Using deep long-read RNAseq in Alzheimer's disease brain to assess clinical relevance of RNA isoform diversity.

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Abstract

Due to alternative splicing, human protein-coding genes average over eight RNA isoforms, resulting in nearly four distinct protein coding sequences per gene**.** Long-read RNAseq (IsoSeq) enables more accurate quantification of isoforms, shedding light on their specific roles. To assess the clinical relevance of measuring RNA isoform expression, we sequenced 12 aged human frontal cortices (6 Alzheimer's disease cases and 6 controls, 50% female) using one Oxford Nanopore PromethION flow cell per sample. Our study uncovered 53 new high-confidence RNA isoforms in clinically relevant genes, including several where the new isoform was one of the most highly expressed for that gene. Specific examples include *WDR4* (61%; microcephaly), *MYL3* (44%; hypertrophic cardiomyopathy), and *MTHFS* (25%; major depression, schizophrenia, bipolar disorder). Other notable genes with new high-confidence isoforms include *CPLX2* (10%; schizophrenia, epilepsy) and *MAOB* (9%; targeted for Parkinson's disease treatment). We identified 1,917 clinically relevant genes expressing multiple isoforms in human frontal cortex, where 1,018 had multiple isoforms with different protein coding sequences, demonstrating the need to better understand how individual isoforms from a "single" gene are involved in human health and disease. Exactly 98 of the 1,917 genes are implicated in brain-related diseases, including Alzheimer's disease genes such as *APP* (Aβ precursor protein; five), *MAPT* (tau protein; four), and *BIN1* (eight). We also found 99 differentially expressed RNA isoforms between Alzheimer's cases and controls, despite the genes themselves not exhibiting differential expression. Our findings highlight the significant knowledge gaps in RNA isoform diversity and their clinical relevance. Deep long-read RNA sequencing will be necessary going forward to fully comprehend the clinical relevance of individual isoforms for a "single" gene.

Key words: RNA isoforms, Clinical relevance, Long reads, Nanopore sequencing, Alzheimer's disease, Human brain.

Main

Due to alternative splicing, human protein-coding genes average over eight RNA isoforms, resulting in nearly four distinct protein coding sequences[1,2], yet because of practical limitations in standard short-read sequencing technologies, researchers have historically been forced to collapse all isoforms into a single gene expression measurement—a major oversimplification of the underlying biology. Given the large number of isoforms derived from a "single" gene, it is possible that many of them have distinct functions. Distinct functions for individual isoforms from a "single" gene have already been demonstrated for a handful of genes[3–5], but detailed analysis of individual isoforms has generally been limited to highly studied genes because studying individual isoforms via high-throughput methods has never been possible. While determining a given RNA isoform's function will ultimately require experimental validation, long-read sequencing enables researchers to begin understanding their role in human health and disease by quantifying expression for each isoform across different tissues and cell types. Knowing which tissues and cell types express each isoform is an important first step in understanding their function. Notably, isoforms can also play quite different, or even opposite roles within a given cell; a classic example includes two well-studied *BCL-X (BCL2L1)* transcripts with opposite functions, where *BCL-*X_L is anti-apoptotic while *BCL-*X_S is pro-apoptotic[5]. Changes in expression ratio between the *BCL-X* isoforms are implicated in cancer and are being studied as therapeutic targets[6], demonstrating the importance of understanding individual RNA isoform function rather than treating them as a "single" gene.

Traditionally, RNAseq studies relied on short-read sequencing approaches that excel at quantifying gene-level expression, but cannot accurately assemble and quantify individual RNA isoforms[7,8] (**Fig. 1a**), forcing researchers to collapse RNA isoforms into a single gene measurement. Long-read sequencing, however, can span entire RNA molecules at once, providing more accurate quantification of RNA isoforms, including *de novo* isoforms and genes (**Fig. 1a**). With this technology researchers can start uncovering individual RNA isoform function and their relationship to human diseases. Recently, two studies demonstrated that long-read sequencing can discover new RNA isoforms across several human tissues, including the brain[9,10]; while both studies revealed important biology, including reporting *de novo* RNA isoforms, they had limited sequencing coverage (averaging <6 million aligned reads per sample). Read depth is essential to accurately quantify individual RNA isoforms, given that a total of more than 250,000 annotated RNA isoforms have been reported, as of 2023[2]. Additionally, neither study focused on the clinical relevance of using long read RNAseq.

In this study, we demonstrate that accurate RNA isoform quantification through deep long-read sequencing can improve our understanding of individual RNA isoform function, and provide insights into how they may impact human health and disease. Specifically, in addition to discovering (1) *de novo* (i.e., unannotated) RNA isoforms in known clinically relevant genes, we also discovered (2) *de novo* spliced mitochondria-encoded RNA isoforms, (3) entirely new gene bodies in nuclear DNA, and (4) demonstrate the complexity of RNA isoform diversity for clinically relevant genes within a single tissue (human frontal cortex from Alzheimer's disease cases and controls). Lastly, (5) we provide a proof of concept for differential RNA isoform expression analysis, demonstrating its potential to reveal disease-relevant transcriptomic signatures unavailable at the gene level (i.e., collapsing all isoforms into a single expression measurement). To do so, we sequenced human frontal cortex brain tissue using Oxford Nanopore Technologies deep long-read sequencing (**Fig. 1b**). Summary data from this study are readily explorable through a public web application to visualize individual RNA isoform expression in aged human frontal cortex tissue (https://ebbertlab.com/brain_rna_isoform_seq.html).

Results

Methodological overview

We sequenced 12 post-mortem aged brain samples (Brodmann area 9/46) from six Alzheimer's disease cases and six cognitively unimpaired controls (50% female; **Fig. 1b**).All samples had postmortem intervals <5hrs and RNA Integrity Score (RIN) ≥9.0; demographics and summary sequencing statistics are shown in **Supplementary Table 1**. Each sample was sequenced using one PromethION flow cell. Sequencing yielded an average of 35.5 million aligned reads per sample after filtering out all reads with a mapping quality <10 (**Extended Data Fig. 1a**). We performed stringent RNA isoform quantification and discovery (including *de novo* gene bodies) using bambu[11]—a tool with emphasis on reducing false-positive RNA isoform discovery compared to other commonly used tools[11] (**Fig. 1b**). Isoform and gene body quantification and discovery were based on version 107 of Ensembl gene annotations (July 2022). For our 12 samples, bambu reported an average of 42.4% reads uniquely assigned to an RNA isoform and 17.5% reads spanning a full-length RNA isoform (**Extended Data Fig. 1c**). We only considered an isoform to be expressed above noise levels if its median counts per million (CPM) was above one (i.e., at least half of the samples had a CPM >1); this threshold is dependent on overall depth, as lower depths will require a higher, more stringent CPM threshold. Using this threshold, we observed 28,989 expressed RNA isoforms from 18,041 gene bodies in our samples (**Extended Data Fig. 2a,b,c**). We used publicly available mass spectrometry data from aged human dorsolateral frontal cortex tissue and human cell-lines to validate new RNA isoforms at the protein level. We included the cell-line data because, as of 2023, it is the largest human proteome with the highest sequence coverage ever reported across multiple digestion enzymes, giving us the best chance to validate our newly discovered RNA isoforms at the protein level. We carefully confirmed isoforms with uniquely matching peptide hits, resulting in a small number of successful validations.

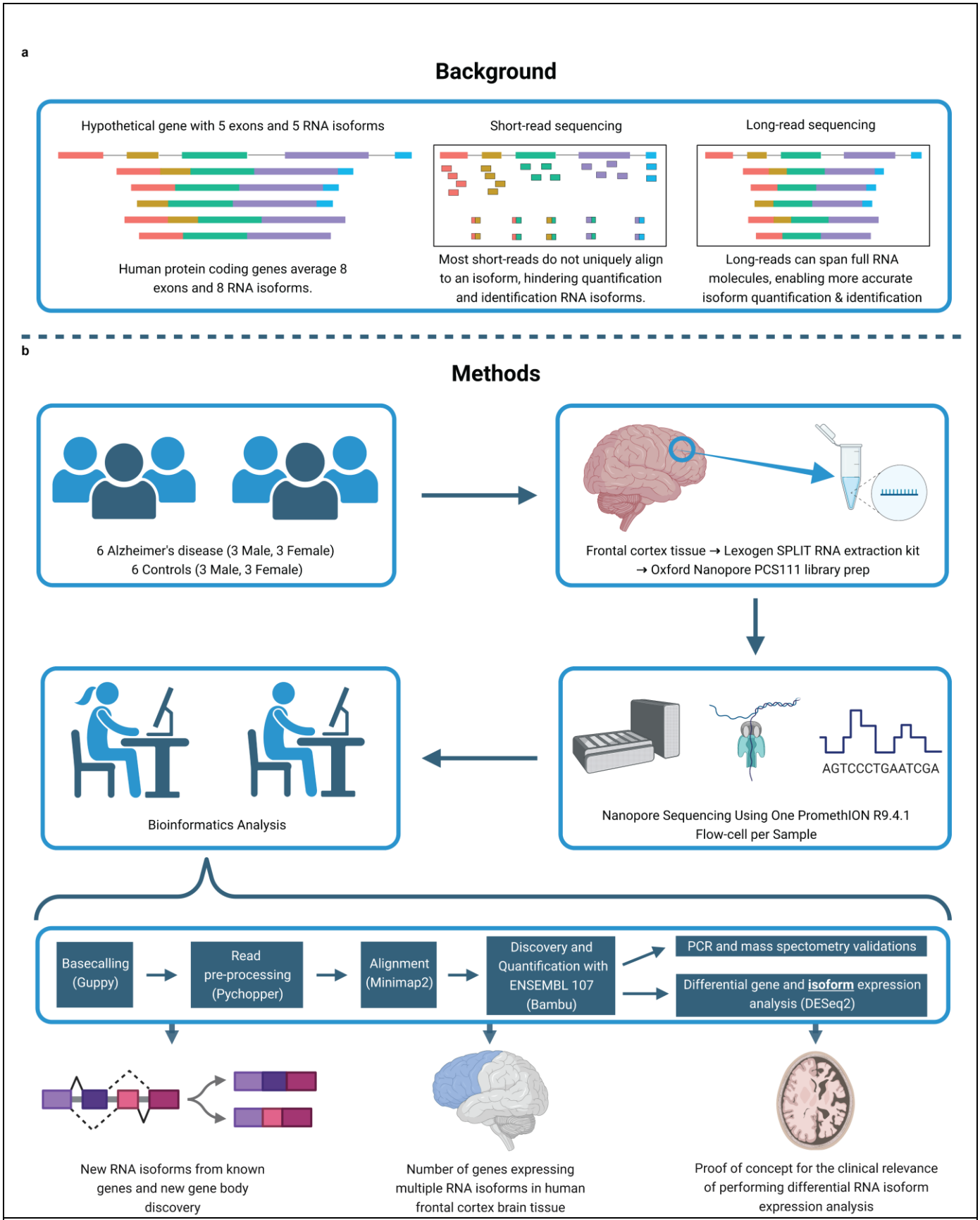

Fig. 1: Study design and rationale.
a, Background explaining the improvements long-read sequencing brings to the study of RNA isoforms. **b,** Details for experimental design, methods, and a summary of the topics explored in this article. Created with BioRender.com.

Discovery of new RNA isoforms from known gene bodies, including clinically relevant genes

Our first goal was to identify and quantify *de novo* RNA isoforms expressed in human frontal cortex. In total, bambu discovered 1,534 new transcripts from known nuclear gene bodies (i.e., annotated nuclear gene bodies). After excluding all isoforms with a median CPM ≤1, 428 remained that we consider high-confidence (**Fig. 2a,b**). Of these 428 isoforms, 303 were from protein-coding genes (**Fig. 2a**). We report significantly fewer new isoforms compared to Glinos et al.[9] (~70,000) and Leung et al.[10] (~12,000) because of (1) differences in the reference database, (2) discovery tool employed, and (3) stringency in what constitutes a new isoform. Specifically, Glinos et al. used gene annotations from 2016 when determining new isoforms. This is likely because they were trying to maintain consistency with previous GTEx releases, but approximately 50,000 new isoforms have already been annotated since then[2]. We also used a recently released tool (bambu) for isoform and gene discovery, which has shown to dramatically reduce false positives[11]. Finally, we set a stricter threshold for what we consider high-confidence isoforms, using a median CPM >1. Given the depth of our data, a CPM = 1 corresponds to an average of 24 observed copies (i.e., counts) per sample. Other tools and papers, however, report certain isoforms as new, even if the isoform is only observed twice, or if it is only slightly different from other isoforms (i.e., within sequencing error rate). Likely because of the depth of our sequencing, approximately 69.4% of our newly discovered isoforms are unique to our data, when compared to Ensembl v107, Glinos et al., and Leung et al. (**Supplementary Table 2,3**).

New high-confidence isoforms had a median of 761.5 nucleotides in length, ranging from 179 to 3089 nucleotides (**Fig. 2c**); the number of exons in new high-confidence isoforms ranged between 2 and 14, with most isoforms falling on the lower end of the distribution (**Fig. 2d**). Our data were enriched for new RNA isoforms containing all new exons and exon-exon boundaries (i.e., exon junctions; **Fig. 2e**). For context, bambu considers modified exons as new (e.g., significantly longer, or shorter), including lengthened UTR regions. The 428 new high-confidence isoforms contained 742 new exon-intron boundaries where 94.9% (356/370) and 100% (367/367) of the 5' and 3' splice site matched canonical splice site motifs, respectively, supporting their biological feasibility (**Fig. 2f**). We attempted to validate five new high-confidence isoforms through PCR and successfully validated three of them (**Fig. 2g, Supplementary Fig. 1-3**). We also validated 11 new isoforms at the protein level using mass spectrometry data from the same brain region and human cell-lines (**Fig. 2h,i**).

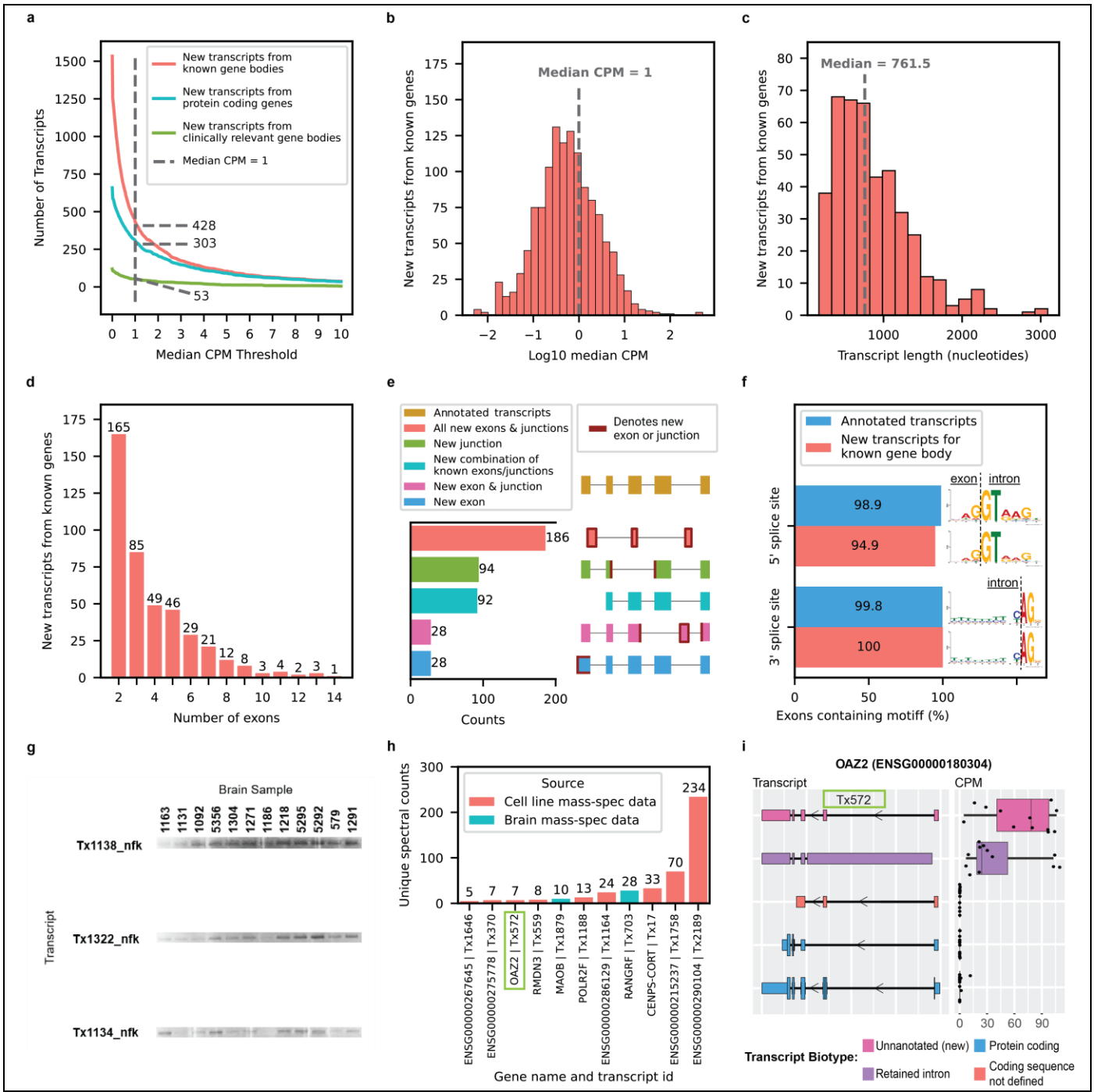

Fig. 2: New high-confidence RNA isoforms and new spliced mitochondrial RNA isoforms expressed in human frontal cortex.

Figures **a-f** refer to new transcripts from annotated gene bodies. **a,** Number of newly discovered transcripts across median CPM threshold. Cutoff shown as the dotted line set at median CPM = 1. **b,** Distribution of $\log_{10}$ median CPM values for newly discovered transcripts from annotated gene bodies, dotted line shows cutoff point of median CPM = 1. Figures **c-f** only include data from transcripts above this expression cutoff. **c,** Histogram showing distribution of transcripts length for new transcripts from annotated gene bodies. **d,** Bar plot showing the distribution of the number of exons for newly discovered transcripts from annotated gene bodies. **e,** Bar plot showing the kinds of events that gave rise to new transcripts from annotated gene bodies. For context, bambu considers modified exons (e.g., significantly longer or shorter) as new exons, including lengthened UTR regions. **f,** Bar plot showing the prevalence of canonical splice site motifs for annotated exons from transcripts with median CPM > 1 versus new exons from new transcripts in annotated gene bodies. **g,** Gel electrophoresis validation using PCR amplification for a subset of new RNA isoforms from known genes. This is an aggregate figure showing bands for several different gels. Individual gel figures are available in **Supplementary Figures 1-3**. **h**, Protein level validation using publicly available mass spectrometry proteomics data. Y-axis shows number of spectral counts from uniquely matching peptides (unique spectral counts); new transcripts from known gene bodies were considered validated at the protein level if they had more than 5 unique spectral counts. **i**, RNA isoform structure and CPM expression for isoforms from *OAZ2* (cellular growth/proliferation). The new isoform Tx572 was most expressed and validated at the protein level (highlighted with the green box).

Clinically relevant genes. Because identifying and quantifying all individual isoforms for a given gene is a critical first step to understanding all possible functions for a "single" gene, we wanted to assess how many new high-confidence isoforms originate from clinically relevant genes. For consistency, we used the list of clinically relevant genes defined in Wagner et. al[12], and we then further annotated with genes relevant to brain-related diseases[13–22]. Identifying and quantifying all isoforms is especially important for known clinically relevant genes because, for example, when clinicians interpret the consequence of a genetic mutation, it is interpreted in the context of a single isoform of the parent gene body. That isoform may not even be expressed in the relevant tissue or cell type, however. Having a firm understanding of which specific tissues and cell types express each isoform for a given gene body is expressed in will be important to fully understanding its function or relevance to human health and disease.

Of the 428 new high-confidence isoforms, 53 originated from 49 clinically relevant genes and we quantified the proportion of total expression for the gene that came from the new isoform(s) (**Fig. 3a**). The genes where the largest percentage of reads came from a newly discovered isoform include *SLC26A1* (86%; kidney stones[23] and musculoskeletal health[24]), *CAMKMT* (61%; hypotonia-cystinuria syndrome, neonatal seizures, severe developmental delay, etc.[25]), *WDR4* (61%; microcephaly[26] and Galloway-Mowat syndrome-6[27]), *MYL3* (44%; hypertrophic cardiomyopathy[28]), and *MTHFS* (25%; major depression, schizophrenia, and bipolar disorder[29]). Other notable genes with new high-confidence isoforms include *CPLX2* (10%; schizophrenia, epilepsy, and synaptic vesicle pathways[30]) and *MAOB* (9%; currently targeted for Parkinson's disease treatment[31]; **Fig. 3c**); the new *MAOB* isoform includes a retained intron. We also found an unannotated RNA isoform for *TREM2* (16%; **Fig. 3b**), one of the top Alzheimer's disease risk genes[32], that skips exon two. This isoform was reported as new in our data because it remains unannotated by Ensembl as of June 2023[2], but it has previously been reported by two groups[33,34]. Notably, the articles identifying this new *TREM2* isoform reported a relative abundance around 10%, corroborating our long-read sequencing results[33,34]. The new isoform for *POLB*—a gene implicated in base-excision repair for nuclear and mitochondrial genomes[35,36]—skipped exon two, accounting for 28% of the gene's expression (**Fig. 3d**). We also discovered new isoforms in *SMN1* and *SMN2* (spinal muscular atrophy); these isoforms do not meet our high-confidence threshold, but we report them for interest because of the genes' direct role in neurodegenerative disease (**Extended Data Fig. 5,6**). The clinically relevant genes where we identified multiple new isoforms were *PSENEN* (3; part of the gamma-secretase protein complex), *CPLX2* (2; part of synaptic vesicles and associated with Schizophrenia), and *DGUOK* (2; associated with mitochondrial DNA depletion syndrome-3, including liver and brain effects).

Spliced mitochondrially encoded isoforms. We identified a new set of spliced mitochondrially encoded isoforms containing two exons (**Fig 3e**), a highly unexpected result given that annotated mitochondrial transcripts only contain one exon. New mitochondrial isoforms were filtered using a count threshold based on full-length reads rather than a median CPM threshold due to technical difficulties in quantification arising from the polycistronic nature of mitochondrial transcription. Bambu identified a total of 34 new spliced mitochondrial isoforms, but after filtering using a strict median full-length count threshold of 40, only five high-confidence isoforms remained. Four of the new high-confidence isoforms span the *MT-RNR2* transcript. Not only does *MT-RNR2* encode the mitochondrial 16S ribosomal RNA, but it is also partially translated into an anti-apoptotic 24 amino acid peptide (humanin; HN) by inhibiting the Bax protein[37]. The fifth new high-confidence isoform spans the *MT-ND1* and *MT-ND2* genes, but on the opposite strand. Our results support previous important work by Herai et al. demonstrating splicing events in mitochondrial RNA[38]. For context, while expression for the new mitochondrial isoforms was low compared to known mitochondrial genes (**Fig. 3f**), their expression is relatively high when compared to all nuclear isoforms. Because we were more stringent in how we estimated expression for the mitochondrial isoforms (using only full-length counts), comparing their overall expression directly to nuclear isoforms is challenging, but all five new high-confidence mitochondrial isoforms are in the top 3,500 most expressed isoforms, including nuclear genome isoforms, when only considering full-length counts (i.e., reads matching all exon-exon boundaries from its assigned transcript). All five exons from new high-confidence mitochondrial isoforms contained the main nucleotides from the canonical 3' splice site motif (AG), while three out of five (60%) contained the main nucleotides from the canonical 5' splice site motif (GT) (**Fig. 3g**).

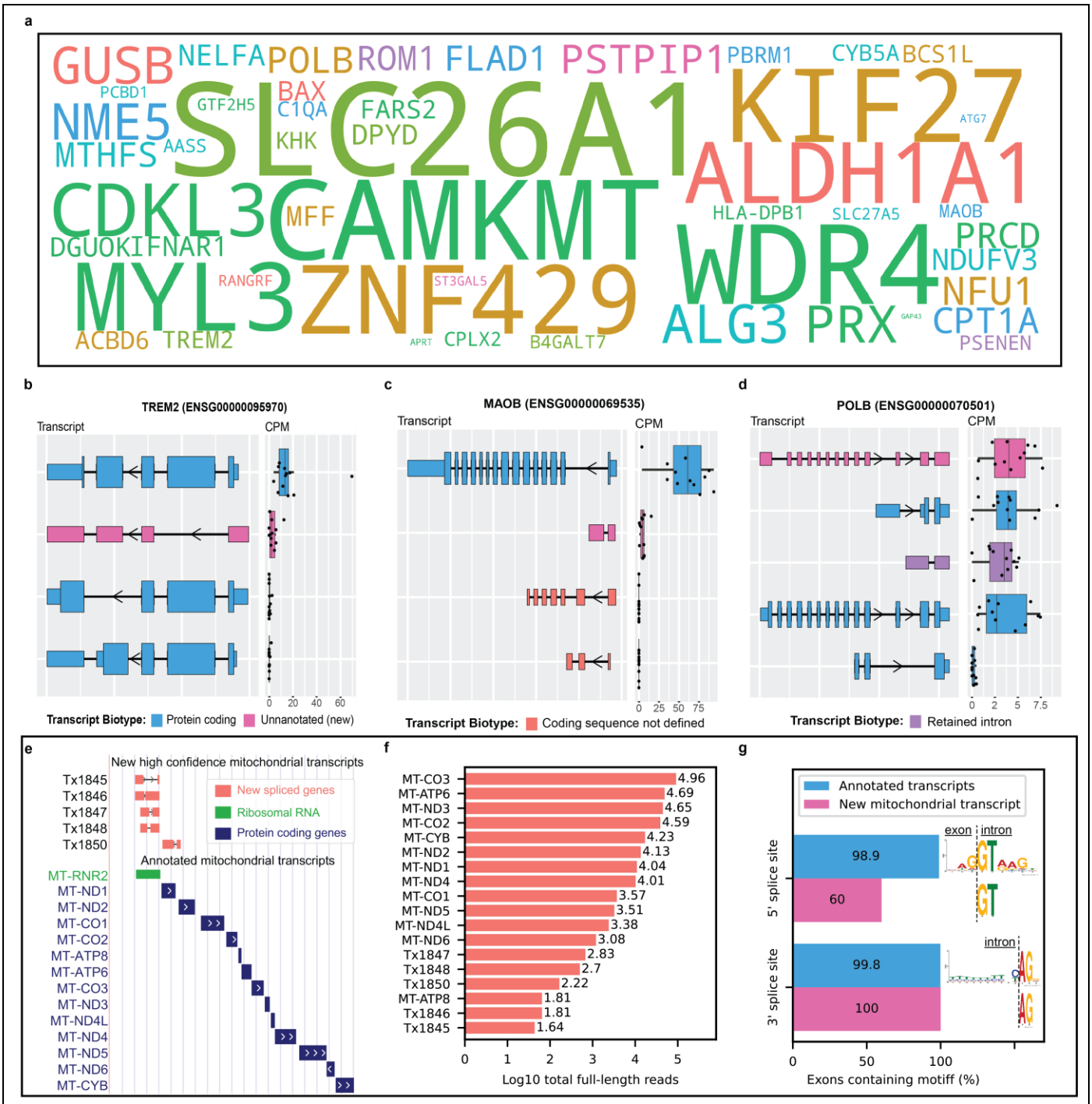

Fig. 3: Clinically relevant genes with new high-confidence RNA isoforms expressed in human frontal cortex.
a, Gene names for clinically relevant genes where we discovered a new RNA isoform that was not annotated in Ensembl version 107. Only included new RNA isoforms with a median CPM > 1. The size of gene name is proportional to relative abundance of the new RNA isoform. *SLC26A1*: 86% of the total gene expression comes from the new isoform. *TREM2*: 16% of the gene expression comes from the new isoform. **b-d,** RNA isoform structure and CPM expression for isoforms from *TREM2*, *MAOB*, and *POLB*. For *TREM2 and MAOB* all isoforms are shown (4 each). For *POLB* only the top 5 most highly expressed isoforms in human frontal cortex are shown. Figures **e-g** refer to new spliced mitochondrial transcripts, we only included new mitochondrial transcripts with median full-length counts > 40. **e**, Structure for new spliced mitochondrial transcripts in red/coral denoted by "Tx", MT-RNR2 ribosomal RNA represented in green (overlapping 4/ out of 5 spliced mitochondrial isoforms), and known protein coding transcripts in blue. **f**, Bar plot showing number of full-length counts (log10) for new spliced mitochondrial transcripts and known protein coding transcripts. **g**, Bar plot showing the prevalence of canonical splice site motifs for annotated exons from nuclear transcripts with median CPM > 1 versus new exon from spliced mitochondrial transcripts.

We attempted to validate three new high-confidence mitochondrially encoded isoforms through PCR and successfully validated two of them (**Supplementary Fig. 7,8**). It was not possible to design specific primers for the other two new high-confidence mitochondrial isoforms because of low sequence complexity or overlap with other lowly expressed (low-confidence) mitochondrial RNA isoforms found in our data; thus, we did not attempt to validate them. Mitochondria have been implicated in a range of human diseases, including seizure disorders[39], ataxias[40], neurodegeneration[41], and other age related diseases[42]. While function for the new isoforms is not clear, mitochondria are essential to human cell life (and most eukaryotes). Thus, if there are any isoforms that must be fully understood, it is isoforms from the mitochondria because these new spliced mitochondrial isoforms could have important biological roles or serve as biomarkers for mitochondrial function.

Discovery of transcripts from new gene bodies

RNA isoforms from new gene bodies refer to polyadenylated RNA species coming from regions of the genome where transcription was unexpected (i.e., unannotated). Bambu identified a total of 1,860 isoforms from 1,676 new gene bodies. After excluding isoforms with a median CPM ≤1, 267 high-confidence isoforms from 245 gene bodies remained (**Fig. 4a,b**). Glinos et al. did not specifically report on new gene bodies, but Leung et al. reported 54 new gene bodies in human cortex where 5 overlapped with our high-confidence isoforms from new genes. The new isoforms from new gene bodies had a median length of 1529 nucleotides, ranging between 109 and 5291 nucleotides (**Fig. 4c**). The number of exons ranged between 2 and 4, with 96.6% of isoforms only having two exons (**Fig. 4d**). Of the 267 new high-confidence isoforms from new gene bodies, 130 overlapped a known gene body on the opposite strand, 97 came from a completely new locus, 40 came from within a known gene body, but did not overlap a known exon (**Fig. 4e**). In new isoforms from new gene bodies, 82.5% (222/269) of exons contained the primary “GT” nucleotides from the canonical 5’ splice site motif, while 90.7% (244/269) contained the primary “AG” nucleotides from the canonical 3’ splice site motif (**Fig. 4f**). Interestingly, the new gene body, *BambuGene290099*, had three high-confidence RNA isoforms (**Fig. 4g**). We attempted to validate eight new high-confidence RNA isoforms from new gene bodies through PCR and successfully validated six of them (**Fig. 4h, Supplementary Fig. 2-6**). We validated three RNA isoforms from new gene bodies at the protein level using mass spectrometry data from the same brain region and human cell-lines (**Fig. 4i**).

We also added External RNA Controls Consortium (ERCC) spike-ins to our samples to benchmark RNA quantification. Mean ERCC CPM values had a strong correlation with their known concentrations, achieving a Spearman correlation coefficient of 0.98 (**Extended Data Fig. 3**). Interestingly, we identified a new low abundance RNA isoform (median CPM < 1) with two exons for ERCC RNA spike-ins (**Extended Data Fig. 4**). We were skeptical about this discovery since ERCCs only contain one exon, but we validated these results by PCR using two different batches of ERCC mix and several sample library preparations (**Supplementary Fig. 9,10**)

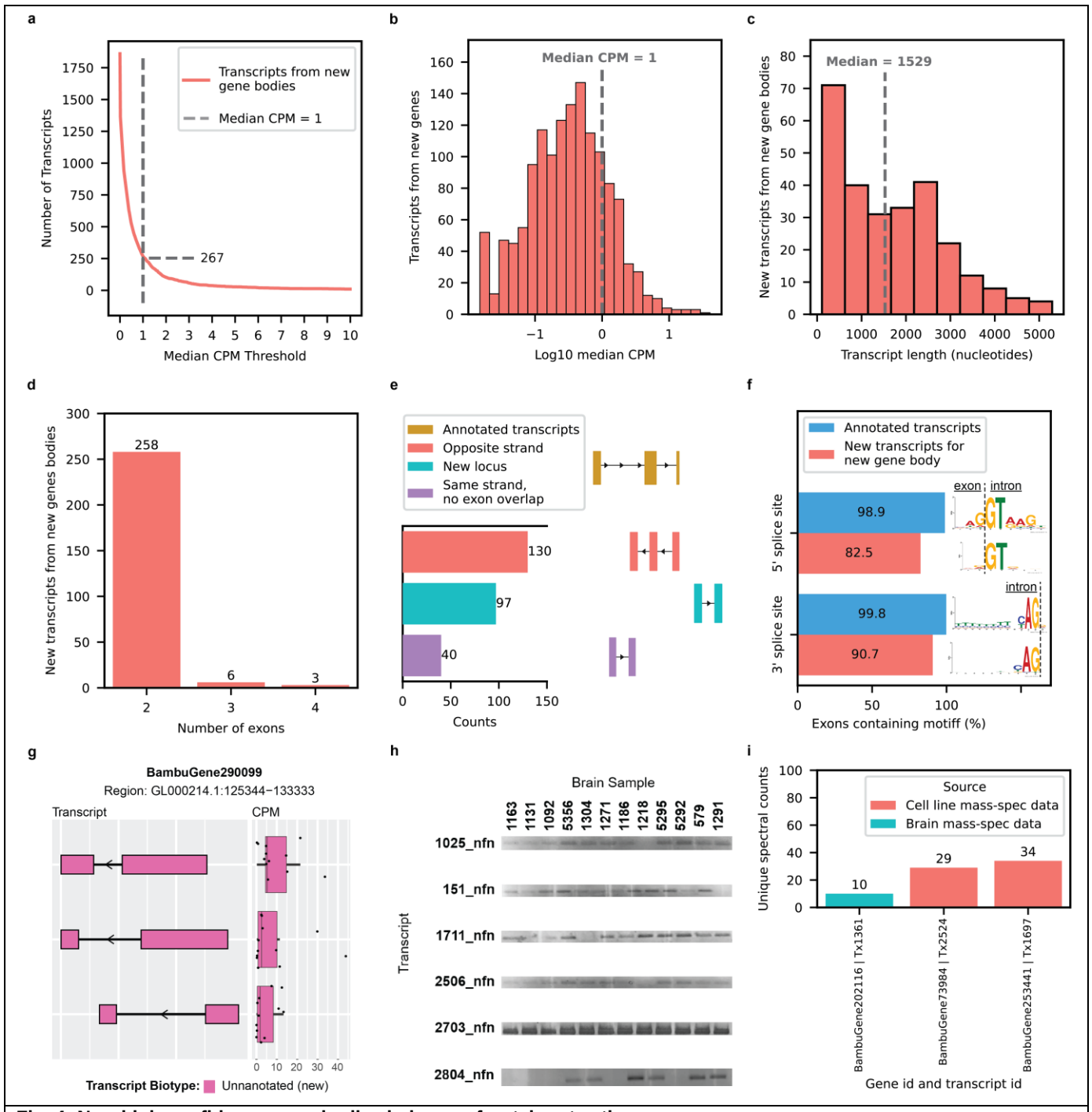

Fig. 4: New high-confidence gene bodies in human frontal cortex tissue.
a, Number of newly discovered transcripts from new gene bodies represented across median CPM threshold. Cutoff shown as the dotted line set at median CPM = 1. **b,** Distribution of $\log_{10}$ median CPM values for new transcripts from new gene bodies, dotted line shows cutoff point of median CPM = 1. Figures **c-g** only include data from transcripts above this expression cutoff. **c,** Histogram showing distribution of transcripts length for new transcripts from new gene bodies. **d,** Bar plot showing the distribution of the number of exons for new transcripts from new gene bodies. **e,** Bar plot showing the kinds of events that gave rise to new transcripts from new gene bodies. **f,** Bar plot showing the prevalence of canonical splice site motifs for annotated exons from transcripts with median CPM > 1 versus new exons from new gene bodies. **g,** RNA isoform structure and CPM expression for isoforms from new gene body (*BambuGene290099)*. **h,** Gel electrophoresis validation using PCR amplification for a subset of new isoforms from new genes. This is an aggregate figure showing bands for several different gels. Individual gel figures are available in **Supplementary Figures 2-6. i,** Protein level validation using publicly available mass spectrometry proteomics data. Y-axis shows number of spectral counts from uniquely matching peptides (unique spectral counts); new transcripts from new genes were considered validated at the protein level if they had more than 5 unique spectral counts.

Clinically relevant genes expressing multiple RNA isoforms in human frontal cortex tissue

As part of understanding the function of individual RNA isoforms, we need to understand the context of their expression, including tissue type. Quantifying the relative abundance of specific isoforms within individual tissues is a critical step to understanding their individual functions. Notably, we found 7,042 genes expressing two or more RNA isoforms with a median CPM >1, where 3,387 genes expressed two or more isoforms with clearly distinct protein sequences (**Fig. 5a,b**). Of the 5,035 clinically relevant genes included here[12] 1,917 expressed multiple isoforms and 1,018 expressed isoforms with different protein coding sequences (**Fig. 5c**), demonstrating the isoform diversity of clinically relevant genes in a single tissue and the importance of interpreting genetic variants in the proper context of tissue-specific isoforms. Isoforms with clearly distinct protein sequences are perhaps most likely to have different functions, but we still need to understand the effects of isoforms that are technically different only in noncoding regions (i.e., modified UTR regions can have large impacts on translation[43,44]).

Interestingly, 98 genes implicated in brain related diseases expressed multiple RNA isoforms in human frontal cortex, including Alzheimer's disease genes such as *APP* (Aβ precursor protein) with five, *MAPT* (tau protein) with four, and *BIN1* with eight (**Fig. 5d,e1-e4**). Notably, we only observed four *MAPT* isoforms with a median CPM > 1, where two were expressed at levels many times greater than the others, while substantial previous research suggests there are six tau proteins expressed in the central nervous system[45–47]. Researchers have been studying these and other top Alzheimer's disease genes for years, yet there is still a lot to learn about most of their isoforms and their clinical relevance. To truly understand how any of the top Alzheimer's disease genes are involved in disease, we need to understand exactly why brain tissue is actively expressing multiple isoforms of the "same" gene and what functions they serve. Similarly, several genes implicated in other neurogenerative diseases and neuropsychiatric disorders expressed multiple isoforms in human frontal cortex, including *SOD1* (amyotrophic lateral sclerosis, ALS; frontotemporal dementia, FTD; **Fig. 5f1**) with two isoforms expressed with a median CPM >1, *SNCA* (Parkinson's disease; **Fig. 5f1**) with four, *TARDBP* (TDP-43 protein; involved in several neurodegenerative diseases; **Fig. 5f1,f2**) with four, and *SHANK3* (autism spectrum disorder; **Fig. 5g1,g2**) with three.

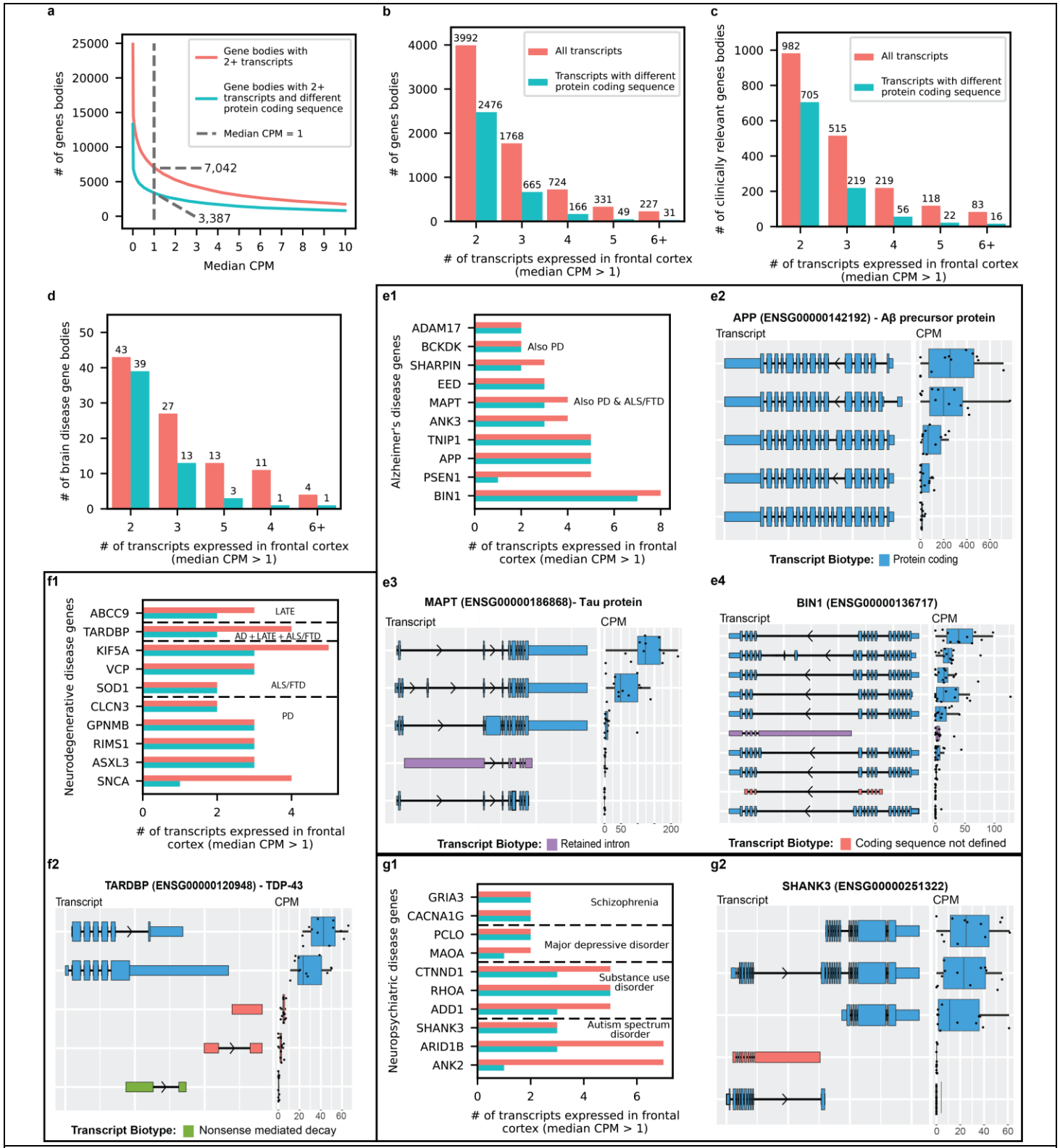

Fig. 5: Gene bodies expressing multiple transcripts in human frontal cortex tissue.
a, Gene bodies with multiple transcripts across median CPM threshold. Dotted line is at median CPM = 1, figures **b-g2** only include gene bodies with multiple transcripts at median CPM > 1. **b,** Gene bodies expressing multiple transcripts. **c,** Clinically relevant gene bodies expressing multiple transcripts. **d,** Brain disease relevant gene bodies expressing multiple transcripts. **e1,** Transcripts expressed in frontal cortex for a subset of genes implicated in Alzheimer's disease. AD: Alzheimer's disease. ALS/FTD: Amyotrophic lateral sclerosis and frontotemporal dementia. PD: Parkinson's disease. **e2,** *APP* transcript expression. **e3,** *MAPT* transcript expression. **e4,** *BIN1* transcript expression. **f1,** Same as **e1** but for a subset of genes implicated in other neurodegenerative diseases. LATE: Limbic-predominant age-related *TDP-43* encephalopathy. **f2,** *TARDBP* transcript expression. **g1,** Same as **e1** but for a subset of genes implicated in neuropsychiatric disorders. **g2,** *SHANK3* transcript expression.

Isoform analysis reveals disease-associated transcriptomic signatures unavailable at gene level

Perhaps the most compelling value in long-read isoform sequencing is the ability to perform differential *isoform* expression analyses. Through these analyses, we can begin to distinguish which isoforms are involved in specific pathways, cell types, tissue types, and ultimately human health and disease, such as Alzheimer's disease. Thus, as proof of principle, we performed differential gene and isoform expression analyses comparing six pathologically-confirmed Alzheimer's disease cases and six cognitively unimpaired controls. The data set is not large enough to draw firm disease-specific conclusions, but it does demonstrate the need for larger studies.

For gene level analysis we included all gene bodies with a median CPM > 1, for a total of 20,448. For RNA isoform level analysis, we included all transcripts with median CPM > 1 coming from genes with more than one transcript expressed at CPM > 1, for a total of 19,423 RNA isoforms from 7,042 genes. To be conservative, the threshold for differential expression was set at a relatively strict |log2foldchange| > 1 and FDR corrected p-value (q-value) < 0.05. We found 176 differentially expressed genes and 105 differentially expressed RNA isoforms (**Fig. 6a,b; Supplementary Table 4,5**). Out of these 105 isoforms, 99 came from genes that were not differentially expressed when collapsing all isoforms into a single gene measurement (**Fig. 6a,b**). Strikingly, there were two differentially expressed isoforms from the same gene (*TNFSF12)*, with opposite trends. The *TNFSF12-219* isoform was upregulated in Alzheimer's disease cases while *TNFSF12-203* was upregulated in controls (**Fig. 6c,d,e**), however, the *TNFSF12* gene was not differentially expressed when collapsing all its transcripts into a single gene measurement (**Fig. 6c**). The *TNFSF12* gene is a pro-inflammatory cytokine that has been associated with both apoptosis and angiogenesis[48], suggesting that this "single" gene has multiple distinct functions; it is also part of the Tumor Necrosis Factor super family that has been implicated in Alzheimer's disease for driving inflammation[49]. These differentially expressed *TNFSF12* RNA isoforms are not thought to be protein coding, but understanding why cells actively transcribe different non-coding RNAs remains an important question in biology. NEDD9-202, NAPB-203, and S100A13-205 are examples of protein coding RNA isoforms that were differentially expressed even though the gene was not. While we cannot make any meaningful conclusions about whether specific genes or isoforms are involved in Alzheimer's disease (because of sample size), we clearly demonstrate the need to perform larger long-read RNA sequencing studies that perform differential *isoform* expression analysis across human cell types, tissues, and disease.

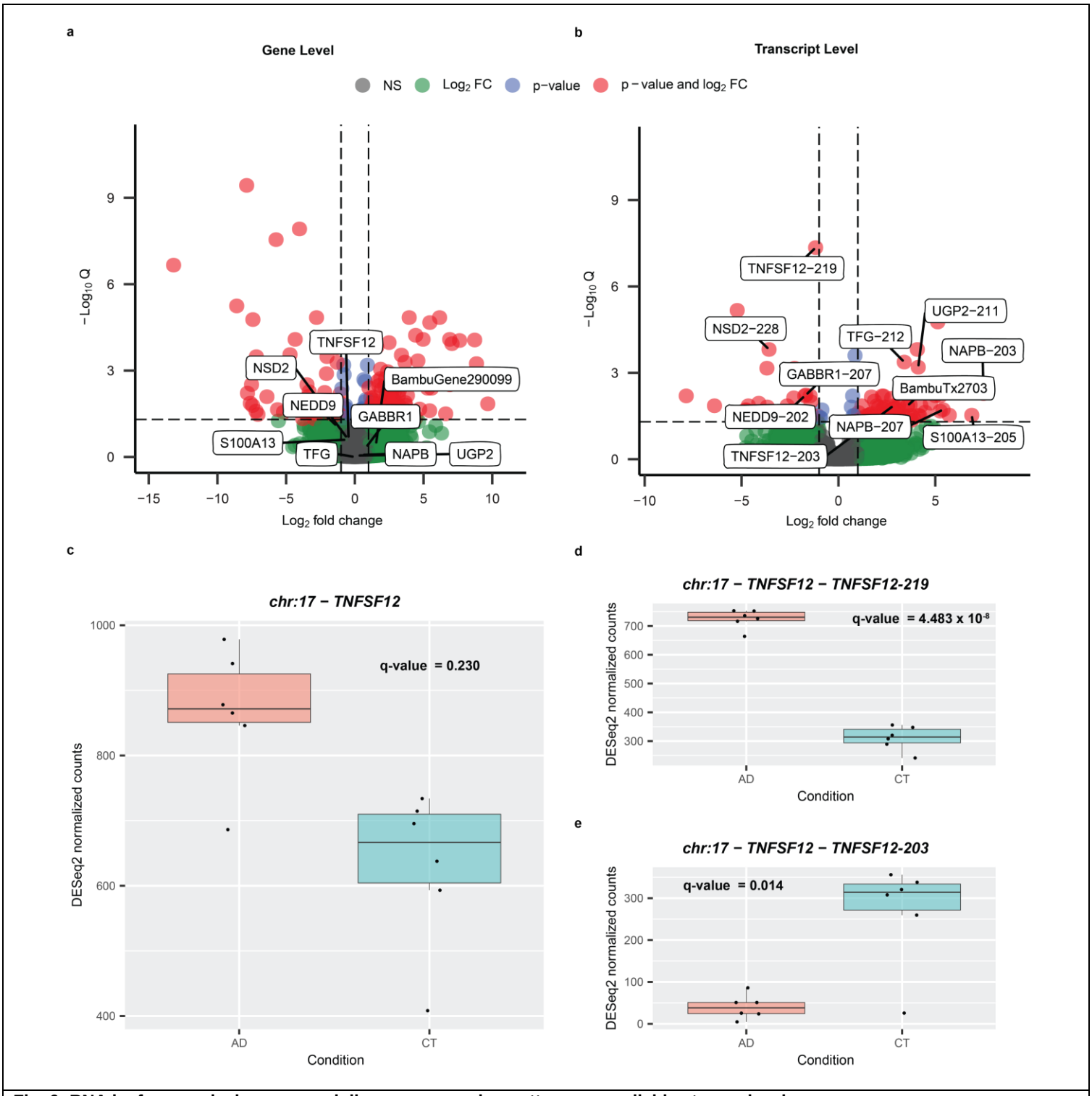

Fig. 6: RNA isoform analysis can reveal disease expression patterns unavailable at gene level.
a, Differential gene expression between Alzheimer's disease cases and cognitively unimpaired controls. The horizontal line is at FDR corrected p-value (q-value) = 0.05. Vertical lines are at Log_2 fold change = -1 and +1. The threshold for differential gene expression was set at q-value < 0.05 and |Log_2 fold change| > 1. Names displayed represent a subset of genes that are not differentially expressed but have at least one RNA isoform that is differentially expressed. **b,** Same as **a** but for differential RNA isoform expression analysis. NEDD9-202, NAPB-203, and S100A13-205 are examples of protein coding RNA isoforms that were differentially expressed even though the gene was not. **c,** Expression for *TNFSF12* between Alzheimer's disease cases and controls. The *TNFSF12* gene does not meet the differential expression threshold. **d,** *TNFSF12-219* transcript expression between cases and controls. *TNFSF12-219* meets the differential expression threshold and is upregulated in cases. **e,** Expression for the *TNFSF12-203* transcript between cases and controls. *TNFSF12-203* meets the differential expression threshold and is upregulated in controls. These differentially expressed *TNFSF12* RNA isoforms are not thought to be protein coding, but understanding why cells actively transcribe non-coding RNAs remains an important question in biology.

Web application for human frontal cortex RNA isoform expression

We used the data from this study to create a web application where other researchers can perform queries and see RNA isoform expression in human frontal cortex for any given gene. The web application can be accessed here: https://ebbertlab.com/brain_rna_isoform_seq.html.

Discussion

We identified hundreds of new human brain-expressed RNA isoforms and new gene bodies, including spliced isoforms in a critical mitochondrial gene (*MT-RNR2*), demonstrating that significant gaps remain in our understanding of RNA isoform diversity (**Fig. 2a,3e,4a**). More importantly, we began to explore RNA isoform function for every gene by quantifying individual RNA isoform expression levels in human frontal cortex. We found 7,042 genes expressing multiple RNA isoforms, with 1,917 being clinically relevant genes (i.e., implicated in human disease) (**Fig. 5a,b,c**). Some of these clinically relevant genes expressing multiple RNA isoforms in human frontal cortex are implicated in brain-related diseases, including Alzheimer's disease, Parkinson's disease, autism spectrum disorder, substance use disorder, and others (**Fig. 5d**). For example, the *APP* gene which encodes the beta-amyloid precursor protein—a defining hallmark of Alzheimer's disease[50]—expressed 5 different RNA isoforms encoding 5 different proteins (**Fig. 5e2**). The *SHANK3* gene—a major autism spectrum disorder risk gene[51,52]—expressed 4 RNA isoforms encoding 3 different proteins (**Fig. 5g2**). Together, these findings highlight the importance of measuring individual RNA isoform expression accurately so we can start to discern the roles of each isoform within human health and disease.

As proof of concept, we performed differential RNA isoform expression analysis to reveal expression patterns associated with disease that are unavailable when performing gene level analysis (**Fig. 6a,b**). We demonstrated that performing differential gene-level expression is important, but insufficient, and suggest that deep long-read RNAseq is necessary to understand the full complexity of transcriptional changes during disease. The gene *TNFSF12* is a key example for the importance of studying RNA isoform expression because the gene itself is not differentially expressed in our data, but in this proof-of-concept study the *TNFSF12-219* isoform is significantly upregulated in Alzheimer's disease cases while *TNFSF12-203* isoform is significantly upregulated in controls (**Fig. 6c,d,e**). While these isoforms are not thought to be protein coding, understanding why cells actively transcribe non-coding isoforms remains an important question in biology, and biology regularly reveals surprises that scientists do not expect. By characterizing and quantifying expression for all isoforms, we can better determine what functions they have, if any. Findings such as this can lead to new and more precise targets for disease treatment and diagnosis across a broad range of complex human diseases because, rather than targeting an entire gene, we can specifically target the isoform(s) that are either promoting cellular health or that are driving cellular dysfunction.

We also identified five new, high-confidence, spliced mitochondrially encoded RNA isoforms with two exons each. This is a surprising finding given that all annotated human mitochondrial transcripts only have one exon (**Fig. 2e,f**). We were skeptical of these spliced mitochondrially encoded isoforms, but validated two of them through PCR (**Supplementary Fig. 7,8**) and previous work by Herai et al. in human cell cultures corroborates our findings[38]. To our knowledge this is the first study identifying spliced mitochondrial RNA isoform expression directly in human tissue. Although their expression was low compared to known mitochondrial protein coding genes, these new isoforms could potentially have important biological roles or serve as biomarkers for mitochondrial function. Given the involvement of mitochondria in many age-related diseases[42], we believe it is important to determine exactly what function these spliced mitochondrial RNA isoforms play, if any. In other words, given the number of diseases associated with mitochondrial dysfunction, we cannot afford to overlook the potential importance of these new isoforms simply because we do not yet know what function they serve.

While long-read sequencing can span entire RNA molecules, most reads are not full-length RNA isoforms despite using template-switching reverse transcription. It is unclear exactly what causes RNAs to not be full-

length, but likely culprits include RNA degradation, mechanical shearing during library preparation and sequencing, and limitations in library preparation protocols. Even with RNA integrity scores > 9, our samples averaged 17.5% full-length reads and 42.4% reads uniquely assigned to an RNA isoform (**Extended Data Fig. 1c**). While long-reads present an improvement over short-read RNAseq, it remains challenging to accurately quantify RNA isoforms in genes with many large and similar isoforms; thus, while this work is a significant improvement over short-read sequencing, the data are not perfect and future improvements in both sequencing and bioinformatic quantification will continue to improve the accuracy of long-read RNA sequencing. The differential RNA isoform expression analysis performed in this study cannot be used to infer RNA expression patterns associated with Alzheimer's disease in the general population due to its small sample size. It only serves as a proof of concept for the value of measuring individual RNA isoform expression in disease tissue. Additionally, this study is based on "bulk" RNA sequencing, rather than single-cell sequencing; bulk sequencing is likely to obscure critical cell type-specific expression patterns that single-cell sequencing can elucidate, though the cost of single-cell sequencing combined with long-read sequencing is still a major hurdle in making a large study of this kind feasible.

Individual RNA isoform expression has been overlooked due to technical limitations of short-read sequencing. Now with long-reads we can more accurately quantify the complete set of RNA isoforms. More importantly, we can start to understand the role of individual RNA isoforms in the development and progression of diseases. We demonstrate a large proportion of clinically relevant genes express multiple RNA isoforms in human frontal cortex, with many encoding different protein coding sequences that could potentially perform different functions. As proof of principle, we demonstrate that differential RNA isoform analysis can reveal transcriptomic signatures in Alzheimer's disease that are not available at the gene level. While we cannot generalize these data to infer differential isoform expression across all Alzheimer's disease cases and controls due to a small sample size, we believe it is worth investigating in a larger cohort. In the long-term, we believe that long-read RNAseq will be the best tool to assess RNA expression patterns in complex human diseases and to identify new molecular targets for treatment and diagnosis.

Methods:

Sample collection, RNA extraction, and quality control

Frozen post-mortem human frontal cortex brain samples were collected at the University of Kentucky Alzheimer's Disease Research Center autopsy cohort[53], snap-frozen in liquid nitrogen at autopsy and stored at -80ºC. Postmortem interval (from death to autopsy) was <5hrs in all samples. Approximately 25mg of grey matter from the frontal cortex was chipped on dry ice into prechilled 1.5ml Low bind tubes (Eppendorf, catalogue number: 022431021), kept frozen throughout the process and stored at -80oC. RNA was extracted using the Lexogen SPLIT RNA extraction kit (Lexogen, catalog number: 008.48) using protocol version 008UG005V0320 (**Supplementary File 1**).

Briefly, ~25mg of tissue was removed from -80oC storage and kept on dry ice until processing began. 400 µL chilled isolation buffer (4ºC), (Lexogen SPLIT RNA kit) was added to each tube and the tissue homogenized using a plastic pestle (Kontes Pellet Pestle, VWR, catalogue number: KT749521-1500). Samples remained on ice to maintain RNA integrity while other samples were homogenized. Samples were then decanted into room-temperature phase lock gel tubes, 400 ul of chilled phenol (4ºC) added and the tube inverted five times by hand. 150 ul acidic buffer (AB, Lexogen) was added to each sample, the tube inverted five times by hand before 200ul of chloroform was added and inverted for 15 seconds. After a two-minute incubation at room temperature, samples were centrifuged for 2 minutes at 12,000g at 18-20oC and the upper phase (approx. 600ul) was decanted in a new 2ml tubes. Total RNA was precipitated by the addition of 1.75x the volume of isopropanol to sample and then loaded onto a silica column by centrifugation (12,000g, 18oC for 20s) with the flow through being discarded. The column was then washed twice with 500 ul of isopropanol and then three times with 500 ul wash buffer (Lexogen) with the column being centrifuged (12,000g, 18oC for 20s) and the flow through being discarded each time. The column was transferred to a new low bind tube, and the RNA eluted by the addition of 30 ul of elution buffer (incubated for one minute and then centrifuged at 12,000g, 18oC for 60s) and the eluted RNA immediately placed on ice to prevent degradation.

RNA quality was determined initially by nanodrop (A260/A280 and A260/A230 ratios) and then via Agilent Fragment Analyzer 5200 using the RNA (15nt) DNF-471 kit (Agilent). All samples achieved Nanodrop ratios of >1.8 and Fragment Analyzer RIN >9.0 prior to sequencing and indicated high quality RNA. (**Supplementary Fig. 11-22; Supplementary Table 1**).

RNA Spike-ins

ERCC RNA Spike-In Controls (Thermofisher, catalogue number: 4456740) were employed to establish a consistent baseline measurement of RNA, ensuring standardization within each experiment and across multiple experiments. ERCC controls were added to the RNA at the point of starting cDNA sample preparation at a final dilution of 1:1000.

Library preparation, sequencing, and base-calling

Isolated RNA was kept on ice until quality control testing was completed as described above. Long read cDNA library preparation commenced, utilizing the Oxford Nanopore Technologies PCR-amplified cDNA kit (Oxford Nanopore Technologies, catalogue number: SQK-PCS111). The protocol, though not available due to a legal embargo, was followed, with two notable modifications being that in the cDNA amplification PCR the expansion time was set to 6 minutes and the PCR was 14 amplification cycles. cDNA quality was determined using Agilent Fragment Analyzer 5200 and Genomic DNA (50kb) kit (Agilent DNF-467). See **Supplementary Figures 23-34** for cDNA traces. The libraries underwent sequencing on the PromethION platform with flow cell R9.4.1. Each sample was allocated one full PromethION flow cell. Sequencing was performed continuously for approximately 60 hours. The Fast5 files obtained were basecalled using the Guppy52 GPU basecaller version 6.3.9 with configuration dna_r9.4.1_450bps_hac_prom.cfg.

Read pre-processing, genomic alignment & quality control

Nanopore long-read sequencing reads were pre-processed using pychopper[54] version 2.7.2 with the PCS111 sequencing kit setting. Pychopper filters out any reads not containing primers on both ends and rescues fused reads containing primers in the middle. Pychopper then orients the reads to their genomic strand and trims the adapters and primers off the reads.

The pre-processed reads were then aligned to the GRCh38 human reference genome (without alternate contigs) with added ERCC sequences using minimap2[55] version 2.22-r1101 with parameters "-ax splice -uf". Full details and scripts are available on our GitHub (**Code Availability**). Aligned reads with a mapping quality (MAPQ) score < 10 were excluded using samtools[56] version 1.6. The resulting bam alignment files were sorted by genomic coordinate and indexed before downstream analysis. Quality control reports and statistics were generated using PycoQC[57] version 2.5.2. Information about mapping rate, read length, and other sequencing statistics can be found in **Supplementary Table 1.**

Transcript Discovery and Quantification:

Filtered aligned reads were utilized for transcript quantification and discovery using bambu[11] version 3.0.5. We ran bambu using Ensembl[2] version 107 Gene transfer format (GTF) annotation file with added annotations for the ERCC spike-in RNAs and the GRCh38 human reference genome sequence with added ERCC sequences. Sample BAM files were individually pre-processed with bambu, and the resulting 12 files were provided as input all at once to perform transcript discovery and quantification using bambu The Novel Discovery Rate (NDR) was determined based on the recommendation by the bambu machine learning model.. The model recommended a novel discovery rate threshold of 0.288. Bambu outputs three transcript level counts matrices, including total counts (all counts including reads that were partially assigned to multiple transcripts), unique counts (only counts from reads that were assigned to a single transcript), and full-length reads (only counts from reads containing all exon-exon boundaries from its respective transcript). Except where specified otherwise, expression values reported in this article come from the total counts matrix.

We used full-length reads for quantification in the mitochondria since the newly discovered spliced mitochondrial transcripts caused issues in quantification. Due to polycistronic mitochondrial transcription, many non-spliced reads were partially assigned to spliced mitochondrial transcripts, resulting in a gross overestimation of spliced mitochondrial transcript expression values. We were able to fix this issue by using full-length counts—only counting reads that match the exon-exon boundaries of newly discovered spliced mitochondrial transcripts.

We only included newly discovered (i.e., unannotated) transcripts with a median counts per millions (CPM) > 1 in downstream analysis (i.e., high-confidence new transcripts). New transcripts from mitochondrial genes were the exception, being filtered using a median full-length reads > 40 threshold. Due to the polycistronic nature of mitochondrial transcription, reads from overlapping transcripts were being erroneously assigned to new spliced mitochondrial transcripts, resulting in large misestimations of expression. To address this error, we only used full-length reads to filter out new spliced mitochondrial transcripts with low expression. We also used full-length counts to compare spliced mitochondrial transcript expression levels to expression from annotated protein coding mitochondrial transcripts.

Data from transcriptomic analysis can be visualized in the web application we created using R version 4.2.1 and Rshiny version 1.7.4 . Link to web application: https://ebbertlab.com/brain_rna_isoform_seq.html

Splice site motif analysis

We utilized the online meme suite tool[58] (https://meme-suite.org/meme/tools/meme) to create canonical 5' and 3' splice site motifs and estimate the percentage of exons containing these motifs. For known genes we only included exons from multi-exonic transcripts that were expressed with a median CPM > 1 in our samples. If

two exons shared a start site or an end site, one of them was excluded from the analysis. For new high-confidence transcripts, we filtered out any exon start or end sites contained in the Ensembl annotation. If two or more exons shared a start site or an end site, we only used one of those sites for downstream analyses. For the 5' splice site analysis we included the last three nucleotides from the exon and the first six nucleotides from the intron. For the 3' splice site analysis we included the last 10 nucleotides from the intron and the first three nucleotides from the exon. The coordinates for 5' and 3' splice site motifs were chosen based on previous studies[59,60]. The percentage of exons containing the canonical 5' splice site motif was calculated using the proportion of 5' splice site sequences containing GT as the two last nucleotides in the intron. The percentage of exons containing the canonical 3' splice site motif was calculated by taking the proportion of 3' splice site sequences containing AG as the first two nucleotides in the intron. Fasta files containing 5' splice site sequences from each category of transcript (1. known transcript from known gene body, 2. new transcript from known gene, 3. new transcript from new gene body, 4. new transcript from mitochondrial gene body) were individually submitted to the online meme suite tool to generate splice site motifs. The same process was repeated for 3' splice site sequences. Due to the small number of transcripts, it was not possible to generate reliable splice site motif memes for new transcripts from mitochondrial transcripts, we instead just used the 5' GT sequence and 3' AG sequence to represent them in **Fig. 2g**.

Comparison between annotations

Annotations from new high-confidence transcripts discovered in this study were compared to annotations from previous studies using gffcompare[61] version 0.11.2. Transcripts were considered overlapping when gffcompare found a complete match of the exon-exon boundaries (i.e., intron chain) between two transcripts. The annotation from Glinos et al.[9] was retrieved from: https://storage.googleapis.com/gtex_analysis_v9/long_read_data/flair_filter_transcripts.gtf.gz. The annotation from Leung et al.[10] was retrieved from: https://zenodo.org/record/7611814/preview/Cupcake_collapse.zip#tree_item12/HumanCTX.collapsed.gff.

Differential gene expression analysis

Although bambu outputs a gene level counts matrix, this matrix includes intronic reads. To create a gene level counts matrix without intronic read, we summed the transcript counts within each gene using a custom Python script (see code on GitHub; Python version 3.10.8). This gene level counts matrix without intronic reads was used for all gene-level analysis in this article. We only performed differential gene expression analysis on genes with a median CPM > 1 (20,448 genes included in the analysis). The counts matrix for genes with CPM > 1 was loaded into R version 4.2.2. We performed differential gene expression analysis with DESeq2[62] version 1.38.3 using default parameters. Differential gene expression analysis was performed between AD samples and cognitively unimpaired controls. We set the threshold for differential expression at |log2 fold change| > 1 and FDR corrected p-value (q-value) < 0.05.

Differential isoform expression analysis

For differential isoform expression analysis, we used the transcript counts matrix output by bambu. We only performed differential isoform expression analysis on transcripts with a median CPM > 1 coming from genes expressing two or more transcripts with CPM > 1 (19,423 transcripts from 7,042 genes included in the analysis). This filtered counts matrix was loaded into R version 4.2.2. We performed differential isoform expression analysis with DESeq2 version 1.38.3 using default parameters. Differential isoform expression analysis was performed using the same methods as the gene-level analysis, comparing AD samples and cognitively unimpaired controls, including the same significance thresholds (|log2 fold change| > 1 and FDR corrected p-value < 0.05).

Figures and tables

Figures and tables were generated using custom R (version 4.2.2) scripts and custom Python (version 3.10.8). We used the following R libraries: tidyverse (version 1.3.2), EnhancedVolcano (version 1.18.0), DESeq2 (version 1.38.3), and ggtranscript[63] (version 0.99.3). We used the following Python libraries: numpy (version 1.24.1), pandas (version 1.5.2), regex (version 2022.10.31), matplotlib (version 3.6.2), seaborn (version 0.12.2), matplotlib_venn (version 0.11.7), wordcloud (version 1.8.2.2), plotly (version 5.11.0), notebook (version 6.5.2). See **Code availability** for access to the custom scripts used to generate figures and tables.

PCR primer design

We used the extended annotations output by bambu to create a reference transcriptome for primer design. This extended annotation contained information for all transcripts contained in Ensembl version 107 with the addition of all newly discovered transcripts by bambu (without applying a median CPM filter). This annotation was converted into a transcriptome sequence fasta file using gffread (version 0.12.7) and the GRCh38 human reference genome. We used the online NCBI primer design tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) to design primers. We utilized default settings for the tool, however, we provided transcriptome described above as the custom database to check for primer pair specificity. We only moved forward with validation when we could generate a primer pair specific to a single new high-confidence transcript. We generated primer pairs and attempted to validate a subset of 11 new high-confidence transcripts—three new transcripts from known/annotated genes, five new transcripts from new gene bodies, three new spliced mitochondrial transcripts. Detailed information about the primers generated can be found in **Supplementary Table 6.**

PCR Validation

New isoform and gene validations were conducted using gel PCR. For this purpose, 2ug of RNA was transcribed into cDNA using the High-Capacity cDNA Reverse Transcription kit (AB Applied Biosystems, catalogue number: 4368814) following the published protocol. The resulting cDNA was quantified using a nanodrop, and its quality was assessed using the Agilent Fragment analyzer 5200 with the DNA (50kb) kit (Agilent, DNF-467). Next, 500ng of the cDNA was combined with primers specific to the newly identified isoforms and genes (**Supplementary Table 6**). The amplification was performed using Invitrogen Platinum II Taq Hot start DNA Polymerase (Invitrogen 14966-005) in the Applied Biosystem ProFlex PCR system. The specific primer sequences, annealing temperatures, and the number of PCR cycles are detailed in **Supplementary Table 6**. After the PCR amplification, the resulting products were analyzed on a 1% agarose TAE gel containing 0.5ug/ml ethidium bromide. The gel was run for 30 minutes at 125v, and the amplified DNA was visualized using a UV light source. Gels from PCR validation for each transcript can be found in **Supplementary Fig. 1-10.**

Proteomics analysis

We utilized publicly available tandem mass spectrometry data from round two of the Religious Orders Study and Rush Memory and Aging Project (ROSMAP) brain proteomics study, previously analyzed by Johnson et al.[64] and Higginbotham et al[65]. We also utilized publicly available deep tandem mass spectrometry data from six human cell lines, processed with six different proteases and three tandem mass spectrometry fragmentation methods, previously analyzed by Sinitcyn et al[66]. This cell line dataset represents the largest human proteome with the highest sequence coverage ever reported. We started the analysis by creating a protein database containing the predicted protein sequence from all three reading frames for the 700 new high-confidence RNA isoforms we discovered, totaling 2100 protein sequences. We translated each high-

confidence RNA isoform in three reading frames using pypGATK[67] version 0.0.23. We also included the protein sequences for known protein coding transcripts that came from genes represented in the 700 new high-confidence RNA isoforms and had a median CPM > 1 in our RNAseq data. We used this reference protein fasta file to process the brain and cell-line proteomics data separately using FragPipe[68–74] version 20.0—a Java-based graphical user interface that facilitates the analysis of mass spectrometry-based proteomics data by providing a suite of computational tools. Detailed parameters used for running FragPipe can be found on GitHub and Zenodo, see **Code Availability** and **Data Availability**.

Mass spectrometry suffers from a similar issue as short-read RNAseq, only being able to detect relatively short peptides that do not cover the entire length of most proteins. This makes it challenging to accurately detect RNA isoforms from the same gene. To avoid false-discoveries, we took measures to ensure that we would only consider an RNA isoform to be validated at the protein level if it had peptide hits that are unique to it (i.e., not contained in other known human proteins). We started by taking the FragPipe output and only keeping peptide hits that mapped to only one of the proteins in the database. We then ran the sequence from those peptides against the database we provided to FragPipe to confirm that they were truly unique. Surprisingly, a small percentage of peptide hits that FragPipe reported as unique were contained in two or more proteins in our database, these hits were excluded from downstream analysis. We then summed the number of unique peptide spectral counts for every protein coming from a new high-confidence RNA isoform. We filtered out any proteins with less than six spectral counts. We took the peptide hits for proteins that had more than 5 spectral counts and used online protein-protein NCBI blast tool (blastp)[75] to search it against the human RefSeq protein database. We used loose thresholds for our blast search to ensure that even short peptides matches would be reported. A detailed description of the blast search parameters can be found on Zenodo. Spectral counts coming from peptides that had a blast match with 100% query coverage and 100% identity to a known human protein were removed from downstream analysis. We took the remaining spectral counts after the blast search filter and summed them by protein id. Proteins from high-confidence RNA isoforms that had more than five spectral counts after blast search filter were considered validated at the protein level. This process was repeated to separately analyze the brain mass spectrometry data and the cell-line mass spectrometry data.

Reproducibility

Read pre-processing, alignment, filtering, transcriptome quantification and discovery, and quality control steps were implemented using a custom NextFlow pipeline. NextFlow enables scalable and reproducible scientific workflows using software containers[76]. Singularity containers were used for most of the analysis in this paper, except for website creation and proteomics analysis due to feasibility issues. Singularity containers enable the creation and employment of containers that package up pieces of software in a way that is portable and reproducible[77]. Instructions on how to access the singularity containers can be found in the GitHub repository for this project. When not forbidden by legal embargo, manufacturer protocols were included as supplementary material. Any changes to standard manufacturer protocols were detailed in **Methods**. All code used for analysis in this article is publicly available on GitHub, see **Code Availability.** All raw data, output from long-read RNAseq and proteomics pipelines, references, and annotations are publicly available, see **Data availability.** Long-read RNAseq results from this article can be easily visualized through this web application: https://ebbertlab.com/brain_rna_isoform_seq.html

Data availability

Raw long-read RNAseq data generated and utilized in this manuscript are publicly available here: https://www.synapse.org/#!Synapse:syn52047893.

Output from long-read RNAseq and proteomics pipelines, reference files, and annotations are publicly available here: https://doi.org/10.5281/zenodo.8180677

Long-read RNAseq results from this article can be easily visualized through this web application: https://ebbertlab.com/brain_rna_isoform_seq.html

Raw cell-line deep proteomics data used utilized in this article are publicly available here: https://proteomecentral.proteomexchange.org/cgi/GetDataset?ID=PXD024364

Raw brain proteomics data from round 2 of the ROSMAP TMT study are publicly available here: https://www.synapse.org/#!Synapse:syn17015098

Code availability

All code used in the manuscript is available at: https://github.com/UK-SBCoA-EbbertLab/brain_cDNA_discovery

Contributions

BAH, JB, and ME developed and designed the study and wrote the manuscript. BAH, MP, BW, KD, MW, EF, and AS performed all analyses. MP developed the RShiny app and KD embedded the RShiny app into ebbertlab.com. JB, KN, LG, GF, PD, SG, EG, RW, and SME helped generate sequencing and supporting data. NS, EF, and AS generated and advised on proteomics analyses. PN provided the invaluable brain samples and pathology. JF, MR, and JM provided important intellectual contributions.

Competing interests

The authors report no competing interests.

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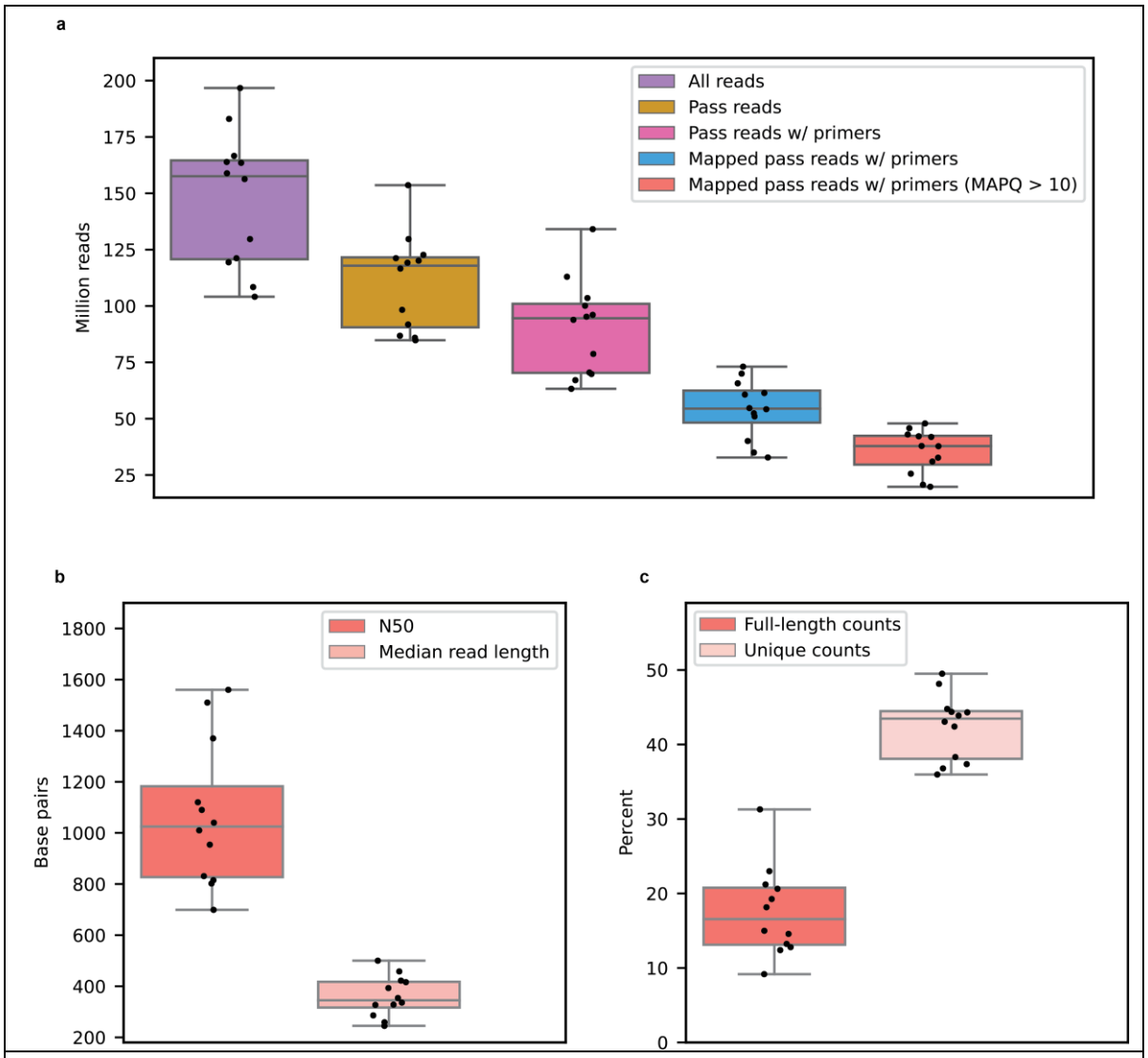
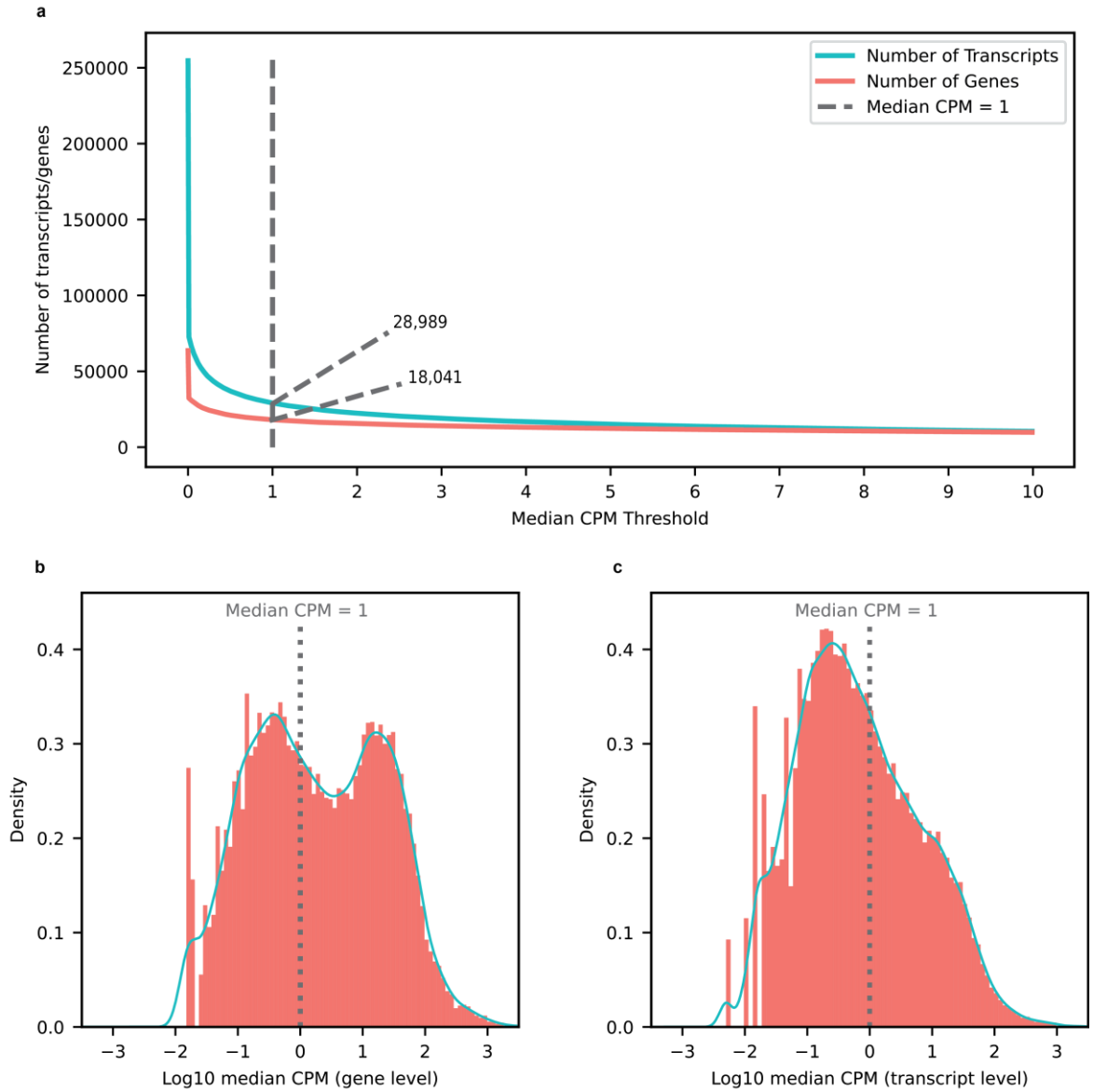
Extended Data:

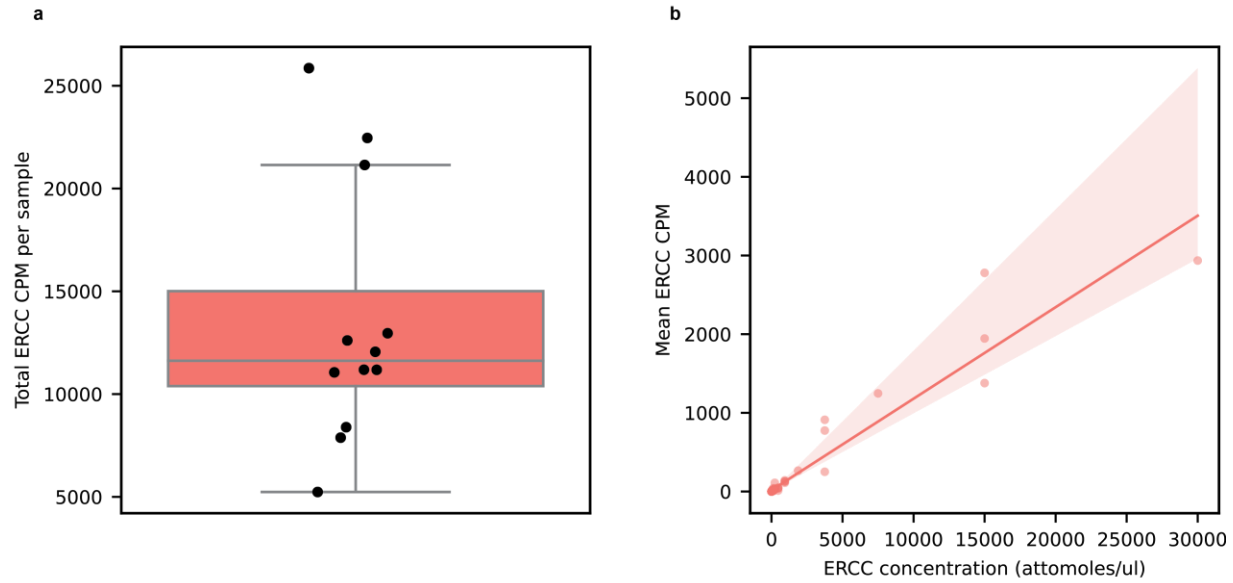


Extended Data Figure 1: Basic sequencing metrics.
a, Number of reads per sample after each step of the analysis. All downstream analysis were done with Mapped pass reads with both primers an MAPQ > 10. **b,** N50 and median read length for Mapped pass reads with both primers and MAPQ > 10. **c,** Percentage of reads that are full-length or unique as determined by bambu. Full-length counts = reads containing all exon-exon boundaries (i.e., intron chain) from its respective transcript. Unique counts = reads that were assigned to a single transcript.

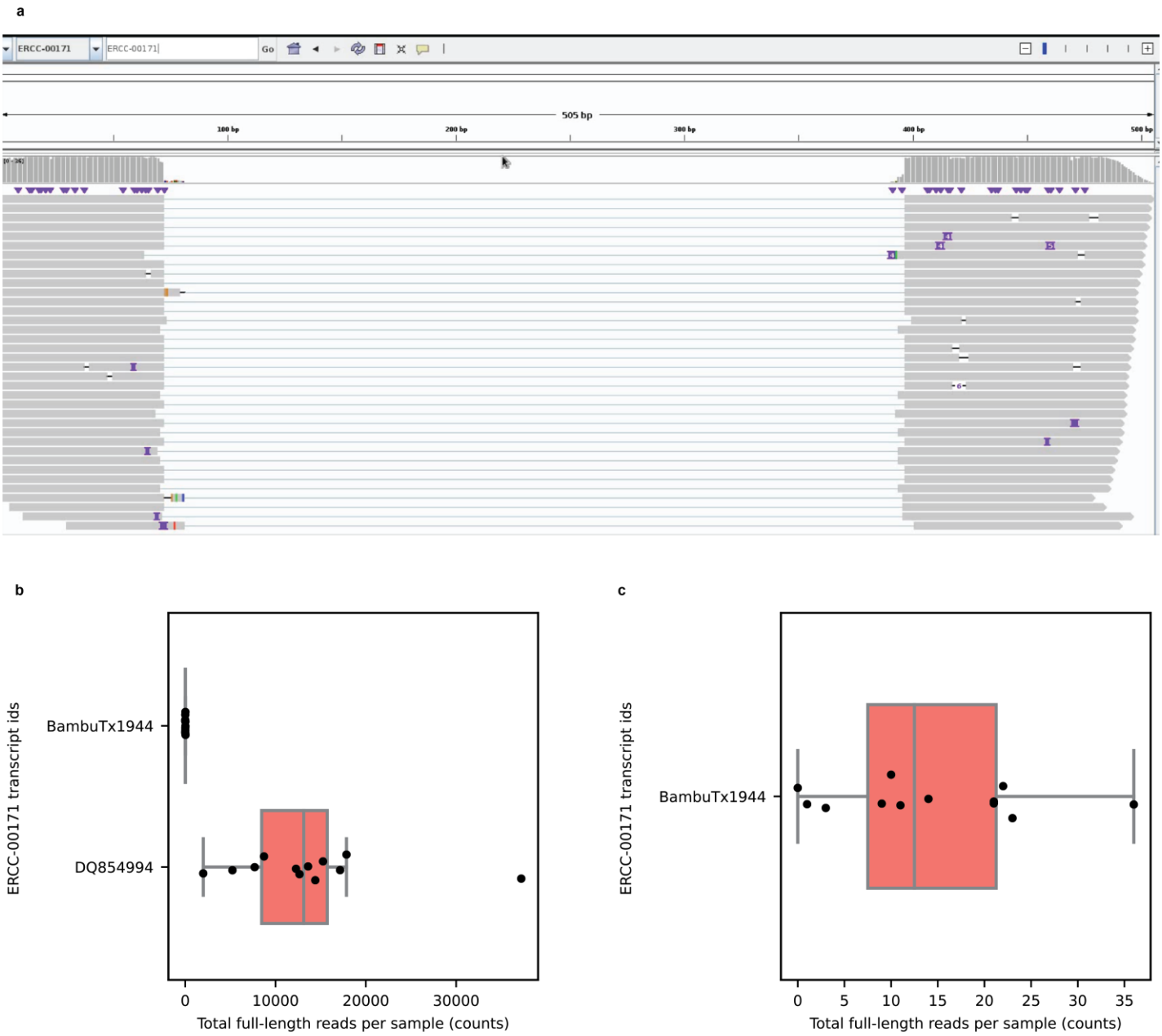


Extended Data Figure 2: Expression distribution and diversity for genes and transcripts.

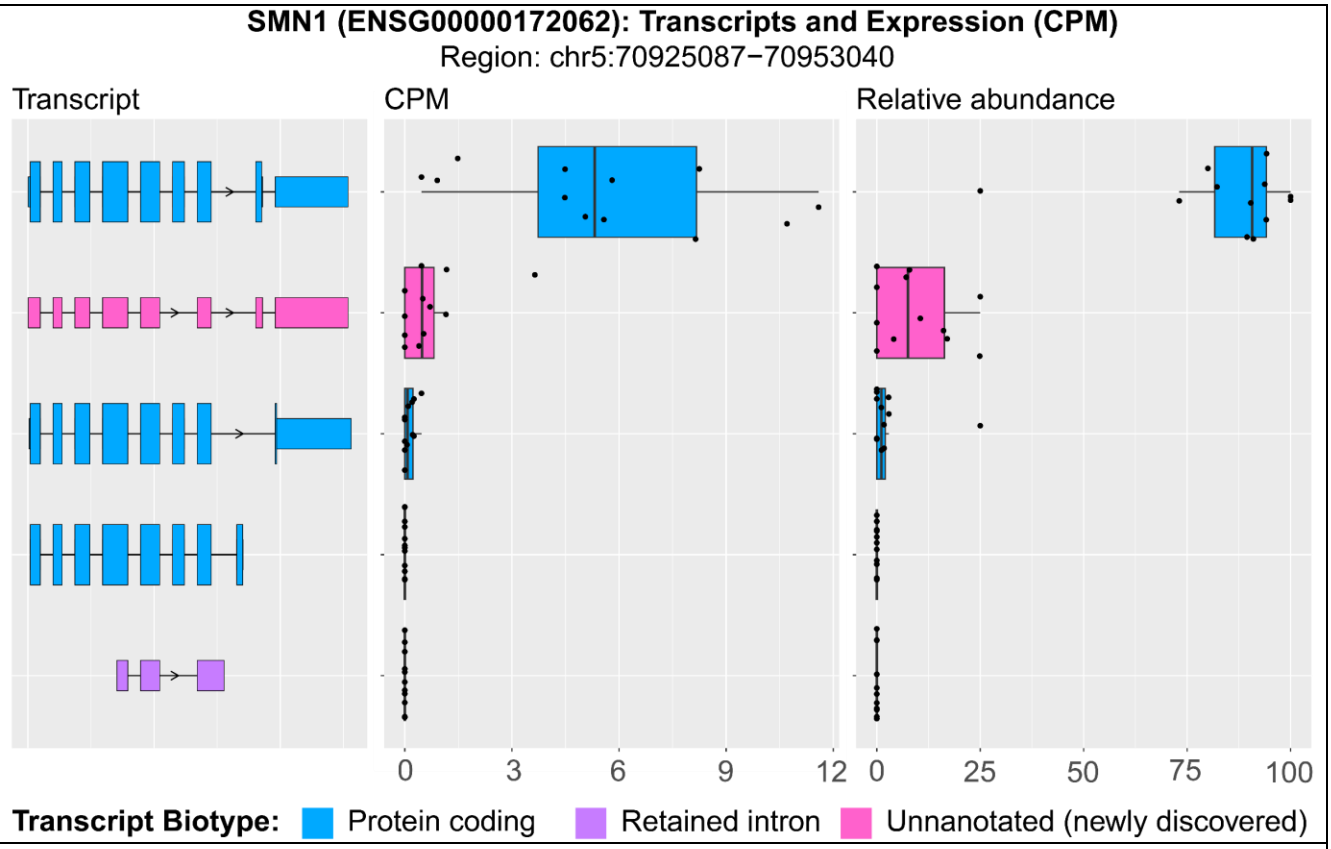
a, Number of genes and transcripts represented across median CPM threshold. Cutoff shown as the dotted line set at median CPM = 1. **b,** Distribution of $\log_{10}$ median CPM values for gene bodies, dotted line shows cutoff point of median CPM = 1. **c,** Distribution of $\log_{10}$ median CPM values for gene bodies, dotted line shows cutoff point of median CPM = 1.



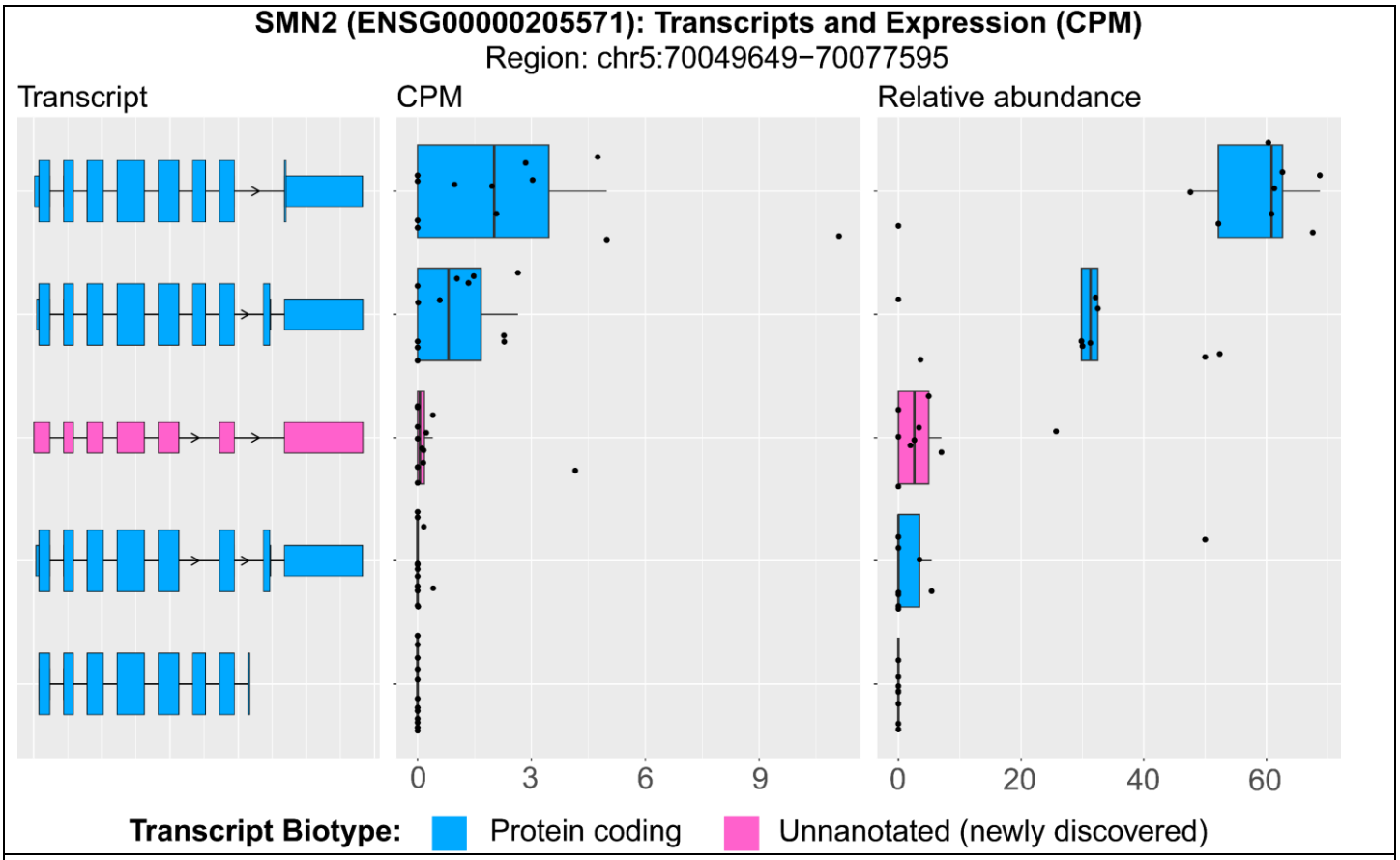
Extended Data Figure 3: Expression distribution and diversity for genes and transcripts. a, Sum of CPM for all ERCC control spike-in RNA transcripts for each sample. **b,** Correlation between ERCC transcript concentration and mean ERCC CPM for that same transcript in all 12 samples. Spearman correlation coefficient = 0.98.



Extended Data Figure 4: Expression distribution and diversity for genes and transcripts.
a, Interactive Genome Viewer (IGV) screenshot showing the “spliced” ERCC reads for one of our samples. **b,** Comparison between total number of full-length reads for the known ERCC transcript and the new spliced ERCC transcript that shares its nucleotide sequence. Each dot on the graph represents a sample. The new spliced ERCC transcript is present at very low concentration in comparison to the non-spliced ERCC transcript that shares its nucleotide sequence. **c,** Number of full-length reads for the new spliced ERCC transcript in each of the 12 samples.



Extended Data Figure 5: RNA isoform expression and structure for SMN1.
RNA isoform structure, CPM normalized expression, and relative abundance for SMN1 RNA isoforms. We only show the top 5 most highly expressed RNA isoforms for this gene. The new RNA isoform is shown in pink. This new isoform did not meet our high-confidence threshold (median CPM > 1), but we report it for interest because of the gene's direct role in neurodegenerative disease.



Extended Data Figure 6: RNA isoform expression and structure for SMN2.
RNA isoform structure, CPM normalized expression, and relative abundance for SMN2 RNA isoforms. We only show the top 5 most highly expressed RNA isoforms for this gene. The new RNA isoform is shown in pink. This new isoform did not meet our high-confidence threshold (median CPM > 1), but we report it for interest because of the gene's direct role in neurodegenerative disease.

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