Supplement 1. Descriptive placeholder input and output filenames (enclosed in <> ) are used in the commands shown. If reusing these commands, please replace placeholder filenames with appropriate ones and omit enclosing "<" and ">".

**rRNA-depleted, total RNAseq genome alignment**

Paired-end reads from five biological replicates (10 samples total) of projection and whole cell RNAseq are provided in the fastq format at GSE129924. Reads from replicates 1-3 are 2 x 100 nt, whereas replicates 4 and 5 are 2 x 125 nt long. Replicates 4 and 5 also contain ERCC RNA spike-in. Reads from all 10 samples were aligned to ERCC, rRNA, repeat elements cataloged by RepeatMasker (*Jurka, 2000*), 7SL or SRP (the RNA component of signal recognition particle), and the mitochondrial genome, serially in that order, using bowtie2 version 2.2.3 (*Langmead and Salzberg, 2012*). Unaligned reads after each step were passed on for the next alignment. The following parameters were used for each alignment:

```
$ bowtie2 -2.2.3/bowtie2 -p 2 --no-unal
--un-conc <unaligned.fastq> --al-conc <aligned.fastq> \\
-1 <read1.fastq> -2 <read2.fastq> -S <alignments.sam>
```

Unaligned or filtered reads were then mapped to the rat genome (Ensembl release 81, Rnor_6.0, annotation downloaded on July 24, 2015) (*Zerbino et al., 2018*) with TopHat version 2.1.1 (*Kim et al., 2013*) using the following parameters:

```
$ tophat/2.1.1/tophat -p 6 --library-type fr-firststrand \\
--b2-sensitive --mate-inner-dist 100 -i 30 -g 10 \\
--max-coverage-intron 5000000 -G <genes.gtf> -o <output_directory> \\
<genome_index_base> <filtered_read1.fastq> <filtered_read2.fastq>
```

High quality read alignments were selected (using SAMtools version 1.4.1) for visualization on the rat genome browser.

```
$ samtools view -bh -q 10 <alignment.bam> > <alignment.q10.bam>
```

**PolyA site identification from PASseq data**

Six samples (three from whole cell and three from projection lysates) were barcoded and sequenced in one lane on NextSeq 500 with 150 cycles. The barcoded reads were parsed using Illumina’s bcl2fastq version 1.8.4 conversion software.

```
$ bcl2fastq --barcode-mismatches 1 -R <run_directory> \\
-o <output_directory> --use-bases-mask 15y*n 2 <output.log>
```

Reads were trimmed using cutadapt version 1.7.1 (*Martin, 2011*) to remove stretches of As from the 3’ end and barcode sequence from the 5’ end, and then selected for minimum 25 nt resulting read length. We used the fastx toolkit version 0.0.14 ([http://hannonlab.cshl.edu/fastx_toolkit/](http://hannonlab.cshl.edu/fastx_toolkit/)) to further select reads with sequencing quality greater than 35.Parsed, trimmed, and filtered fastq files are provided on GSE129924.

```
$ cutadapt -a AAAAAAAA -o <A.trimmed.fastq> <input.fastq> \\
2> <output.log>
$ cutadapt -u 7 -m 25 -o <A.barcode.trimmed.fastq> \\
< A.trimmed.fastq> 2> <output.log>
```
For PAS site identification, we used cleanUpdTSeq v.1.0.2 (Sheppard et al., 2013), which calculates the probability of a genomic locus to be a true polyadenylation site. The reads were first aligned to the rat genome using TopHat version 2.1.1 (Langmead and Salzberg, 2012), then reads with mapping quality greater than 10 were selected using SAMtools version 1.4.1 (Li et al., 2009).

```
$ tophat/2.1.1/tophat -p 4 --library-type fr-secondstrand \n--b2-very-sensitive --no-novel-juncs -i 30 -g 10 \n-G <genes.gtf> -o <output_directory>\n<genome_index_base> <PASseq_reads.fastq>
```

Only those genomic loci with more than 5 read alignments were considered for further analyses.

```
$ awk '{ ofs="\t"; { print $1, $2, $2, "SampleID_"NR, $3, "+"} } ' <*.3 cov.bed > <*.3 cov.plus.bed>
$ awk '{ ofs="\t"; { print $1, $2, $2, "SampleID_"NR, $3, "-"} } ' <*.3 cov.bed > <*.3 cov.minus.bed>
```

Bed files from the plus and minus strands were combined and sorted, and then they were split by chromosome.

```
$ cat <3cov.plus.bed> <3cov.minus.bed> | sort -k 1,1 -k 2,2n \n> output.bed
```

A custom R script was used to run cleanUpdTSeq to identify polyA sites. Genomic loci with a high probability of being a true polyadenylation site (p-value < 0.001) were selected for further analysis or visualization on the rat genome browser.

```
$ Rscript ./cleanUpdtseq.R $FILE $NAME.nbp rat
```
Cytoplasmic polyA+ RNAseq and ribosome profiling genome alignment

The adapter was trimmed from fastq files, and only reads longer than 24 nt were kept. Reads were filtered for RNA, tRNA, repeat elements, and mitochondrial genome following the steps described in the PASseq methods section. Reads were aligned to the genome with TopHat using RefSeq annotation (RGSC 6.0/rn6, Jul. 2014, downloaded on July 7, 2016) as a reference. The alignment is reported in bam file format.

```
$ tophat/2.0.14/tophat -p 4 --library-type fr-secondstrand \
   --b2-sensitive -g 10 --keep-tmp -G <genes.gtf> \
   -o <output_directory> <genome_index_base> <filtered_reads.fastq>
```

Differential expression analysis

To quantify annotated transcript abundance, we used Kallisto version 0.44.0 (Bray et al., 2016) with the following parameters:

```
$ kallisto quant -i genes.idx -o ./kallisto_output \
   -b 100 --rf-stranded <read1.fastq.gz> <read2.fastq.gz>
```

Reference sequences (fasta format) of protein coding and noncoding RNAs were downloaded from ensembl.org (Zerbino et al., 2018) in January, 2018. Transcripts from the mitochondrial genome were omitted from this analysis. The sequence of nuclear noncoding RNA, Xist, was imported from RefSeq (NR_132635.1) (O’Leary et al., 2016) because it was not annotated in the rat genome reference data downloaded on July 24, 2015 (Ensembl release 81, Rnor_6.0).

To identify RNAs enriched in projections, we compared transcript abundances in projections to whole cells using Sleuth version 0.30.0 (Pimentel et al., 2017). The code used to run sleuth provided is SuppCode_sleuth_RNA_diffexp.Rmd and the output is provided as Table S2 (TableS2_sleuth_RNA_diffexp_WC_vs_P.tsv).

Gene ontology analysis

To identify gene families enriched in projections or whole cells, we used GeneCodis http://genecodis.cnb.csic.es/ (accessed on November 5, 2018) (Carmona-Saez et al., 2007) with default parameters, focusing on the cellular component for gene ontology classification. RNAs enriched in projections (n = 1,440) or whole cells (n = 1,297) were selected using a q-value cutoff of < 0.01 and log_{2} mean read counts across all libraries > 1 and compared against a background comprising all RNAs that had log_{2} mean read counts > 1 (n = 19,461).

Intron quantification

To define intron regions, we first extracted the genomic coordinates of all annotated exons in any isoform of a gene as a .gff file using dексseq_prepare_annotation.py (provided as supplementary code) from DEXSeq (Anders et al., 2012). Intron regions were defined as the non-exon regions of each gene. Intron region coordinates were derived from exons using a program written by Alejandro Reyes (ORCID: 0000-0001-8717-66) copied from http://seqanswers.com/forums/showpost.php?p=137918&postcount=4.

To count the number of reads aligning to intron regions, exon-intron (EI) boundaries, intron-exon (IE) boundaries, and exon-exon (EE) junctions, we used BEDTools version 2.26.0 (Quinlan and Hall, 2010). Reads were aligned to the genome using TopHat, a splice-sensitive alignment algorithm, as described above. Only reads with high quality alignments were counted on regions of interest.

The intron reference file was first converted from .gff to .bed file format using BEDOPS (Neph et al., 2012). EI and IE regions span 50 nt, 25 nt from the exon and 25 nt from the intron. The EE region coordinates include the intron region and 50 nt of each flanking exon. To count reads crossing the EE junction, only those read alignments that started and ended at the EE coordinates were considered. Since EE regions are longer than 100 nt because they include the intron, the only
way a 100 nt long read would start and end at the EE coordinates is if the alignment splits. In case of ribosome profiling and polyA+ RNAseq data, where the minimum read length is 24 nt, the EE regions included the intron region and 12 nt of each flanking exon. The commands used to align the reads to the rat genome, define regions of interest, and count reads on them are shown in Figure 1–Figure Supplement 3 and Figure 3–Figure Supplement 1.

Circularly permuted read alignments

To align reads allowing circular permutation, we used find_circ.py version 1.2 (Memczak et al., 2013) following instructions provided on the github repository https://github.com/marvin-jens/find_circ. Reads that failed to align to the rat genome using TopHat (see above) were used to search for circularly permuted alignments. The following commands were executed:

```bash
$ unmapped2anchors.py <unmappedreads.bam> > unmapped_anchors.qfa

$ bowtie2 -p 8 --score-min=C,-15,0 --reorder --mm -q \n-x <Bowtie2Index/genome> -U <unmapped_anchors.qfa> > \nfind_circ.py -G <genome.fa> -B <anchors.bam> --noncanonical \n-R <spliced_reads.txt> -s <stats.txt> > circs.txt
```

Sequence conservation

To assess sequence conservation of regions of interest (free intron regions enriched in and depleted from projections, retained introns in projections, and all annotated exons), we used the PhyloP sequence conservation scores from 20 aligned vertebrate genomes (Pollard et al., 2010) downloaded from the UCSC database (Kent et al., 2002). Each nucleotide has a PhyloP conservation score. For every region of interest, we calculated the average PhyloP score across 50 nt windows with a 10 nt interval (using bigWigAverageOverBed version 2 from Kent et al. (2010)) and took the median score across all windows in the region.

```bash
$ bedtools makewindows -w 50 -s 10 -b <region.bed> \n-i srcwinnum > windows.bed

$ bigWigAverageOverBed -bedOut=avg_phyloscores.bed \n-rn6.phyloP20way.bw <windows.bed> <avg_phyloscores.tab>
```

Data and code availability

All raw data (fastq format) and corresponding coverage files (bigwig format) are available at NCBI GEO under accession number GSE129924, and a tarball of supplementary tables and code can be found on http://eddylab.org/publications.html#Saini19

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References


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,4UNG9#,,0IPER-,GUMY,,&AWCETT*7,9EOG3(,(OLT#%. 3UBCELLULAR0RO

919

LING2EVEALSDISTINCT

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ANDDEVELOPMENTALLY2EGULATED2EPERTOIREOFGROWTH#ONEM2.AS. *OURNALOF.EUROSCIENCE.201011;

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Figure 1–Figure supplement 1. (A) GFAP and Vimentin (VIM) immunopositive projections pass through the semipermeable membrane and are visible on the bottom surface by immunofluorescence imaging. (B) Percentage of total RNAseq reads from whole-cells and projections aligning to various genomic regions. A majority of reads from projection RNAseq data align to the mitochondrial genome. (C) Genome browser plot of read densities and sequence conservation for Srsf5, an mRNA with a known detained intron (highlighted in pink).
**Figure 1-Figure supplement 2.** Microcapillary electrophoresis of total RNA (Bioanalyzer RNA pico assay) from whole cells and projections of three biological replicates. Only RNAs > ~150 nt were sequenced for this study. In projections, the band at ~100 nt was striking. By sequencing RNAs within 20-150 nt size range, we found that they were mostly full length and fragmented tRNAs (unpublished data).
**Paired-end Illumina HiSeq sequencing**

**rRNA-depleted total RNAseq**
Whole-cells
5 biological replicates
*.fastq files (R1, R2)

$ tophat/2.1.1/tophat -p 6
--library-type fr-firststrand
--b2-sensitive
--mate-inner-dist 100 -i 30 -g 10
--max-coverage-intron 5000000 -G
<genes.gtf> -o <output_directory>
<genome_index_base>
<filtered_read1.fastq>
<filtered_read2.fastq>

**Ribosome profiling**
5 biological replicates
*.fastq files (R1, R2)

$ tophat/2.0.14/tophat
-p 4 --library-type fr-secondstrand
--b2-sensitive
--keep-tmp -G <genes.gtf>
-i <read1.fastq> -2 <read2.fastq>

**PolyA+ RNAseq**
3 biological replicates
*.fastq files (single read)

$ bowtie2-2.2.3/bowtie2
-p 2 -N 1 --no-unal
--un-conc <unaligned.fastq> -l <read1.fastq> -2 <read2.fastq>
--al-conc <aligned.fastq>
<alignments.sam>

**Single-read Illumina HiSeq sequencing**

**Ribosome profiling**
3 biological replicates
*.fastq files (single read)

$ bowtie2-2.2.3/bowtie2
-p 2 -N 1 --no-unal
--un-conc <unaligned.fastq> -l <read1.fastq> -2 <read2.fastq>
--al-conc <aligned.fastq>
<alignments.sam>

$ samtools view -bh -f 0x40 <alignment.q10.bam>
<alignment.q10.bed>

**PolysA+ RNAseq**
3 biological replicates
*.fastq files (single read)

$ samtools merge <output.bam> <file1.bam> <file2.bam> <file3.bam>

Converted alignments in bam format

$ bamToBed -split -i <alignment.q10.bam> <alignment.q10.bed>
<alignment.q10.bed>

$ samtools view -bh -f 0x40 <alignment.q10.bam>
<alignment.q10.R1.bam>
<alignment.q10.R1.bed>

$ bamToBed -split -i <alignment.q10.bam> <alignment.q10.bed>
<alignment.q10.bed>

$ samtools merge <output.bam> <file1.bam> <file2.bam> <file3.bam>

Merged alignments in bam format

$ bamCoverage --binSize 10 --p 2 --b <output.bw>

Bigwig files are available at GSE129924

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**Figure 1—Figure supplement 3.** Data analysis workflow for sequence alignments and RNA abundance quantitation. Files provided as supplementary data or available at GSE129924 are in red text.
Figure 2–Figure supplement 1. Correlation between biological replicates (TPM values) of whole cell and projection RNAseq datasets. The color gradient corresponds to the value of Spearman’s correlation coefficient shown in each cell of the heatmap.

Figure 2–Figure supplement 2. Gene ontology classes significantly represented by genes enriched in projections or whole cells. CC = Cellular Component sub-ontology classification.
### Counting the number of reads on regions of interest

<table>
<thead>
<tr>
<th>Library type</th>
<th>Intron regions</th>
<th>EI and IE boundary regions</th>
<th>EE junction regions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total RNA-seq</td>
<td>$\text{coverageBed -sorted -s -nobuf -a Intron_regions_bed -b alignment.q10_bed}$</td>
<td>$\text{coverageBed -sorted -s -f 0.60 -nobuf -a EIIE_bed -b alignment.q10_bed}$</td>
<td>$\text{coverageBed -s -f 1.0 -counts -sorted -a EE_100nt_bed -b alignment.q10_bam}$</td>
</tr>
<tr>
<td>PolyA+ RNA-seq</td>
<td>$\text{coverageBed -sorted -s -nobuf -a Intron_regions_bed -b alignment.q10_bed}$</td>
<td>$\text{coverageBed -sorted -s -f 0.60 -nobuf -a EIIE_bed -b alignment.q10_bed}$</td>
<td>$\text{coverageBed -s -f 1.0 -counts -sorted -a EE_100nt_bed -b alignment.q10_bam}$</td>
</tr>
<tr>
<td>Ribosome profiling</td>
<td>$\text{coverageBed -sorted -s -nobuf -a Intron_regions_bed -b alignment.q10_bed}$</td>
<td>$\text{coverageBed -sorted -s -f 0.60 -nobuf -a EIIE_bed -b alignment.q10_bed}$</td>
<td>$\text{coverageBed -s -f 1.0 -counts -sorted -a EE_24nt_bed -b alignment.q10_bam}$</td>
</tr>
<tr>
<td>PAS-seq</td>
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<td>$\text{coverageBed -sorted -s -f 0.60 -nobuf -a EIIE_bed -b alignment.q10_bed}$</td>
<td>$\text{coverageBed -s -f 1.0 -counts -sorted -a EE_24nt_bed -b alignment.q10_bam}$</td>
</tr>
</tbody>
</table>

Output provided in: TableS3_intron_master.tsv

**Figure 3–Figure supplement 1.** Data analysis workflow to count reads on intron regions. Files provided as supplementary data or available at GSE129924 are in red text.
Intron regions with read counts above threshold in:
- Whole cells only
- Projections and whole cells
- Neither
- Intron regions of genes containing CIRTs

Figure 3—Figure supplement 2. (A) In Buckley et al. (2011), 27 genes with cytoplasmic intron retaining sequences (CIRTs) were reported. All introns (n=436, black dots) of genes containing CIRTs (n=25) are overlaid on the scatter plot from Figure 3. Six intron regions pass the detection threshold in our projection RNAseq data and are labeled. (B) Genome browser plots for introns labeled in (A) with the relevant intron highlighted in pink. A retained intron in the 3' UTR of Stx1b was also observed by Yap et al. (2012). Gabbr1 has multiple annotated isoforms, of which only one is shown. RNAseq reads on Map2-i4 (not shown here) come from an unannotated exon.
(i) $\text{EI} \geq 1$

Calm2: Retained intron in 3' UTR

(ii) $\text{EI} < 1$ and $\text{IE} > 1$

Mts1l: Unannotated 3' splice site

Nell2: Unannotated transcription start site

(iii) $\text{EI} \geq 1$ and $\text{IE} < 1$

Aplp1: Unannotated 5' splice site

Cxadr: Unannotated polyA site

(iv) $\text{EI}$ and $\text{IE} < 1$

Abi2: Unannotated internal exon

Map4: Unannotated terminal exon

Ncapd2: snoRNA within intron

Gatc: Another gene within intron

Figure 4-Figure supplement 1. Genome browser views of introns labeled in Figure 4.
A  Free intron versus mRNA enrichment in projections

![Free intron versus mRNA enrichment in projections](image)

B  Retained intron versus mRNA enrichment in projections

![Retained intron versus mRNA enrichment in projections](image)

**Figure 4—Figure supplement 2.** Comparison of intron enrichment versus mRNA enrichment in projections.
Figure 4—Figure supplement 3. Subcellular location of regular, retained, detained, and free introns validated by smFISH.
**Kcnma1**: Intron reported to undergo splicing in dendrites by *Bell et al., 2010*

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**Figure 4-Figure supplement 4.** Genome browser view of *Kcnma1* shows only the spliced isoform in projections. The intron reported to undergo splicing in dendrites is highlighted in pink, and red bars indicate the genomic locus targeted by ISH probes in the study by *Bell et al. (2010).*