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Problem solving protocol

ExonSkipAD provides the functional genomic landscape of exon skipping events in Alzheimer's disease

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Abstract

Exon skipping (ES), the most common alternative splicing event, has been reported to contribute to diverse human diseases due to the loss of functional domains/sites or frameshifting of the open reading frame (ORF) and noticed as therapeutic targets. Accumulating transcriptomic studies of aging brains show the splicing disruption is a widespread hallmark of neurodegenerative diseases such as Alzheimer's disease (AD). Here, we built ExonSkipAD, the ES annotation database aiming to provide a resource/reference for functional annotation of ES events in AD and identify therapeutic targets in exon units. We identified 16 414 genes that have ∼156 K, ∼ 69 K, ∼ 231 K ES events from the three representative AD cohorts of ROSMAP, MSBB and Mayo, respectively. For these ES events, we performed multiple functional annotations relating to ES mechanisms or downstream. Specifically, through the functional feature retention studies followed by the open reading frames (ORFs), we identified 275 important cellular regulators that might lose their cellular regulator roles due to exon skipping in AD. ExonSkipAD provides twelve categories of annotations: gene summary, gene structures and expression levels, exon skipping events with PSIs, ORF annotation, exon skipping events in the canonical protein sequence, 3′-UTR located exon skipping events lost miRNA-binding sites, SNversus in the skipped exons with a depth of coverage, AD stage-associated exon skipping events, splicing quantitative trait loci (sQTLs) in the skipped exons, correlation with RNA-binding proteins, and related drugs & diseases. ExonSkipAD will be a unique resource of transcriptomic diversity research for understanding the mechanisms of neurodegenerative disease development and identifying potential therapeutic targets in AD.
Significance: AS the first comprehensive resource of the functional genomics of the alternative splicing events in AD, ExonSkipAD will be useful for many researchers in the fields of pathology, AD genomics and precision medicine, and pharmaceutical and therapeutic researches.

Key words: alternative splicing; exon skipping; protein domain retention; mutation; RNA-binding protein; transcriptome; resource; Alzheimer's disease

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Introduction

Exon skipping is reported to be the most common alternative splicing (AS) event due to loss of functional domains/sites or shifting of open reading frame (ORF), leading to a variety of human diseases and considered therapeutic targets. For example, apolipoprotein E receptor 2 (*ApoER2*) is an apolipoprotein E receptor involved in long-term potentiation, learning and memory [1]. *ApoER2* protein isoforms that lack exon 19 are associated with defects in long-term memory storage and spatial learning, perhaps through a dominant negative effect on the exon 19-included active isoform [2, 3]. Alzheimer's disease (AD) mice with a single dose of antisense oligonucleotide (ASO) [4] targeting exon 19 corrected *ApoER2* splicing for up to 6 months and improved synaptic function and learning and memory [5]. Recently, the FDA approved the ASO therapy for the Duchenne muscular dystrophy patients who have exon 53 skipping in DMD gene to restore its normal function. To date, there were studies showing that the brain expresses more alternatively spliced genes than other tissues [4]. Specifically, recent studies showed that AS event is involved in the key AD genes so that the cells in AD patients' brain move forward to AD pathogenesis [6]. However, still, the molecular mechanisms that can explain the detailed cellular processes of AS in AD remain largely unknown. There are no systematic analyses of the multiple functional aspects of the alternative splicing events in big AD patient data. Exon skipping is the most common and useful AS event as the therapeutic targets. To resolve these urgent needs, we built ExonSkipAD, the exon skipping annotation database for nine different brain regions of AD patients. First, we analyzed the three representative AD cohorts that have multi-omics data on nine different brain regions of AD and identified 16 414 genes that have ∼156 K, ∼ 69 K, ∼ 231 K exon skipping events in ROSMAP, MSBB and Mayo, respectively. Through our integrative analyses, we identified 1530 disease-specific and 2415 disease stage–associated ES events, 2000 SNP-induced ES events, 450 genes that will affect microRNA(miRNA) regulations due to 3′-untranslated regions (3′-UTR) ES events, and RNA-binding protein (RBP)-associated ES regulations. We also studied the ORF after exon skipping and following potential functional feature retention of individual ES events. This work generates a knowledgebase that proves an instrumental platform for assessing the role of exon skipping events in AD. Figure 1 is an overview of our analyses. All entries and annotation data are available for browsing and downloading on the ExonSkipAD web site, https://ccsm.uth.edu/ExonSkipAD.

Material and methods

Subjects and samples

We downloaded the RNA-seq bam file, whole genome and whole exome sequencing-based genomic variants information, and clinical information of three representative AD cohorts (ROSMAP [7], MayoRNAseq [8] and MSBB [9]) from Accelerating Medicines Partnership - Alzheimer's Disease (AMP-AD) Knowledge Portal (https://www.synapse.org/#!Synapse:syn2580853/wiki/409840) [10]. According to the different diagnosis annotations of each dataset, we chose 1697 AD and control samples from nine different regions (Supplementary Table S1), such as the dorsolateral prefrontal cortex (DLPFC), head of caudate nucleus (HCC), posterior cingulate cortex (PCC), cerebellum (CB), temporal cortex (TC), frontal pole (FL), inferior frontal gyrus (IFG), parahippocampal gyrus (PG) and superior temporal gyrus (STG). To utilize these findings, we also analyzed the mouse AD model data from the previous study with GEO id of GSE65159 [11]. The raw RNA-seq data of adult female double-transgenic CK-p25 mice and their respective control littermates were downloaded for the analysis.

Analyses

Identification of exon skipping events

For the consistent alignment results of three AD cohorts, we realigned all RNA-seq data to GENCODE v32 using STAR [12]. To identify alternative splicing events, we used SplAdder with default parameters [13]. The parameter for each software is in Supplementary Table S2. We only used the exon skipping events that had four conserved loci among the six splice sites of three exons (upstream, skipped and downstream exons). The four sites are the upstream exon's donor site, the skipped exons' acceptor and donor sites and the acceptor site of the downstream exon. Based on GENCODE v32, we identified exon skipping events in 72 095, 39 530 and 80 241 annotated gene structures of ROSMAP, MSBB and Mayo, respectively.

Analysis of differential exon skipping and gene isoforms

We performed the Wilcoxon test and used the Benjamini-Hochberg false discovery rate for multiple testing to study the differential exon skipping events based on percent spliced in (PSIs) values ($|\Delta PSI| > 10\%$ and p.adjusted < 0.05). To estimate the expression level of genes and isoforms, we ran RSEM [14] with default parameters from the RNA-seq alignments by STAR. To identify the differentially expressed genes and isoforms, we included gender and sequencing library batch information in the analysis as covariates. DESeq2 was used to perform the differential analysis between AD patients and control samples in each brain region (p.adjusted ≤ 0.05 and $|\log2FC| \geq 0.8$).

Analysis of disease stage–associated ES events

Braak semi-quantitative measure of neurofibrillary tangles (Braak stage) is a semi-quantitative measurement of severity of neurofibrillary tangle (NFT) pathology [15]. We performed the association studies between Braak stage information and PSI values using Spearman correlation. Benjamini-Hochberg false discovery rate was used for multiple testing ($|\rho| > 0.3$ and p.adjusted < 0.05).

Analysis of RNA-binding protein–associated ES events

oRNAment, a database of putative RBP target sites in the transcriptomes of model species, provides the binding sites of 113 RNA-binding proteins (RBPs) [16]. We chose the exon skipping events targeted by these113 RBPs. Then, we performed the Spearman correlation between the expression of individual RBPs and PSI values of individual ES events. Benjamini-Hochberg false discovery rate was used for multiple testing ($|\rho| > 0.3$ and p.adjusted <0.05).

Analysis of AD-specific mutation-associated ES events

To identify the single nucleotide variants (SNVs)/polymorphisms (SNPs) that might induce the exon skipping events, we overlapped the genomic coordinates of the skipped exons with high-impact mutation (e.g. splice acceptor variant, splice donor variant and frameshift variant). The impact of mutations was annotated by SnpEff from all SNVs/SNPs in three AD cohorts. Then, for these variants, we investigated if there were at least

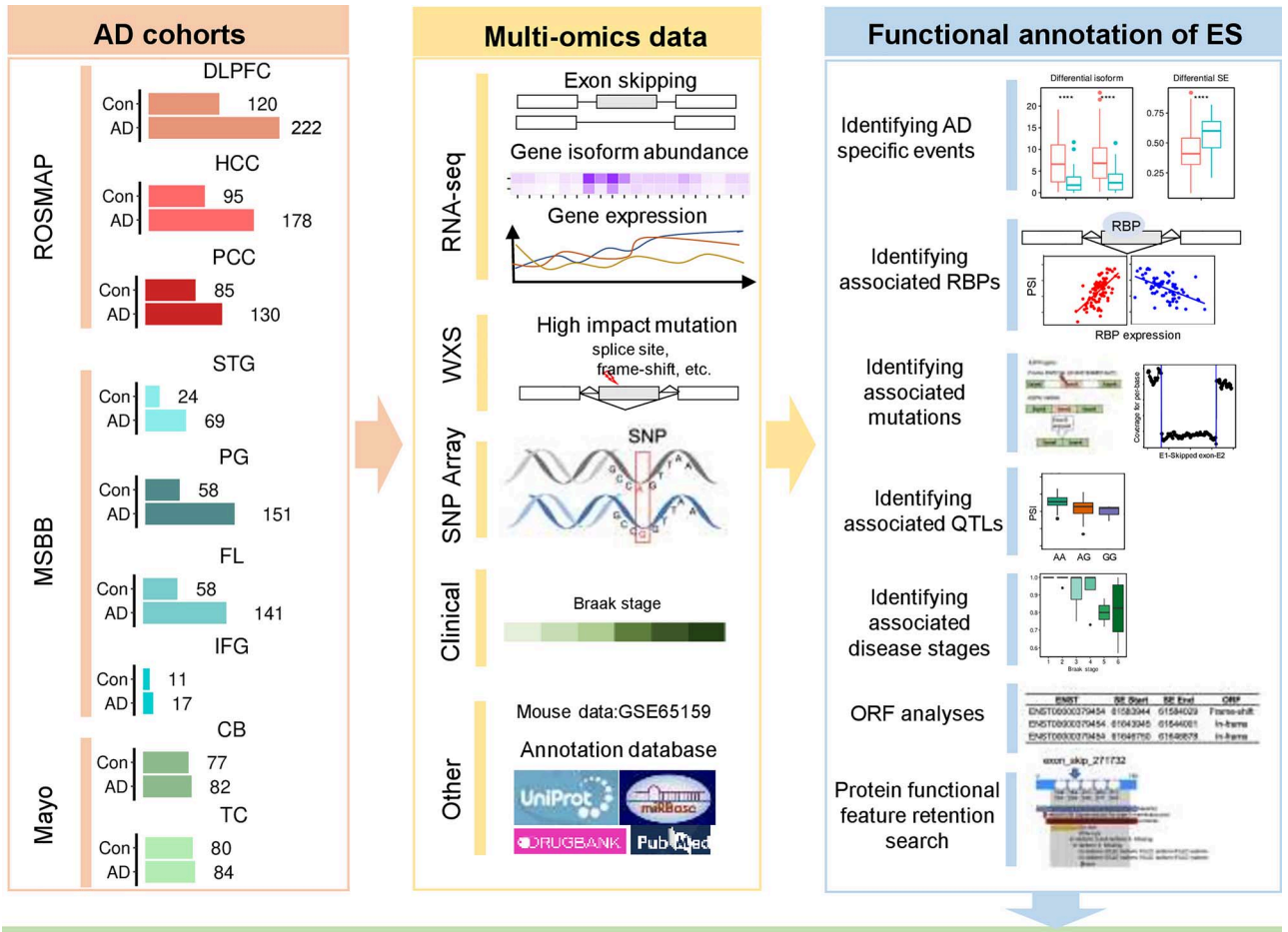

Figure 1. Overview of ExonSkipAD.

three AD patients that have a specific mutation, but there was no control with that variant (AD-specific variants). We chose the exon skipping events by checking the difference of PSIs (ΔPSI < -0.1 and p.adjusted <0.05) between AD patients with variant and control groups.

Analysis of mutation-associated ES events in AD patients

To identify the mutation-associated exon skipping events, we focused on the high-impact variants. To do this, we defined the non-recurrent and recurrent mutation as the mutation that only happened in one patient and the mutation that happened at least in two patients. We selected the mutation if the difference of PSIs (ΔPSI) is smaller than minus 0.1 with p.adjusted <0.05 between mutated and wild-type AD samples. Among these, if the averaged PSI value of the mutated samples is less than or equal to 0.4 and one of the wild-type samples is more than or equal to 0.5, then, we remained these cases for the next step. We also checked the read depth change whether the difference of mean read counts of the skipped exon is bigger than or equal to 10.0 compared to the ones of the two neighbor exons. Then, there were 21 exon skipping events in 18 genes that have recurrent mutations and 12 exon skipping events in 10 genes that have non-recurrent mutations. In this way, ExonSkipAD provides detailed evidence of the association between SNVs and exon skipping events with read depth coverage plot, differential PSI box plots and sashimi plots [17] between non-mutated and mutated samples.

miRNA-binding sites in 3′-UTR located exon skipping events

We downloaded wild-type miRNA mature sequences from miRbase [18] and used the skipped exon sequences located in the 3′-UTR based on the GENCODE v32 reference genome. Using TargetScan [19] and andmiRanda [20] to predict the miRNA-binding sites across 3′-UTR located exon skipping events. We only adopted the miRNA-binding site if it was called using both tools.

Exon skipping–specific splicing quantitative trait loci (sQTL)

To identify the ES-specific sQTLs, we used a fast eQTL analysis R package via large matrix operations, MatrixeQTL [21], with the ANOVA model. To control potential confounding factors such as gender, age of death, brain pH, RNA integrity number and sequencing library batch information, we included these variables in the analysis as covariates. ES-specific sQTL are required P-value < 0.01 and FDR < 0.05. When a distance between the genomic positions of the spanning exons was less than 100 kb, then we regarded it as the cis-sQTL.

Open reading frame (ORF) annotation

For specific exon skipping events based on the GENCODE v32, we examined the ORFs of major isoform transcript sequences, which encode the canonical protein sequences defined by UniProt [22]. When both of the nucleotide start and end positions of exon skipping were located inside of the coding region (also known as the coding sequencing, CDS) and the number of transcript sequences after exclusion of the skipped exon sequence was a multiple of three, then we reported such exon skipped gene isoform as 'in-frame'. When one or two nucleotide insertions are present, we reported such transcripts as 'frameshift'.

Retention analysis of 39 protein features from UniProt

Firstly, we created the exon skipped transcript sequence based on the canonical transcript sequence. Then, this sequence was mapped to the non-redundant protein sequence database using BLASTX, and the mapped proteins with 100% identity were selected. Through this process, we obtained the loci of skipped exon on the genomic, transcriptomic and protein sequences for the ES genes in ROSMAP/MSBB/Mayo. We screened the retention of 39 protein features of UniProt at the canonical amino acid sequence level with skipped exon loci information, including 6 molecule processing features, 13 region features, 4 site features, 6 amino acid modification features, 2 natural variation features, 5 experimental info features and 3 secondary structure features.

Drug and disease information

Drug-target interactions (DTIs) were extracted from DrugBank [23], and duplicated DTI pairs were excluded. All drugs were grouped using Anatomical Therapeutic Chemical (ATC) classification system codes. Disease-genetic information was extracted from a database of gene-disease associations.

Manual curation of PubMed articles

For the 1023 exon skipped genes among ~1500 AD genes, AD genes were collected from the International Genomics of Alzheimer's Project (IGAP) [24], AMP-AD [25], and several relevant literatures [26–31]. PubMed's literature query was performed in June 2020 using the search expression applied to each gene. Taking *ASPH* as an example, it is '((*ASPH* [Title/Abstract]) AND exon [Title/Abstract]) AND (Alzheimer's disease [Title/Abstract])'. After a manual review of the abstracts, we found that there was 396 documentary evidence supporting 417 genes (Supplementary Table S7).

Results

AD-specific exon skipping events and disease stage–associated exon skipping events

To understand the landscape of exon skipping events, we performed differential PSI values between AD and controls across nine brain regions. For this analysis, we only focused on the identified exon skipping events from the SplAdder. Then, we identified 1530 differential ES events in 1103 genes that showed a significant change in PSI values. Out of these, 93 genes were the ones known as AD genes, and 1387 differential ES events are the brain region–specific exon skipping events (Figure 2A and D). These 1530 genes were mainly involved in the biological processes of vesicle-mediated transport, regulation of Ras protein signal transduction and regulation of ARF protein signal transduction (Figure 2C) [32]. Furthermore, through the Braak stage association studies, we identified 1701 genes that have 2415 ES events associated with disease stages (see Methods 2.2.3). These genes were mainly enriched in biological pathways such as AD, vesicle-mediated transport and axon guidance (Figure 2B and D, and Supplementary Table S4) [32]. In summary, there were 304 ES events associated with both disease stage and differential expression between AD and control (Supplementary Table S5). For example, we identified a degraded myelin basic protein (*MBP*) in AD patients that are known as associated in part with vesicles particularly in a periventricular white matter, which is adjacent to areas of ependymal injury [33]. Exon 5 skipping of *MBP* (exon_skip_164743 in chr18:76988495–76988527) showed significant differential PSI values in CB, which is also associated with the Braak stage (Figure 2E). The *MBP* exon 5 skipping was anticipated to make the in-frame exon skipped protein with the loss of part of the original protein, and eventually, the expression/function of protein will not be the same as the normal level and might be beneficial for aggravation of AD progression.

Aspartate beta-hydroxylase (*ASPH*) is known as playing an important role in calcium homeostasis [34]. *ASPH* has three brain region–specific exon skipping events. *ASPH* exon 15 (exon_skip_238494 in chr8:61583944:61584029) was skipped out in IFG in AD patients compared to control, and it showed a negative correlation with disease stages (Figure 2F). On the other hand, exon 8 (exon_skip_251058 in chr8:61643945–61644001) and exon 5 (exon_skip_271732 in chr8:61646750–61646878) of this gene were skipped out in CB in AD patients (Supplementary Figure 2A). Exon 5 (exon_skip_271732) is located from 163 to 206 of the canonical protein amino acid sequence (Q12797). From retention search of the protein functional features, exon 5 skipping was anticipated to lose the normal function due to partial loss of 'luminal domain' and 'Glu-rich' region. Exon 8 (exon_skip_251058) would lose part of the *TPR1* domain, which might cause the loss of function and aggravate AD progression.

To utilize these findings, we analyzed the mouse AD model data. By analyzing the RNA-seq data of two pairs of mouse AD and control data, we identified 24 human differential ES events that were also differentially expressed in mouse AD model data (Supplementary Table S6, Figure 1B). By checking the read depth coverage change, we found an interesting exon skipping event in the disheveled-associated activator of morphogenesis 1 (*Daam1*), which is the exon 22 skipping (exon_skip_33352 in chr12:71958746–71958773 for mouse) (Figure 2G). One study

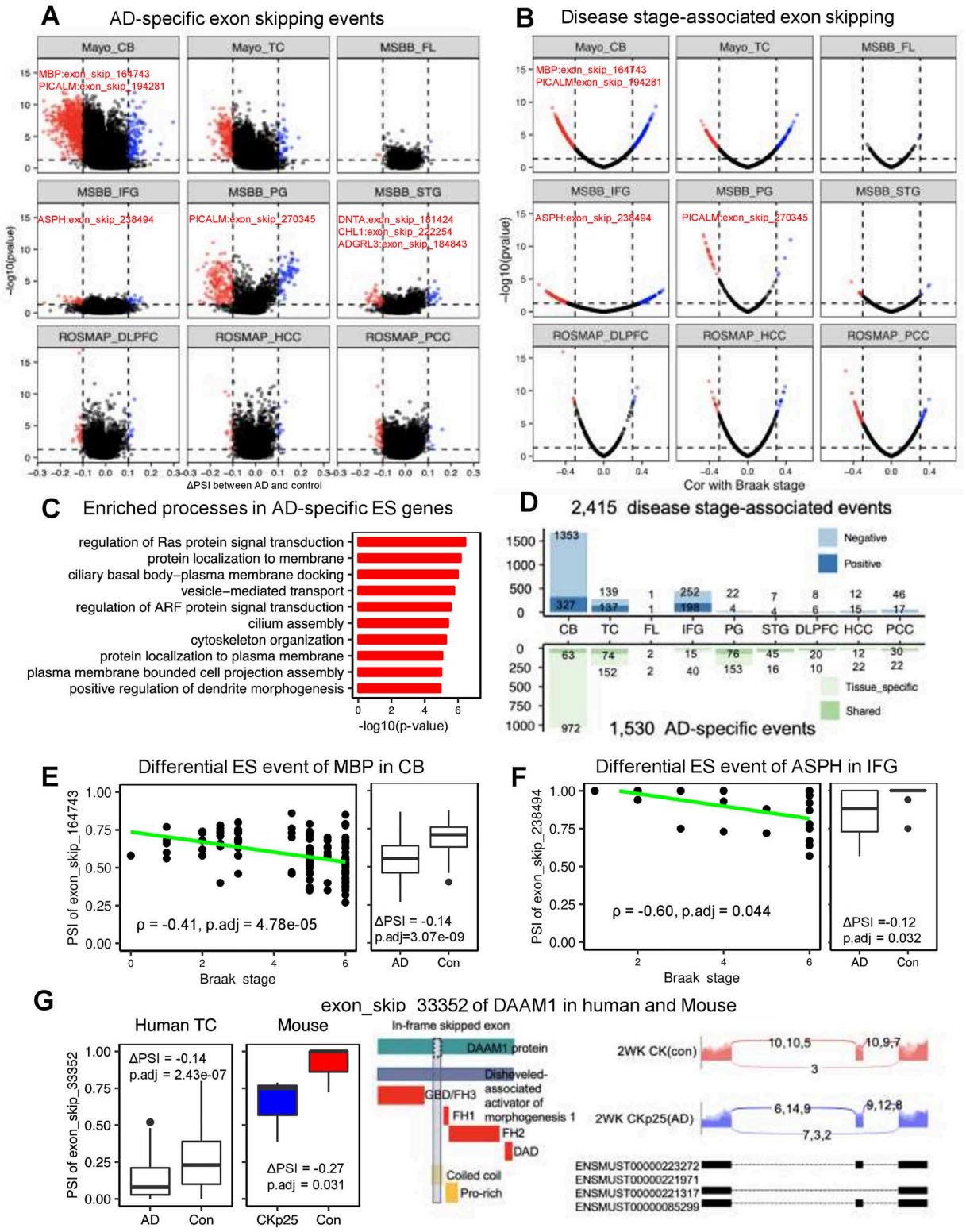

Figure 2. Differential ES events and AD stage–associated ES events. (**A**) The volcano plot of differential SE for each region. Red marked as ΔPSI < –0.1 and p.adjusted < 0.05, blue marked as ΔPSI > 0.1 and p.adjusted < 0.05. (**B**) The volcano plot of disease stage–associated exon skipping events. Red marked as $\rho < -0.3$ and p.adjusted < 0.05, blue marked as $\rho > 0.3$ and p.adjusted < 0.05. (**C**) The top 10 biological progress of genes with differential ES events. (**D**) The number of differential ES and disease stage–associated ES events for each region. (**E**) Differential ES event of *MBP* in CB. Left: the spearman correlation between Braak stage and PSI of exon_skip_164347. Right: the boxplot of PSI in AD and control group. (**F**) Differential ES event of *ASPH* in IFG. Left: the spearman correlation between Braak stage and PSI of exon_skip_238494. Right: the boxplot of PSI in AD and control group. (**G**) exon_skip_33352 of *DAAM1* in humans and mice. From the left to right, the figures are the boxplot of exon_skip_33352 in human TC, the boxplot of exon_skip_33352 in mouse, the protein function annotation and the sashimi plot for exon_skip_33352 in mouse data. The number in sashimi plot represents the junction reads for each sample.

demonstrated that the activity of the *Daam1/RhoA/ROCK* arm is necessary for Aβ-driven synaptotoxicity [35]. Daam1 is an intermediate gene in the Wnt-β-catenin pathway but the current understanding of the effects of its exon 22 skippings is lack. Our protein functional feature annotation shows that exon 22 skip of *DAAM1* would cause the frameshifting ORF so that affects the protein function.

Systematic study on RBPs identified RBM3 and targeted ES events related to AD pathogenesis

Since RBPs are one of the main regulators of splicing events, we studied the effects of these on ES events. oRNAment provides the putative RBP target sites of 113 RBPs [36]. From our association studies, we identified 15 556 RBP-associated ES events with these 113 RBPs (see Methods 2.2.4). 1206 exons were skipped out in AD patients and 101 events happened in AD genes (Figure 3A). We performed the differentially expressed genes analysis for 1542 genes encoding RBPs from the previous study on a census of human RBPs [37]. Then, we found 4 RBPs that were significantly differentially expressed in STG. Out of those, ribosomal protein S16(*RPS16*), alpha-2-glycoprotein 1, zinc-binding (*AZGP1*) and RBP, MRNA processing factor 2 (*RBPMS2*) showed up-regulation and RNA-binding motif protein 3 (*RBM3*) showed down-regulation in STG. We checked 113 RBPs, which have the binding target pair information from oRNAment. Then, *RBM3* was the only gene that showed a differential pattern in STG (Figure 3B). *RBM3*, a member of a small family of cold-inducible RBPs, is known for promoting global protein synthesis, the stability of mRNAs bearing AU-rich elements and the biogenesis of many microRNAs at the Dicer step. In neural cells, *RBM3* protects against cell death induced by ER stress and/or hypoxia/ischemia [38]. The knockdown of *RBM3* has been reported as accelerating AD condition and also inhibits the neuroprotective effects of cooling [39]. From our analysis, there were 107 ES events that are positively correlated with *RBM3* in STG and 4 of them were skipped in AD patients specifically (Figures 3C, E and 1A). Skipping events of cell adhesion molecule L1-like (*CHL1*) exon 25 (exon_skip_222254 in chr3:398227–398385), dystrobrevin alpha (*DTNA*) exon 11 (exon_skip_181424 in chr18:34825267–34825275) and adhesion G protein–coupled receptor L3(*ADGRL3*) exon 25 skipping (exon_skip_184843 in chr4:62068166–62068217) were positively correlated with *RBM3* in the same brain region (Figure 3D). We also identified *ASPH* exon 5 skipping (exon_skip_271732 in chr8:61646750–61646878) was highly correlated with *RBM3*'s expression in 8 of 9 regions (Figure 3C and D and Supplementary Figure S2). From the protein functional feature retention screening, *CHL1* exon 25 skipping was anticipated as retaining the ORF but seemed to affect the functional domains including 'extracellular', 'helical' and 'cytoplasmic' domain. *ASPH* exon 5 skipping in AD patients might let canonical protein amino acid (Q12797) lose normal function due to partial loss of 'luminal domain' and 'Glu-rich' region. Therefore, we assume that the down-regulation of *RBM3* in STG of AD patients might cause the loss of normal function of *CHL1* and *ASPH* and proceed with AD (Figure 3F).

Identification of mutation-associated exon skipping events may provide an insight into the potential action of the mechanism of exon skipping events

To identify SNPs/SNversus that might induce the exon skipping events, we overlapped the genomic coordinates of skipped exons with high-impact mutation (e.g. splice site, nonsense and frameshift mutations) or moderate-impact mutations (e.g. splice site branch U12) annotated by a program for annotating and predicting the effects of SNPs, SnpEff [40]. Then, we identified two genes [3′(2′), 5′-bisphosphate nucleotidase 1 (*BPNT1*) and ciliogenesis and planar polarity effector 1 (*CPLANE1*)] that had the AD-specific mutations associated with ES events (Supplementary Table S3). Recent work on the homolog of *BPNT1* in *Caenorhabditis elegans* shows that *BPNT1* can modulate phenotypes associated with lithium [41]. A mutation in exon7 (chr1:220062792:C > T, exon_skip_285244 in chr1:220062757–220062954) shows the association between with exon 7 skipping. The exon7 skipping of BPNT1 is anticipated as inducing the loss of magnesium binding site in *BPNT1* (see Methods 2.2.5, Supplementary Figure 3A).

From integrative transcriptome level studies relating to exon skipping, we identified 32 ES events from 27 genes that showed the decreased read coverage depth at the skipped exon and differential in PSI values between mutated and non-mutated samples in AD patients (see Methods 2.2.6, Table 1). Figure 4A shows the evidence of chr8:61646851delT mutation might cause *ASPH* exon 5 skipping (exon_skip_271732 in chr8:61646750–61646878) in FL including the difference of PSI values between mutated samples and wild-type samples, and sashimi plot of the junction split reads between the two flanking exons. We performed the differential analysis between AD patients with and without *ASPH* exon 5 skipping in FL, and nine genes show the differential expression. Among these, two RBP [ribosomal protein S4 Y-linked 1 (*RPS4Y1*) and eukaryotic translation initiation factor 1A Y-linked, (*EIF1AY*)] and one DNA-binding protein (DBP), zinc finger protein Y-linked (ZFY), are down-regulation in AD. What's more, *ZFX* as an upstream factor regulating SET gene expression might activate the SET nuclear proto-oncogene (*SET*) overexpression in brain neurons of patients suffering from AD [42].

Another example is ATP-binding cassette subfamily B member 7 (*ABCB7*) encoding a protein involved in the transport of heme from the mitochondria to the cytosol. Genome-wide association studies originally identified *ABCA7* as risk gene of AD [43]. There are also reports about premature termination codon variants in *ABCA7* that are associated with an increased risk for AD, and *ABCA7* deficiency exacerbates brain Aβ accumulation and AD-related phenotype [44, 45]. The variant in the splice donor site (chrX:75114753) was associated with *ABCB7* exon 2 skipping (exon_skip_54884,chrX: 75114754–75114831), which is anticipated as inducing the loss of function in TC and CB regions (Supplementary Figure 3B). Our result might provide a new sign of how *ABCB7* increases the risk of AD.

Survey of splicing quantitative trait loci (sQTL) in AD

SNPs can affect the alternative splicing events through modifying the sequence-specific binding affinity of the splicing factors to the pre-mRNAs, resulting in splicing quantitative trait locus (sQTL). From our analysis of sQTL using MatrixeQTL, we identified 8699 potential cis-sQTL pairs between 7107 SNPs and 2000 ES events in 1511 genes across 9 brain regions from three AD cohorts. Out of these, there were 141 AD genes that have the ES-specific cis-sQTLs. There are also 427 and 536 cis-sQTL pairs in Mayo (TC and CB, Supplementary Figure 3C) and ROSMAP cohorts, respectively (DLPFC, HCC and PCC, Supplementary Figure 3D). Specifically, 81 cis-sQTL pairs were identified in both ROSMAP and Mayo cohorts (Figure 4B). For example, there was a report on the association between SNP rs10791097 and down-regulation of sorting nexin 19 (*SNX19*) [46]. This genetic risk variant was known as strongly associated with the expression

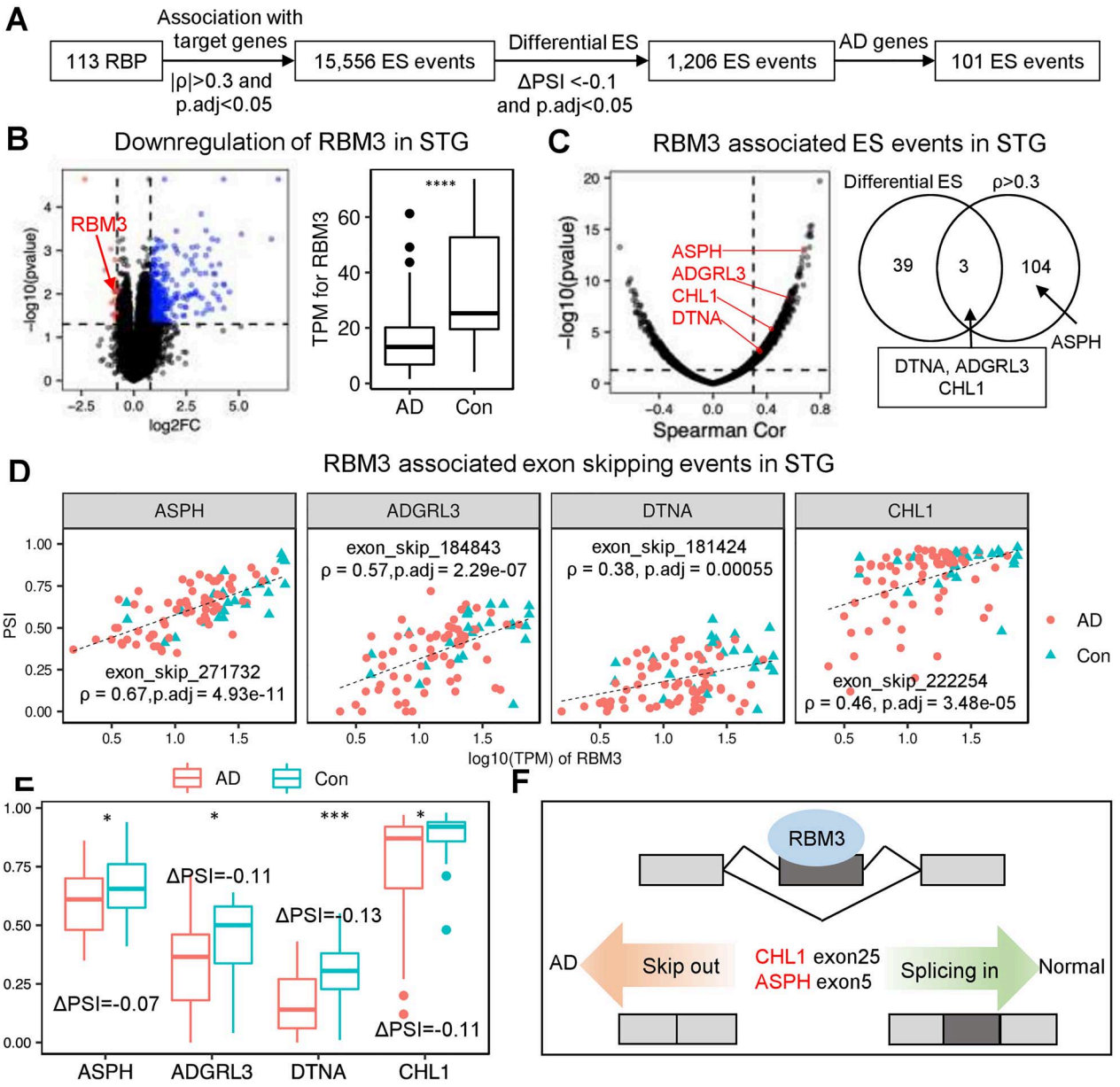

Figure 3. RBP-associated ES events. (**A**) The analysis of RBP-associated ES events. (**B**) Down-regulation of *RBM3* in STG. Left: the volcano plot of the differential gene in STG. Right: the boxplot of *RBM3*'s expression in the AD and control group. (**C**) *RBM3*-associated ES events in STG. Left: the volcano plot of *RBM3*-associated ES events. Right: the Venn diagram of skip out events in AD (ΔPSI < –0.1, p.adjusted < 0.05) and *RBM3*-positive associated ES events in STG. (**D**) Five *RBM3*-associated exon skipping events in STG. (**E**) The boxplot of five *RBM3*-associated exon skipping events' PSI in AD and control group. (**F**) The regulation of *RBM3* in STG.

of *SNX19*, which is important to schizophrenia [47]. Interestingly, in our study, we identified that this SNP rs10791097 has been significantly associated with the PSI values of the ratio of *SNX19* exon 9 skipping (exon_skip_108661 in chr11:130880622–130880806) (Figure 4C). rs7107595 and rs948085 also showed a high correlation with the exon 9 skipping in AD patients. The exon 9 skipping out of *SNX19* might cause the frameshift ORF.

3′-UTR region exon skips will affect the miRNA binding

MicroRNAs usually bind to the 3′-UTR of mRNAs and down-regulate their expressions [48]. There were several studies focusing on the effects of shortened length of 3′-UTR. For example, a widespread shortening of 3′-UTRs was reported in cancer cells by the alternative cleavage and polyadenylation, and it results in the activation of oncogenes [49]. Alterations in mRNA 3′-UTR isoform abundance accompany gene expression changes in human Huntington's disease brains [50]. From ORF checking based on the reference genome, we found 450 genes that have exon skipping events located in the 3′-UTR (see Methods 2.2.7). Among these, there were 34 AD-related genes. Further checking of the differential PSI values identified 12 exon skipping events that showed a significant difference in PSIs between AD and control (Supplementary Table S8). The exon_skip_141130 (chrX: 103678499–103678600, p.adjusted = 3.00e-06, ΔPSI = –0.147) of mortality factor 4 like 2, (*MORF4L2*). This exon skipping event showed higher PSI values in AD patients in PG and was predicted as causing a gain of miRNA-binding site (like

Table 1. Exon skipping events that are associated with the variants

SE	Gene	Chrom	Skipped exon start	Skipped exon end	Mutation start	Mutation end	Reference seq	Mutation seq
exon_skip_100621	ANKRD20A11P	chr21	13916747	13916958	13916746	13916747	C	T
exon_skip_103814	RGS14	chr5	177366177	177366355	177366175	177366176	A	T
exon_skip_105385	FAM153A	chr5	177714054	177714177	177714053	177714054	C	A
exon_skip_105787	ABCC8	chr11	17430814	17431032	17431033	17431034	C	T
exon_skip_10756	CCDC7	chr10	32518416	32518505	32518506	32518507	G	T
exon_skip_111127	DECR1	chr8	90017124	90017326	90017131	90017132	G	A
exon_skip_11858	CEP89	chr19	32933451	32933669	32933493	32933494	T	A
exon_skip_119899	MICAL2	chr11	12249184	12249246	12249246	12249249	GGT	G
exon_skip_124436	CCDC7	chr10	32518416	32518505	32518506	32518507	G	T
exon_skip_132375	MYEF2	chr15	48152234	48152284	48152233	48152234	C	A
exon_skip_171500	MRPL2	chr6	43055897	43056007	43055895	43055896	A	G
exon_skip_172170	USP54	chr10	73504850	73504990	73504978	73504979	G	A
exon_skip_189220	DECR1	chr8	90017124	90017326	90017131	90017132	G	A
exon_skip_190554	RP11-33B1.1	chr4	119543753	119543845	119543846	119543847	G	A
exon_skip_200426	USP16	chr21	29050092	29050178	29050091	29050092	G	A
exon_skip_206719	HERPUD1	chr16	56935404	56935475	56935459	56935461	CA	C
exon_skip_20836	C5orf42	chr5	37157313	37157420	37157322	37157323	G	A
exon_skip_267811	SNHG5	chr6	85677954	85678032	85677953	85677954	C	T
exon_skip_269095	SNHG5	chr6	85677954	85678032	85677953	85677954	C	T
exon_skip_270565	ABCB7	chrX	75114754	75114834	75114753	75114754	C	T
exon_skip_270947	TBC1D31	chr8	123142262	123142456	123142457	123142458	G	T
exon_skip_271732	ASPH	chr8	61646750	61646878	61646850	61646852	AT	A
exon_skip_278770	GDE1	chr16	19517014	19517185	19517013	19517014	C	T
exon_skip_287394	RP11-481J2.2	chr16	58433810	58433948	58433949	58433950	G	A
exon_skip_54884	ABCB7	chrX	75114754	75114831	75114753	75114754	C	T
exon_skip_56341	ENDOV	chr17	80422206	80422245	80422204	80422205	A	G
exon_skip_58850	KLHL24	chr3	183636221	183636311	183636220	183636221	G	A
exon_skip_62643	BROX	chr1	222727168	222727257	222727166	222727167	A	G
exon_skip_85382	IFIH1	chr2	162268087	162268277	162268086	162268087	C	T
exon_skip_85668	P2RX5	chr17	3690956	3691027	3690982	3690984	TG	T
exon_skip_96014	ABCC8	chr11	17430814	17431032	17431033	17431034	C	T
exon_skip_122956	HINT3	chr6	125974847	125974973	125974812	125974851	TGGTTTGTCAATCATCTCTTTACTTTCTATTTTAGGATG	T

hsa-miR-1343-3p, hsa-miR-19a-5p, hsa-miR-19b-1-5p, hsa-miR-6783-3p, hsa-miR-3185, hsa-miR-6852-5p, and hsa-miR-19b-2-5p, hsa-miR-450b-5p, hsa-miR-424-3p).

ORF analysis of individual exon skipping events will be helpful for screening potential therapeutic candidates

The systematic analyses of ORF of individual exon skipping events in AD patients across three big cohorts can identify potential candidates that need to be targeted by ASO therapies in case their abnormally diminished protein functional features are essential for normal brain functions. Overall, we identified 8670 and 7505 genes that have exon skipping events made in-frame and frameshift ORFs, respectively. We systematically investigated the retention of important cellular regulators/domains in case of in-frame exon skipping events of kinases, transcription factors (TFs), drug targets in IUPHAR, AD genes and cancer gene census (CGC) genes, and then, we identified 449, 1037, 2021, 1023 and 579 genes lost their main domains due to the in-frame exon skipping events, respectively. All these genes had other exon skipping events that create frameshift ORFs in their gene bodies. Among 1452 known AD-related genes, 1023 genes (70.45%) lost normal functions by exon skipping mechanisms. The anticipated loss of functional effects of our identified exon skipping events in these AD genes seems that it will likely be related to the AD pathogenesis. Among these, 376 genes had AD-specific exon skipping events, and 275 genes belong to the important cellular regulators mentioned above including 41 kinases, 42 TFs, 139 drug targets, 80 AD genes and 45 CGC genes (Supplementary Table S9). Figure 5 shows the most enriched GO biological processes of individual gene groups. We can see the most significant potential loss of functional genes due to exon skipping events was the peptidyl-serine phosphorylation. Rosenberger et al. observed an overall decrease of protein kinase activity (e.g. phosphorylation of 96.7% of the serine/threonine peptides and 37.5% of the tyrosine peptides) associated with the increased Braak stage [51]. It is also shown that 45 TFs are mainly involved in the negative regulation of diverse cellular processes including macromolecule biosynthesis. Here, we can understand the macromolecule would also include the aggregated Aβ peptides. IUPHAR drug target genes were enriched in calcium ion transporting. It is reported that the disturbances in calcium homeostasis are also one of the earliest molecular changes that occur in AD patients, alongside alterations in calcium-dependent enzymes in the post-mortem brain [52]. Eighty-six AD genes that lost partial functional domains due

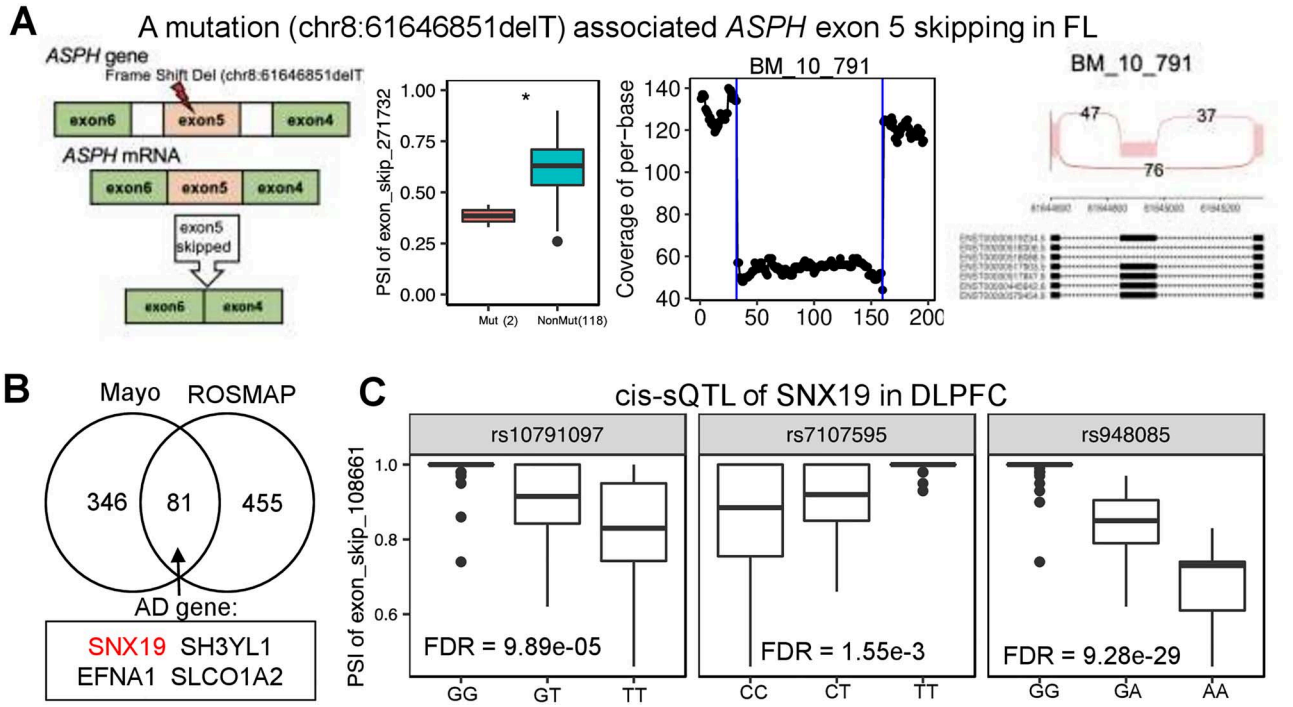

Figure 4. The mutation associated with ES events. (**A**) A mutation (chr8:61646851delT)-associated *ASPH* exon 5 skipping in FL. From the left to right, the Figs are the mutation in exon_skip_271732 (upstream exon: chr8:61644600–61644632, skipped exon: chr8:61646750–61646878, downstream exon: chr8:61651050–61651086), the boxplot of PSI in mutated and non-mutated AD patients in FL, the reads coverage for exon_skip_271732 of BM_10_791 and the sashimi plot for exon_skip_271732. BM_10_791 is patient id for MSBB FL region. (**B**) The Venn diagram of cis-sQTL in Mayo and ROSMAP. (**C**) The cis-sQTL of *SNX19* in DLPFC.

to exon skipping events were mainly the genes working for neuronal cell developments. Last, the roles of 45 CGC genes that lost their partial domains were relating to the positive regulation of neuron differentiation and microtubule. Reduced microtubule stability is well-known in AD relating to the inhibited neuronal activity [53]. As shown in these genes' anticipated loss of functional aspects, we can better understand the genomic functional effects of the exon skipping events in AD.

Discussion

This study provides a comprehensive and quantitative functional annotation of exon skipping events in AD across nine brain regions from three AD cohorts. We identified exon skipping events and performed multiple functional annotation studies such as AD-specific ES events, disease stage–associated ES events, SNP-induced ES events studies, 3′-UTR events with different miRNA regulations, RBP-associated ES events, ORF annotation and functional feature retention screening of individual ES events. To our knowledge, this is the first study providing a systematic functional annotation of exon skipping events in AD. Our study will be helpful to have insights into the roles of ES events in the pathogenesis of AD. Comparison of PSI values of the identified exon skipping events between AD and control samples identified 1530 differential ES events. 143 of these ES events happened in more than one brain region type and 1387 ES events were brain region–specific events in AD. Ninety-three out of these events happened in the known AD-related genes (i.e. *MBP* and *ABCB9*) with in-frame/frameshifting ORFs (Supplementary Table S9). Furthermore, we identified 304 differential ES events that are associated with disease stages, which might involve in AD pathogenesis (Supplementary Table S5). Among the AD-specific events, 40 genes were reported in the previous AD studies (see Methods 2.2.12). Interestingly, *ASPH* has brain region–specific exon skipping events in three brain regions. These exon skipping events were anticipated to cause the loss of function and aggravate AD progression. From analyzing the mouse AD model data, we identified 24 overlapped ES events that showed a significant difference between AD and control in both humans and mice. *DAAM1* exon 22 skipping in AD disease would cause the frameshifting of ORF and might affect its normal protein function. Our functional annotations of individual ES events provide potential stories on ORF change and protein functional feature loss, and these will enable the assessment of how the specific exon skipping event would influence the biological functions in AD.

Most of all, our integrative analyses of multi-omics data provide a landscape of the functional aspects of exon skipping–related cellular regulations including SNV/SNP-induced ES events, 3′-UTR events with different miRNA regulations and RBP-associated ES events. From the association study between SNVs and exon skipping events, we identified two exon skipping events associated with high-impact mutations in AD (see Methods 2.2.5). We also found 32 exon skipping events associated with high-impact mutations across 27 genes in AD patients with read depth and PSI difference between mutated and wild-type samples (see Methods 2.2.6). From our previous study of ExonSkipDB providing a systematic functional annotation of exon skipping events in pan-cancer, we noticed that there are not many splicing site–associated mutations even in pan-cancer. However, the mutation-induced exon skipping should not be ignored since the FDA approved capmatinib as the treatment of lung cancer patients who have the MET

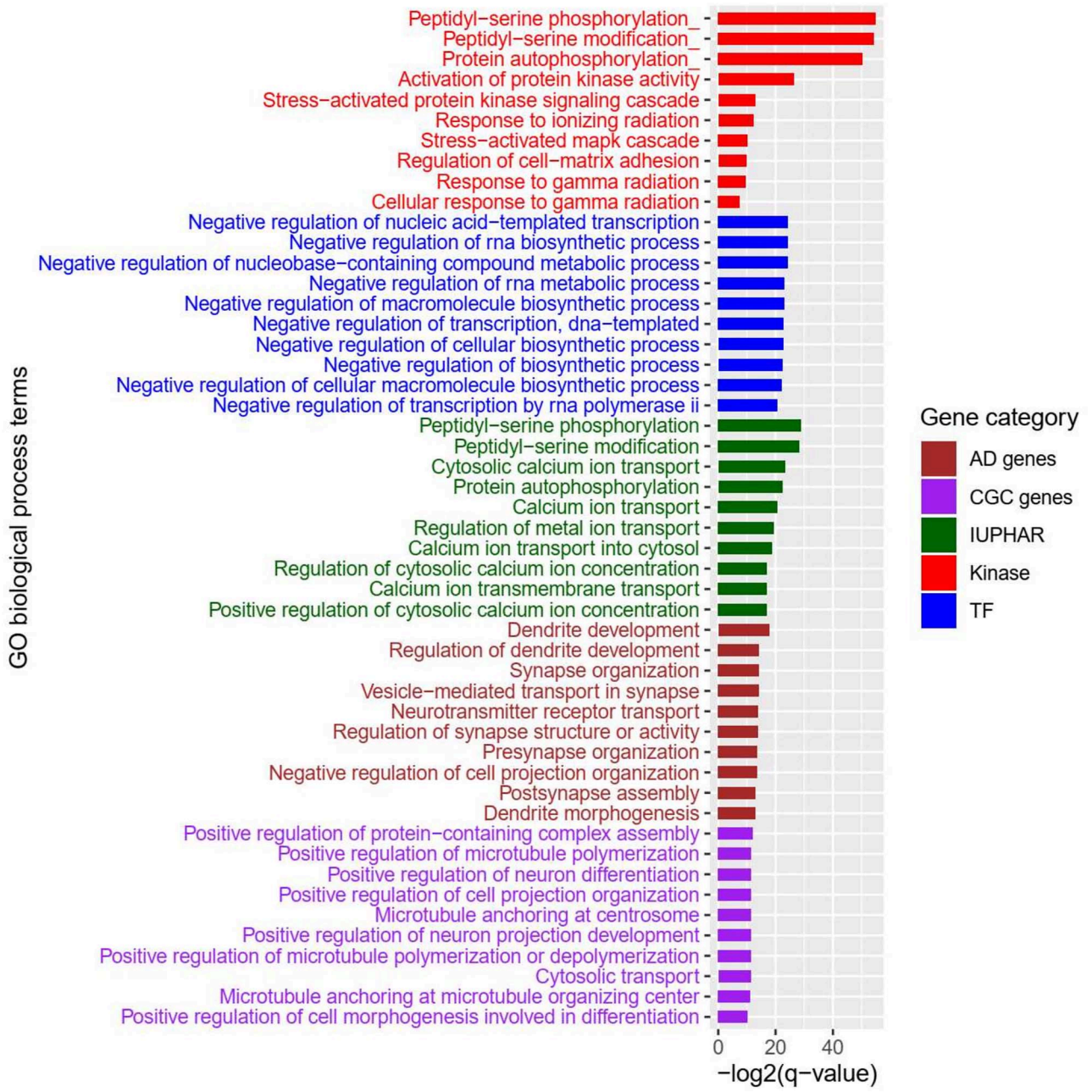

Figure 5. Top 10 enriched GO biological processes of the genes that lost partial domains due to exon skipping events per individual gene groups.

exon 14 skipping variants induced by SNV. Among the three potential types of mutations that can affect the alternative splicing such as splice site, nonsense and frameshift mutations, the most effective and frequent mutation type was the splice site mutations, which located +/− 2 nt from the exon junction site. After checking the differential PSIs and RNA-seq read depth coverage at the three exons composing the skipped exon and two neighbor exons, we were able to identify 136 exon skipping events only from The Cancer Genome Atlas (TCGA) samples. Our work of ExonSkipAD focuses on AD relying on the SNPs of ~1500 AD patients, not somatic mutations. However, we also expected to identify similar mechanisms in AD as a recent study showed a greater contribution of germ line alterations to the transformation process while late-onset cancers are more driven by somatic mutations [2]. Furthermore, even though the frequency of variants is low (Supplementary Table S3), the variants still can play a role in disease. For example, the biggest frequency case of the somatic mutation associated with exon skipping in cancer, which is the recently approved drug target of *MET* exon 14 skipping, was only four samples in TCGA's ~500 lung adenocarcinoma patients. Therefore, we needed to reflect these small frequency contexts to identify the mutation-associated exon skipping event in this work. So, we deeply and systematically checked their mutation types and effects on the read depth change patterns rather than relying on the association study with PSI values only. The small sample frequency is usually problematic statistically. However, in this context of our samples' nature, we intended to identify novel

Table 2. Summary of the significant exon skipping events identified from the individual analysis categories

Analysis category	Brain region	ES events id	Gene	Spearman correlation		Wilcoxon test (AD versus control)	
				ρ	p.adj	ΔPSI	p.adj
Disease stage–associated ES events	CB	exon_skip_164743	*MBP*	−0.41	4.78e-05	−0.14	3.07e-09
	IFG	exon_skip_238494	*ASPH*	−0.60	0.0447	−0.12	0.032
RBM3-associated ES events	STG	exon_skip_184843	*ADGRL3*	0.57	2.29e-07	−0.11	0.0329
	STG	exon_skip_181424	*DTNA*	0.38	5.56-e-04	−0.13	3.03e-04
	STG	exon_skip_222254	*CHL1*	0.46	3.48e-05	−0.11	0.033
	STG	exon_skip_271732	*ASPH*	0.67	4.93e-11	−0.07	0.042
3′-UTR region exon skips	PG	exon_skip_141130	*MORF4L2*	NA	NA	−0.15 (mutation AD versus non-mutation control)	3.00e-06
AD-specific mutation-associated ES events	TC	exon_skip_285244	*BPNT1*	NA	NA	−0.27	6.8e-14
	TC	exon_skip_20836	*CPLANE1*	NA	NA	−0.28 (mutation AD versus non-mutation AD)	7.0e-04
Mutation-associated ES events	FL	exon_skip_271732	*ASPH*	NA	NA	−0.22	0.037
	CB	exon_skip_54884	*ABCB7*	NA	NA	−0.68	0.0095
	TC	exon_skip_54884	*ABCB7*	NA	NA	−0.73	0.017

case, and even the pattern was shown in only one sample by compensating the low frequency with the relevant screening of other information as we did. As a result, we found these cases. chr8:61646851delT mutation in two AD patients is associated with *ASPH* exon 5 skipping in FL, which might be related to the down-regulation of two RBPs (*RPS4Y1* and *EIF1AY*) and one DBP (*ZFY*). The known AD risk gene, *ABCB7* has the variant in the splice donor site, which was associated with exon 2 skipping event. From these results, our study might provide the potential mechanism of creating ES events with mutations. From the sQTL analysis, we identified 536 and 427 cis-sQTL pairs in ROSMAP and Mayo, respectively. Out of these, 81 cis-sQTLs were common in both cohorts. *SNX19* exon 9 skipping was significantly associated with three SNPs (rs10791097, rs7107595 and rs948085), which were reported as affecting splicing events. Through investigating 3′-UTR located exon skipping events, we found 450 genes that might affect miRNA regulations due to exon skipping, which provides insights into the regulation mechanism between miRNA and exon skipping. Last, from the association study between RBP and exon skipping events, we identified 1206 exon skipped events that have the RBP-associated ES events in AD patients. And 101 of them are in the AD genes. Specifically, we found the down-regulation of *RBM3* in the STG region of AD patients. This was further analyzed as it might cause the loss of function of *CHL1* and *ASPH* and might be helpful for proceeding into AD. On the whole, the ES events we found in each category provide the potential biomarker for AD treatments (Table 2).

ExonSkipAD is the first database to systematically annotate the function of exon skipping events for AD. To serve the broad biomedical research communities, we will continually update and curate exon skipping events by routinely checking newly released RNA-seq data of AD. To make better use of ExonSkipAD, we will add the analysis results for the exon skipping events of multiple mouse AD model data in the near future. We will continue to expand our research and improve our approaches to find clinically significant exon skip events and downstream target genes. The user-friendly web site provides multiple annotations and facilitates a comprehensive functional study of exon skipping. Therefore, ExonSkipAD will be a useful resource for many researchers in the fields of pathology, AD genomics and precision medicine, and pharmaceutical and therapeutic researches.

Key Points

- ExonSkipAD provides multiple functional annotations of exon skipping events in AD such as AD-specific ES events, RBP-associated events, SNV/SNP-associated events, ORF annotation, functional feature retention study, 3′-UTR events with different miRNA regulations and association study with disease stages.
- Through ExonSkipAD, we identified AD-specific, disease stage–associated ES events in *ASPH* and *MBP*. We also identified RBP, *RBM3*-associated events in MBP exon 5. Through studying the lost functional domains due to AD-specific events, we identified 275 important cellular regulators that might lose their cellular regulator roles.
- ExonSkipAD is a unique resource for an understanding of exon skipping events in AD.

Supplementary data

Supplementary data are available online at *Briefings in Bioinformatics*.

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Data availability

The data involved in the study is available at https://ccsm.uth.edu/ExonSkipAD/.

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