Transcriptomic changes resulting from *STK32B* overexpression identifies pathways potentially relevant to essential tremor

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Abstract

Essential tremor (ET) is a common movement disorder that has a high heritability. A number of genetic studies have associated different genes and loci with ET, but few have investigated the biology of any of these genes. *STK32B* was significantly associated with ET in a large GWAS study and was found to be overexpressed in ET cerebellar tissue. Here, we overexpressed *STK32B* in human cerebellar DAOY cells and used an RNA-Seq approach to identify differentially expressed genes by comparing the transcriptome profile of these cells to the one of control DAOY cells. Pathway and gene ontology enrichment identified axon guidance, olfactory signalling and calcium-voltage channels as significant. Additionally, we show that overexpressing *STK32B* affects transcript levels of previously implicated ET genes such as *FUS*. Our results investigate the effects of overexpressed *STK32B* and suggest that it may be involved in relevant ET pathways and genes.

Introduction

Essential tremor (ET) is one of the most common movement disorders and is typically characterized by a kinetic tremor in the hand or arms[1]. The severity tends to increase with age and may involve different regions such as the head, voice or jaw[2]. Previous studies investigating the histology of post-mortem tissue have pinpointed the cerebellum as a region of interest for ET[3]. Specifically, abnormalities with Purkinje cell axons and dendrites were found in ET brains[4]. Furthermore, the olivocerebellar circuitry has been implicated in ET pathology and many calcium voltage channels are highly expressed in this circuitry[5].

The genetic etiology of ET has remained largely elusive and most studies have focused on common or rare variants and on looking for genetic overlap with other disorders[6,7]. Twin studies have shown that ET has a concordance of 69–93% in monozygotic twins and 27–29% in dizygotic twins, which suggests that both genetic and environmental factors drive the onset and development of this complex trait[8]. A recent genome-wide association study (GWAS) identified a significant locus in *STK32B* and found that ET patients overexpressed *STK32B* in cerebellar tissue by comparison to healthy controls[9]. *STK32B* is transcribed and translated into YANK2, a serine/threonine kinase, that has not been well characterized. There have been several exome-wide studies that implicated different genetic variants as causes of ET. The first ET-implicated gene found through exome

sequencing was the Fused in Sarcoma gene (*FUS*)[10]. However, it is unclear whether these "ET" genes interact or have indirect effects on the expression of each other.

To understand the effects of overexpressed *STK32B* and identify pathways potentially relevant to ET, we overexpressed the gene in human cerebellar DAOY cells and compared transcriptomic changes in overexpressed cells and empty-vector controls using RNA sequencing. Several interesting pathways such as axon guidance, calcium ion transmembrane transport, and olfactory transduction were significantly enriched after overexpression of *STK32B*. We also identified previously implicated ET genes whose expression is dysregulated through the overexpression of *STK32B*, suggesting that overexpressed *STK32B* may have relevant downstream effects.

Results

Confirming overexpression of *STK32B*

The *STK32B* stable cell lines had higher RNA expression of *STK32B*, which was detected by reverse-transcriptase qPCR (Supplementary Figure 1). RNA sequencing (RNA-Seq) also confirmed that the stable cell lines have higher *STK32B* RNA levels compared to controls; it was the most significantly differentially expressed gene (Figure 1–2).

Quality control of RNA-seq analyses

The QQ-plot for differentially expressed genes did not show stratification or potential biases (Supplementary Figure 2). The M-A plot showed slight enrichment of downregulated genes compared to upregulated genes (Supplementary Figure 3). A principal component (PC) plot showed separation of controls and *STK32B* cell lines (Figure 3). Additionally, the dendrogram with heatmap shows that controls and *STK32B* cell lines are distinct (Supplementary Figure 4). The mean-variance plot shows the shrinkage under the sleuth model (Supplementary Figure 5).

Overexpression of *STK32B* affects expression of previously identified ET-associated genes

A total of 3,794 genes were found to be differentially expressed with a q-value <0.05. There were 425 genes with a $\beta > |0.5|$ and q-value <0.05. Amongst the 425 genes, three potentially relevant ET genes were dysregulated: *FUS* (q-value = 0.007, β=0.812) and two calcium voltage channel genes that are enriched in the olivocerebellar circuitry, *CACNA1C* (q-value = 0.011, β= 0.503) and

CACNA1A (q-value = 0.030, β= 0.702). Several pathways and gene ontologies were enriched for differentially expressed genes (DEGs) due to the overexpression of *STK32B*, including olfactory transduction (P=2.68E-39), axon guidance (P=9.50E-36), and calcium ion transmembrane transport (P=3.02E-31) (Table 1).

Gene network analyses

To identify which cluster of DEGs drove the significant pathways, gene network analysis based on co-expression was done and identified 14 different clusters within the differentially expressed genes (Figure 4). Analysis of the top 3 largest clusters for pathway enrichments found several similar pathways in different databases including axon guidance (P<1.09E-12) in cluster 1 and calcium signalling pathway in cluster 3 (2.06E-05).

Discussion

The genetic etiology of ET is complex and likely explained by a combination of copy number variants, rare variants, gene-gene interactions and common variant drivers. One of the most significantly enriched pathways we identified was olfactory signalling and transduction. Previous reports have shown conflicting evidence about olfactory loss in ET[11,12]. It may be that ET patients with dysregulated olfactory signalling have overexpressed *STK32B*, which may contribute to the subset of ET patients with olfactory loss.

Interestingly, *FUS* was amongst the genes dysregulated when *STK32B* was overexpressed. In the exome familial ET study that identified *FUS*, we also found reduced mRNA levels for *FUS*[10]. Similarly, the expression of *FUS* is lower when *STK32B* is overexpressed, suggesting an indirect relationship between the two genes. Additionally, two calcium voltage-gated channel genes, *CACNA1C* AND *CACNA1A* were overexpressed. Enrichment analyses found calcium ion transmembrane transport to be highly significant in GO Processes, suggesting that *STK32B* may play a role upstream of these genes. In the olivocerebellar circuitry, a system implicated in ET, is enriched for both of these genes[5,13]. *CACNA1A* has been shown to be predominantly expressed in Purkinje cells, a cell type relevant to ET[13]. Another enriched pathway was axon guidance. A previous study identified the *TENM4* missense variant segregating within ET families[14]. *TENM4* is a regulator of axon guidance and myelination and Tenm4 knockout mice showed an ET

115 phenotype, suggesting that axon guidance is important in ET[14]. Similarly, overexpressed *STK32B*
116 could be dysregulating important pathways relevant to axon guidance.
117
118 By sub-stratifying the overexpressed genes into different clusters that are co-expressed, several
119 interesting pathways involving the cardiovascular system were identified. Pathways such as right
120 ventricular cardiomyopathy in KEGG and angiogenesis in GO Processes were found among the
121 top five significant pathways. In certain ET patients, beta-blockers can reduce tremor magnitude
122 and frequency. Beta-blockers lower blood pressure and are used to treat irregular heart rhythm and
123 other cardiovascular phenotypes. A common treatment for ET is propranolol, a beta-blocker. This
124 was a drug developed for cardiovascular health that affects adrenergic activity, but still reduces
125 tremor in ET individuals. This could suggest that overexpressed *STK32B* affects cardiovascular
126 health, which may in turn affect or lead to the ET phenotype or that *STK32B* may be pleiotropic
127 and affect the nervous and cardiovascular system differently.
128
129 Due to the heterogeneity of ET, it is likely that not all ET-affected individuals have overexpression
130 of *STK32B*. One limitation of this study is that DAOY cells are a cancerous cell line and may not
131 be the ideal model for understanding the transcriptome of Purkinje cells. Future studies on the
132 effects of *STK32B* overexpression could use induced pluripotent stem cells differentiated into cells
133 with Purkinje cells properties; such cells would likely have less biological noise. However, our
134 findings support the idea that *STK32B* is a gene of interest for a subset of ET cases, and further
135 investigations into how *STK32B* may interact with other genes are warranted.
136

137 Methods and Materials

138 Cell Culture and plasmid construction

139 The DAOY cell line was cultured in Eagle's Minimum Essential Medium (EMEM) with 10% fetal
140 bovine serum (FBS) at 37 degrees Celsius and 5% CO_2 with the Glico Pen-Strep-Glutamine
141 cocktail at 1X. Cells were passaged every 2 days at 80-90% confluence and at the same time. The
142 cDNA of *STK32B* (NM_001306082.1) was inserted into a pcDNA3.1(+) vector containing a
143 neomycin resistance gene, used as a selectable marker. This transcript was picked because it had
144 the highest expression in the cerebellum based on GTEx 53 v7. The plasmid was transformed and

145 expanded in XL10-Gold *Escherichia coli* strain (Stratagene). The cDNA of the plasmid was sent
146 for Sanger sequencing to validate the gene sequence.
147

148 ## Transfection and deriving stable cell lines

149 The cells were transfected for 48 hours with 8 ug of DNA of using the jetPRIME transfection
150 reagent (Polyplus). A green fluorescent protein (GFP) control vector was used to assess and
151 estimate transfection efficiency and optimize transfection parameters. Stable cell lines were
152 established by subjecting transfected cells to G418 antibiotic at 1 µg/mL for 10 days and maintained
153 at a concentration of 400 µg/mL for an additional 2 weeks. A kill curve was done to determine the
154 ideal antibiotic selection. In parallel, empty pcDNA3.1(+) vector control lines were also grown and
155 underwent antibiotic selection.
156

157 ## RNA extraction and sequencing

158 RNA was extracted using the RNeasy Mini Kit (Qiagen). The RNA concentration was measured
159 using the Synergy H4 microplate reader. RNA for the top three overexpressed *STK32B* cell lines
160 and three empty-vector controls was sent to Macrogen Inc. for sequencing. RT-qPCR was used to
161 confirm overexpression in the cell liens compared to controls. Library preparation was done with
162 the TruSeq Stranded Total RNA Kit (Ilumina) with Ribo-Zero depletion. Sequencing was done on
163 the NovaSeq 6000 at 150bp paired-end reads with a total of 200M reads. Samples were randomized
164 for cell lysis, RNA extraction, Ribo-Zero depletion, library preparation and sequencing to account
165 for potential batch effects.
166

167 ## Data processing and quality control

168 The FASTQ files were pseudo-aligned with Salmon using the Ensembl v94 annotation of the
169 human genome[16]. The parameters of Salmon included the following: 200 bootstraps with mapping
170 validation, GC bias correction, sequencing bias correction, 4 range factorization bins, a minimum
171 score fraction of 0.95 and VB optimization with a VB prior of 1e-5. The estimated counts were
172 analyzed using sleuth to identify DEGs[17]. A likelihood ratio test was used to identify differentially
173 expressed transcripts and a Wald test was used to get β values ($\log_2(x + 0.5)$). Additionally,
174 transcript-level aggregation was done against the Ensembl v94 annotation to determine gene-level
175 differential expression. P-values were corrected using the Benjamini-Hochberg procedure to

account for false discovery rate (FDR). Q-value (p-values corrected for FDR) significance was set for an FDR of <0.05. A principal component (PC) plot and heatmap was done to identify clusters.

Pathway and gene set enrichment analysis

Pathway and gene set enrichment for relevant databases was done using Gene Network 2.0. To investigate genes with larger fold changes, only differentially expressed genes with a $\beta > |0.5|$ were analyzed for pathway enrichments. Network clustering was done by grouping genes based on co-expression. Pathway analyses were also conducted with Gene Network 2.0 for the clusters using a Wilcoxin test.

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Data availability statement

Any data is available upon request of the corresponding author.

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Data availability statement

C.L. conducted the experiments, analyses and drafted the manuscript. F.S., V.V., D.R., F.A., S.D., Q.H., H.C. helped with experiments. A.D.L. and D.S. helped with bioinformatic analyses. P.A.D., G.A.R oversaw the study and drafting the manuscript.

Figures and Tables

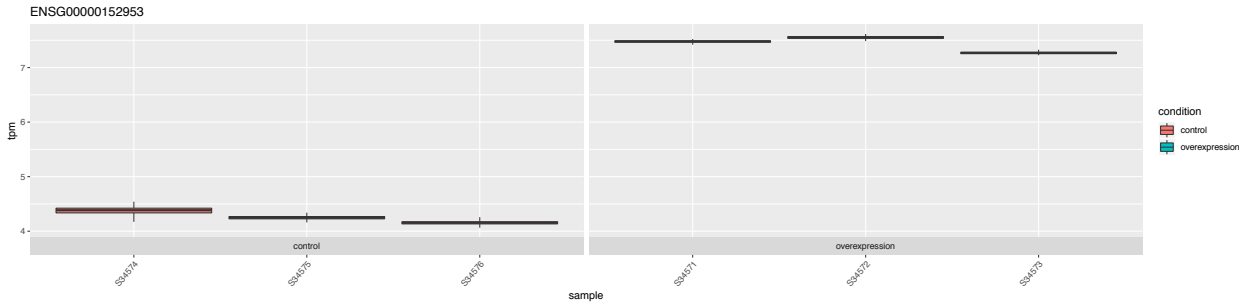

Figure 1. The RNA expression of *STK32B* overexpressed cells compared to empty-vector controls based on RNA sequencing data. Error bars show the technical variance determined with 200 bootstraps. Abbreviations: TPM, transcript per million.

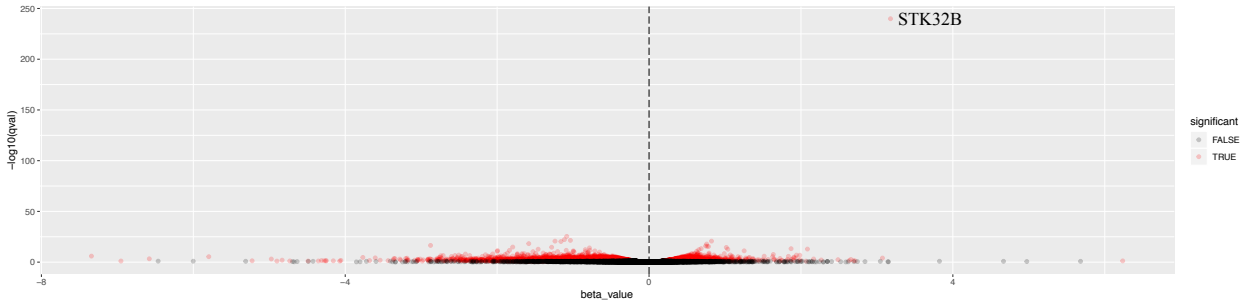

Figure 2. Volcano plot of comparing overexpressed *STK32B* to empty-vector controls. Differentially expressed genes (q < 0.05) are shown in red. Beta value was determined with a Wald's test.

Figure 3. Principle component analysis plot of RNA sequencing data of *STK32B* overexpressed cells and empty-vector controls. Overexpressed cells shown in blue and controls shown in red.

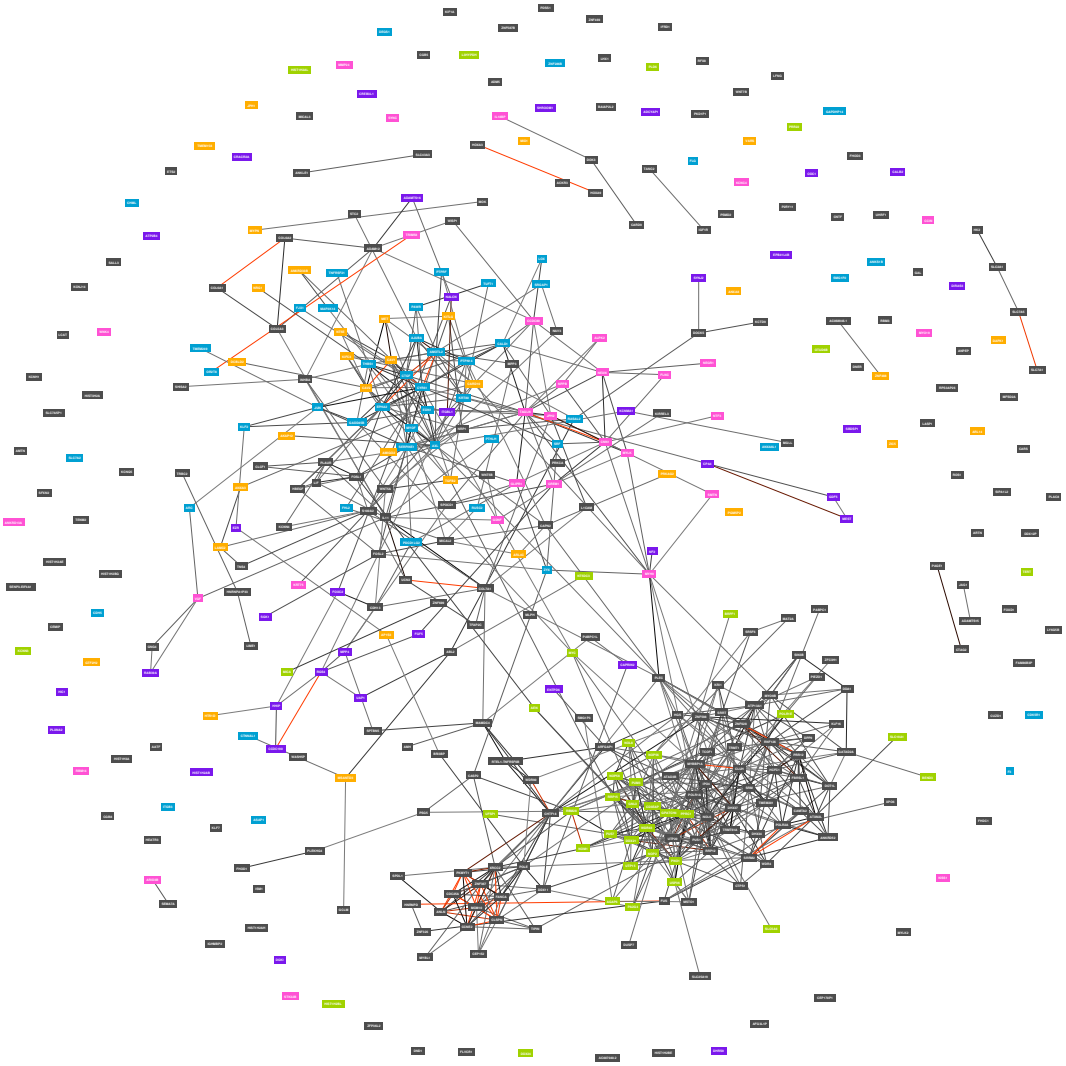

Figure 4. Gene network of differentially expressed genes based on co-expression of RNA sequencing data for 31,499 samples. Each unique colour represents a cluster.

261 **Table 1. Gene ontology and pathway analyses of differentially expressed genes.**

Gene Set ID	Gene Set Name	*P*	Database
KEGG_OLFACTORY_TRANSDUCTION	Olfactory transduction	2.68E-39	KEGG
KEGG_PATHWAYS_IN_CANCER	Pathways in cancer	2.96E-28	
KEGG_FOCAL_ADHESION	Focal adhesion	1.19E-26	
KEGG_BLADDER_CANCER	Bladder cancer	6.53E-25	
KEGG_ECM_RECEPTOR_INTERACTION	Ecm-receptor interaction	8.38E-25	
GO:0001650	fibrillar center	1.13E-29	GO Cellular
GO:0031012	extracellular matrix	8.57E-27	
GO:0001725	stress fiber	1.24E-26	
GO:0005925	focal adhesion	2.82E-23	
GO:0015629	actin cytoskeleton	4.02E-22	
REACTOME:R-HSA-381753	Olfactory Signaling Pathway	3.22E-46	Reactome
REACTOME:R-HSA-418555	G alpha (s) signalling events	1.19E-29	
REACTOME:R-HSA-3000170	Syndecan interactions	1.53E-27	
REACTOME:R-HSA-2022090	Assembly of collagen fibrils and other multimeric structures	4.31E-25	
REACTOME:R-HSA-1474290	Collagen formation	2.35E-24	
GO:0050911	detection of chemical stimulus involved in sensory perception of smell	1.00E-44	Go Processes
GO:0007411	axon guidance	9.50E-36	
GO:0048146	positive regulation of fibroblast proliferation	1.31E-33	
GO:0070588	calcium ion transmembrane transport	3.02E-31	
GO:0050907	detection of chemical stimulus involved in sensory perception	3.14E-31	

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265 **Table 2. Gene ontology and pathway analyses of differentially expressed gene for the three largest clusters based on co-**
266 **expression.**

Gene Set Name	P	Database	Cluster
Regulation of actin cytoskeleton	1.03E-13	KEGG	1
Pathways in cancer	1.23E-13		
Axon guidance	1.09E-12		
Focal adhesion	4.96E-12		
Arrhythmogenic right ventricular cardiomyopathy (ARVC)	4.63E-09		
fibrillar center	1.13E-29	GO Cellular	
extracellular matrix	8.57E-27		
stress fiber	1.24E-26		
focal adhesion	2.82E-23		
actin cytoskeleton	4.02E-22		
actin binding	3.66E-15	GO Function	
laminin binding	2.37E-13		
insulin-like growth factor binding	9.72E-13		
collagen binding	3.73E-12		
protein heterodimerization activity	5.54E-12		
cell migration involved in sprouting angiogenesis	7.07E-18	GO Processes	
cell migration	2.94E-16		
inactivation of MAPK activity	3.31E-15		
cytoskeleton organization	6.34E-15		
negative regulation of cell migration	9.12E-15		
Cell-extracellular matrix interactions	7.16E-15	Reactome	
Signal Transduction	1.98E-13		
Non-integrin membrane-ECM interactions	2.58E-12		
Signaling by Rho GTPases	1.84E-11		

EPHA-mediated growth cone collapse	2.30E-11		
Rna polymerase	4.57E-13	KEGG	2
Pyrimidine metabolism	1.37E-12		
Alzheimer's disease	3.55E-11		
Purine metabolism	4.11E-11		
Gnrh signaling pathway	1.84E-09		
nucleolus	5.96E-19	GO Cellular	
preribosome, large subunit precursor	2.21E-18		
small-subunit processome	5.13E-18		
DNA-directed RNA polymerase III complex	5.10E-15		
DNA-directed RNA polymerase I complex	1.85E-14		
pseudouridine synthase activity	7.75E-16	GO Function	
RNA binding	1.25E-15		
snoRNA binding	4.90E-14		
rRNA binding	3.55E-13		
ATP-dependent RNA helicase activity	5.23E-13		
RNA secondary structure unwinding	1.57E-16	GO Processes	
tRNA modification	7.73E-16		
positive regulation of gene expression, epigenetic	3.09E-15		
ribosomal large subunit biogenesis	3.17E-15		
maturation of SSU-rRNA from tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA)	3.32E-15		
rRNA modification in the nucleus and cytosol	3.38E-18	Reactome	
tRNA processing	1.65E-17		
tRNA modification in the nucleus and cytosol	1.66E-16		
rRNA processing	9.91E-15		
tRNA processing in the nucleus	1.69E-14		
RIG-I-like receptor signaling pathway	1.24E-10	KEGG	

Cytosolic DNA-sensing pathway	1.92E-07		
O-glycan biosynthesis	1.163E-06		
Focal adhesion	1.36893E-06		
Calcium signaling pathway	2.06986E-05		
caveola	2.00E-08	GO Cellular	
proteinaceous extracellular matrix	7.41E-08		
postsynaptic membrane	2.45E-06		
microfibril	4.99E-06		
extracellular matrix	1.12E-05		3
type I interferon receptor binding	3.64E-07	GO Function	
neuropeptide binding	5.89E-07		
scavenger receptor activity	6.23E-07		
calcium-dependent phospholipid binding	9.44E-07		
RNA binding	2.46E-06		
response to mechanical stimulus	8.42E-09	GO Processes	
lung development	1.55E-08		
negative regulation of neuron apoptotic process	5.29E-08		
cellular response to hypoxia	7.29E-08		
regulation of type I interferon-mediated signaling pathway	1.11E-07		
O-glycosylation of TSR domain-containing proteins	3.73E-10	Reactome	
Defective B3GALTL causes Peters-plus syndrome (PpS)	5.15E-10		
DDX58/IFIH1-mediated induction of interferon-alpha/beta	1.29E-09		
O-linked glycosylation	4.91E-08		
Diseases associated with O-glycosylation of proteins	1.04E-07		

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