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A Heterogeneous Population of Nuclear-Encoded Mitochondrial mRNAs Is Present in the Axons of Primary Sympathetic Neurons

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Abstract

Mitochondria are enriched in subcellular regions of high energy consumption, such as axons and pre-synaptic nerve endings. Accumulating evidence suggests that mitochondrial maintenance in these distal structural/functional domains of the neuron depends on the “in-situ” translation of nuclear-encoded mitochondrial mRNAs. In support of this notion, we recently provided evidence for the axonal targeting of several nuclear-encoded mRNAs, such as cytochrome c oxidase, subunit 4 (COXIV), ATP synthase, H⁺ transporting and mitochondrial Fo complex, subunit C1 (ATP5G1). Furthermore, we showed that axonal trafficking and local translation of these mRNAs plays a critical role in the generation of axonal ATP. Using a global gene expression analysis, this study identified a highly diverse population of nuclear-encoded mRNAs that were enriched in the axon and presynaptic nerve terminals. Among this population of mRNAs, fifty seven were found to be at least two fold more abundant in distal axons, as compared with the parental cell bodies. Gene ontology analysis of the nuclear-encoded mitochondrial mRNAs suggested functions for these gene products in molecular and biological processes, including but not limited to oxidoreductase and electron carrier activity and proton transport. Based on these results, we postulate that local translation of nuclear-encoded mitochondrial mRNAs present in the axons may play an essential role in local energy production and maintenance of mitochondrial function.

Keywords

Microarray analyses; mRNAs; subcellular localization; superior cervical; ganglion; Campenot cell culture chambers

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1. INTRODUCTION

It is now well-established that the synaptic nerve terminals contain large numbers of highly active mitochondria, and that these organelles play a fundamental role in synaptic function (Sheng and Cai, 2012). For example, synaptic transmission requires mitochondrial ATP generation and control of local $[Ca^{2+}]$ levels for neurotransmitter exocytosis, and potentiation of neurotransmitter release (Picard, 2015). Moreover, it is becoming well-recognized that mitochondrial dysfunction plays an important role in the pathophysiology of several major psychiatric disorders, while mitochondrial fragmentation has been shown to be one of the hallmarks of neurodegeneration (Lin and Beal, 2006). Historically, it has been accepted that nuclear-encoded proteins destined for mitochondria are translated in the cytoplasm of the neuronal cell soma and subsequently transported into the organelles located in the distal parts of cells by complex translocation machinery (Lin and Sheng, 2015). However, recent data suggest that nuclear-encoded mRNAs encoding mitochondrial proteins, such as COXIV and ATP5GI are targeted to the distal axon, and that the local synthesis of these proteins plays a key role in the maintenance of mitochondrial activity (Aschrafi et al., 2010; Natera-Naranjo et al., 2012). Hence, the local synthesis of mitochondrial proteins establishes an intimate link between translation and translocation to ensure the viability of the organelle located in these distal structural / functional neuronal domains (Gioio et al., 2001; Margeot et al., 2002). These observations also add to previous findings that axonal growth is dependent on the local synthesis of a multitude of proteins including metabolic as well as structural and cytoskeletal proteins (Holt and Schuman, 2013; Shane SS et al., 2015). In this regard, extracellular cues direct growth cones by inducing rapid changes in local protein expression, and developing axons contain the necessary translational machinery and specific mRNAs to support axonal growth and differentiation. Indeed, some of the first identified functions of axonal protein synthesis were intricately linked to its ability to mediate local translation of mRNAs that encode the cytoskeletal proteins in growing axons (Scott et al., 2015). Given the significance of the local translation of mitochondrial mRNAs in axonal outgrowth and maintenance, the present study was designed to identify the composition of nuclear-encoded mitochondrial mRNAs in the distal axons. Following a microarray approach and bioinformatic analysis, we identified 57 mRNAs that are highly enriched in these neuronal compartments. Quantitative PCR and *in situ* hybridization facilitated the validation and visualization of a subset of these mRNAs to the axons of SCG neurons.

2. MATERIAL AND METHODS

2.1 Neuronal Cell Culture

Cells were prepared from superior cervical ganglia (SCG) and were cultured as previously described (Hillefors et al., 2007). Briefly, SCG were dissected from three-day-old Sprague Dawley rat pups and dissociated using the Miltenyi Biotec gentleMACS Dissociator and Neuronal Tissue Dissociation Kit according to the manufacturer's protocol. Dissociated primary neurons were plated into the center compartment of a Campenot culture chamber. Neurons were cultured for 14 days in serum-free Neurobasal medium (Invitrogen) containing nerve growth factor (NGF; 50 ng/ml), 20 mM KCl, and 20 U/ml penicillin and 20

mg/ml streptomycin (Hyclone). Growth medium was changed every 3–4 days. After two DIV, 5-fluoro-2'-deoxyuridine (FUDR, 50 μ M) was added to the growth medium to inhibit the proliferation of non-neuronal cells and remained in the medium for the duration of the experiments. Phase-contrast microscopy was used to determine whether the side compartments, which contained distal axons, were devoid of neural soma and non-neuronal cells. After 14 DIV, distal axons were harvested from side compartments and parental soma were harvested from center compartments. For in situ hybridization histochemistry (ISH) studies, dissociated SCG neurons were plated on collagen coated Nunc® Lab-Tek® II-CC2™ glass chamber slides (Sigma-Aldrich) and cultured for 6 days before being processed for staining.

2.2 Microarray processing and analysis

RNA samples were prepared from three independent SCG axons and parental soma. Samples were prepared according to Affymetrix protocols (Affymetrix). RNA quality and quantity was monitored using the Bioanalyzer (Agilent) and NanoDrop (ThermoFisher Scientific) respectively. Per RNA labeling, 200 nanograms of total RNA was used in conjunction with the Affymetrix recommended protocol for the GeneChip 2.0 ST chips. The hybridization cocktail containing the fragmented and labeled cDNAs was hybridized to the Affymetrix Rat Genome 2.0 ST GeneChip. The chips were washed and stained by the Affymetrix Fluidics Station using the standard format and protocols as described by Affymetrix. The probe arrays were stained with streptavidin phycoerythrin solution (Molecular Probes, Carlsbad, CA) and enhanced by using an antibody solution containing 0.5 mg/mL of biotinylated anti-streptavidin (Vector Laboratories, Burlingame, CA). An Affymetrix Gene Chip Scanner 3000 was used to scan the probe arrays. Gene expression intensities were calculated using Affymetrix AGCC software. Partek Genomic Suite was used to normalize for Robust Multi-array Average (Robust Multichip Analysis), summarize the probes, log2 transform the data and conduct ANOVA analysis.

2.3 RNA isolation and quantitative reverse transcription PCR (qRT-PCR)

Total RNA was isolated from SCG axons and parental soma using Direct-Zol™ RNA MiniPrep (Zymo Research) according to the manufacturer's instructions. RNA was DNase treated using DNase I (New England Biolabs) according to the manufacturer's instructions. The purity of the total RNA preparations from both axons and cell bodies was monitored using the A260/280 ratio of *uv*-absorbance. The ratios for the RNA preparations were 1.9–2, indicating that the samples were highly pure. Total RNA was reversed transcribed into cDNA using a High Capacity RNA-to-cDNA Kit (Applied Biosystems). qRT-PCR was performed with gene specific primers designed using Primer-BLAST (Ye et al., 2012) and purchased from Invitrogen with the following sequences: Scn3b sense (FW): 5'-TGTGGTGTGACTTGAGGTGAT-3'; Scn3b antisense (RV): 5'-TGTTGGCTCTTCGGTTCAGG-3'; Glrb FW: 5'-CTTTCAGGTTGGTGAGACC-3'; Glrb RV: 5'-CGGAGGCTTCTTGTCTTTGC-3'; Cryab FW: 5'-ACTCCCTGAGCCCCCTTCTA-3'; Cryab RV: 5'-TGCTTCACGTCCAGGTT-CAC-3'; Atp5i FW: 5'-GTCACGGACAAAATGGTGCC-3'; Atp5i RV: 5'-AGTACCGGCCGAACCTTGATG-3'; Ndufa1 FW 5'-CGGGGGCAGGAAAAGA-GAG-3'; Ndufa1 RV: 5'-AGATGCGTCTA-TCGCGTTCC-3'; Lym2 FW: 5'-AGGCAACAAGTT-

CTCCTCCTC-3'; Lym2 RV: 5'-GATTGTATCCTCCTCGGTGGC-3'. qRT-PCR analysis of Prss35 was performed with a gene specific QuantiTect® primer (Qiagen). Quantitative RT-PCR was performed using VeriQuest SYBR Green quantitative PCR Master Mix (Affymetrix) and a StepOne Real-Time PCR system (Applied Biosystems). For each experimental RNA sample, the PCR reactions were run in triplicate. Target mRNA levels were normalized to the geometric mean of β -actin mRNA and 18S mRNA levels as suggested in (Ruijter et al., 2013).

2.4 In situ hybridization histochemistry (ISH)

ISH was performed on fixed SCG neuronal cultures, using the RNAscope® 2.0 High Definition (HD) - RED Assay (Advanced Cell Diagnostics) according to manufacturer's instructions. Cultured neurons were fixed in 10% formalin for 30 min, dehydrated in increasing concentrations of ethanol (50%, 70%, 100%) and rehydrated (70% ethanol, 50% ethanol, PBS) before applying Pretreat 1 and Pretreat 3 reagents. Target specific riboprobes (rat Cox4i1, RefSeq:NM_017202.1; rat AADAT, RefSeq:NM_017193.1) or a control riboprobe (DapB of *Bacillus subtilis* strain, GenBank: EF191515.1) (Advanced Cell Diagnostics) were hybridized for 2 h at 40°C, followed by incubation with signal amplification reagents. The hybridized probes were visualized under either bright field or fluorescence microscopy using a Texas Red filter.

2.5 Gene Ontology

Gene categories in the axonally-enriched mitochondrial genes were detected using STRING analysis (Jensen et al., 2009). The STRING database extracts experimental data from BIND, DIP, GRID, HPRD, IntAct, MINT, and PID databases and extracts curated data from Biocarta, BioCyc, GO, KEGG, and Reactome databases. All included GO categories had p-values of less than 0.05 after the Bonferroni correction for multiple comparisons.

2.6 Statistical Analysis

All statistical analyses were performed in Excel. Results are expressed as mean $\pm$ SEM. p-values were calculated by Student's two-tailed t test, except where Pearson's correlation is noted.

3. RESULTS

Previously, we have used cultured rat primary SCG neurons as a model system to study aspects of axonal nuclear-encoded mitochondrial mRNA functions and localization (Hillefors et al., 2007; Kaplan et al., 2009). Using a Campenot multi-compartment cell culture chamber, we purified mRNA from distal axons and their parental cell bodies 13 days in culture, and identified the composition of the nuclear-encoded mitochondrial mRNA population using a microarray analysis. Although we routinely monitor the side compartments of the Campenot cell culture chambers, where the distal axons of SCG neurons are localized, for the presence of neuronal cell soma and non-neuronal cells using light microscopy, trace cellular contamination of the axonal RNA preparation might still be detectable at the level of resolution afforded by microarray analysis or real-time PCR methodology. To address this issue, the purity of the RNA samples prepared from the distal

axons was assessed by RT-PCR using gene-specific primer sets for mRNAs present in neuronal cell soma. As shown in Fig. 1, amplicons for Sodium Channel, Voltage Gated, Type III Beta Subunit (SCN3B) and Glycine Receptor, Beta (GLRB), were readily detected in RNA prepared from the neurons present in the central compartment, but were not observed in RNA obtained from distal axons. These two genes were chosen as controls as they exhibited low axonal abundance levels in the microarray analysis, being more than thirty times more enriched in the cell body. This finding was consistent with previous results, which established the purity of the axonal mRNA preparations (Natera-Naranjo et al., 2010).

Two hundred nanograms of each RNA, isolated from the axons and cell bodies from three independent samples were fluorescently labeled and hybridized on a set of three Affymetrix rat microarray chips. All microarray chips showed successful and reproducible hybridization; therefore the data were processed for further analyses. Following microarray data annotation and normalization, a threshold value of 1.25-fold (p-value = 0.05) was chosen to identify transcripts that were significantly enriched in the axon. This analysis yielded 2844 unique axon enriched mRNAs. To confirm that a threshold value of 1.25-fold enrichment represented a reasonable estimate of enrichment, we visualized the presence of COXIV mRNA (fold change (fc): 3.74) and (AADAT), an aminotransferase with a 1.26 fc, a value, which was just above the cutoff threshold. COXIV mRNA was also chosen, since previous studies indicated that disruption of the axonal trafficking and local translation of this mRNA had profound importance for the regulation of mitochondrial activity, as well as axonal growth and function (Aschrafi et al., 2008; Kar et al., 2014). DapB, an mRNA present in *Bacillus subtilis*, was used as a negative control as this mRNA is not present or trafficked into axons (Fig. 2). Using ISH with target specific riboprobes strong fluorescent COXIV signals in the cell bodies, proximal and distal axons were detected (Fig. 2). These fluorescent signals appeared as puncta along the entire length of the axon. In contrast, very few fluorescent puncta were detectable when target riboprobes were hybridized to AADAT mRNA, suggesting a fc threshold value of 1.25 to be a valid cutoff value. No fluorescent DapB-specific fluorescent signal was observed in axons confirming that the axonal localization of these mRNAs was neither nonspecific nor the result of passive diffusion.

The list of 2844 enriched axonal mRNAs was annotated using MitoMiner, a database of the mitochondrial proteome (Smith and Robinson, 2016). The database identified 108 of these mRNAs as nuclear-encoded mitochondrial mRNAs (3.8% of the 1.25 fold enriched total axonal mRNAs). Additional information on the differentially expressed genes is summarized in Supplementary Table I. Since some messages were highly abundant in SCG neurons, we examined whether the mRNAs extracted from distal axons would be biased toward abundant messages. Toward this end, a Pearson product-moment correlation coefficient was computed to assess the relationship between the intensity of mRNAs in cell bodies and the intensity of these same mRNAs in axons (Fig. 3A). This inquiry revealed that the distribution of the mRNA levels in the axons and cell bodies is highly correlated ($r=0.967$, $n=108$, $p < 0.001$), suggesting that the hybridization of equal amounts of RNA probes from axonal and cell body samples should identify axon-enriched RNAs based on relative signal strength. Indeed, when fold-change was plotted against axonal mRNA intensity, the mRNAs most enriched in axons were not the most highly expressed messages in the neuronal cell soma (Fig. 3B). To

validate the microarray analysis, the expression of the five mRNAs most enriched in the axon was assessed by qRT-PCR. This analysis confirmed an axon enrichment of Prss35, Cryab, Atp5i, Ndufa1, Lyrm2 (Fig. 4).

To examine the biological processes in which axonal enriched nuclear-encoded mitochondrial mRNAs are involved, we chose a gene ontology approach. Toward this end, STRING pathway analysis was employed to investigate protein-protein interactions. This analysis yielded a number of biologically relevant molecular and biological processes (Table 1), namely oxidation-reduction, as well as cation, hydrogen ion transmembrane transport and proton transport. These processes are vital for energy production, and mRNAs associated with these processes may be enriched due to the high energy demands of axons. Interestingly, KEGG pathway mapping suggests a significant number of axonally-enriched nuclear-encoded mitochondrial mRNAs may be involved in the pathophysiology of neurodegenerative diseases, such as Huntington's, Alzheimer's, and Parkinson's disease. This analysis raises the possibility that the localization, translation, and regulation of the mRNAs play important roles in both axonal health and neurological disease.

4. DISCUSSION

In this communication, we identify greater than 100 nuclear-encoded mitochondrial mRNAs that are present in the distal axons of sympathetic neurons. In more than half of these cases, the relative abundance of the mRNA in question is at least 2-fold greater than the levels of corresponding mRNAs in the parental cell bodies. The extant literature has established that the asymmetric subcellular distribution and local translation of mRNA is critical for proper neuronal development and function (for review see (Holt and Schuman, 2013; Shane SS et al., 2015)). The molecular mechanisms underlying the sorting of mRNA to the axon involves the recognition of *cis*-acting elements present in the mRNA by RNA binding proteins that may function in both the regulation of mRNA transport and translation (for review see (Doxakis, 2014)). At present, little is known regarding the *cis*-acting regulatory elements that target nuclear-encoded mitochondrial mRNAs to the axon. Recently, we identified a 38-nt sequence in the 3' UTR of COXIV mRNA that is essential for the axonal trafficking of this messenger (Aschrafi et al., 2010). Our preliminary data also suggest that this putative stem-loop structure present in the 3' UTR of the mRNA contains sequence information that targets the mRNA to the organelles itself. In addition, we have previously demonstrated that the inhibition of the trafficking and local translation of this mRNA has marked inhibitory effects on mitochondrial activity, as well as on axonal growth and function (Aschrafi et al., 2010; Natera-Naranjo et al., 2012). This finding is consistent with the results of Corral-Debrinski and associates who reported that greater than 100 mRNAs encoding mitochondrial proteins are localized to the vicinity of mitochondria in both yeast and human HeLa cells (Marc et al., 2002; Sylvestre et al., 2003). Taken together, these findings support the idea that subcellular mRNA distribution and translational control are key mechanisms for post-transcriptional regulation of the expression of nuclear-encoded mitochondrial proteins. Previous large-scale experimental approaches to analyze the composition of mRNA in dendrites or axons relied on subcellular fractionation techniques and subsequent analysis with microarrays or PCR methodology. Unfortunately, fractionation methods are highly susceptible to contamination by fragments of glial or neuronal soma that may confound the

experimental findings. The utility of compartmentalized culture systems provides a versatile approach for the isolation and quantitation of functionally relevant transcripts in distal axons that are devoid of cell body and/or non-neuronal cell contaminants. Therefore, the constituents of the mRNAs we identified in this investigation are highly likely to represent endogenous axonal mRNAs. In addition, the limited resolution inherent with microarray analysis and the stringent cut-off criteria employed in this study of the diversity of the axonal nuclear-encoded mitochondrial mRNAs provides for a conservative estimate of the diversity of the axonal nuclear-encoded mitochondrial mRNA population. Taylor et al. (2009) identified a subset of nuclear-encoded mitochondrial mRNAs enriched in CNS axons, suggesting that the axonal enrichment identified in SCG neurons may hold true for CNS axons (Taylor et al., 2009). Interestingly, many mitochondrial mRNAs are downregulated in regenerating axons following axotomy, suggesting a modification of mitochondrial function in axons following injury. Gummy et al. (2011) employed microarray analysis of transcriptome present in embryonic and adult sensory axons. This study revealed that many nuclear-encoded and mitochondrial mRNAs were enriched in both the embryonic and adult axons. These mRNAs were also found to be enriched in the axons of SCG neurons in this study. Lastly, extracellular ligands can regulate protein generation within subcellular regions by specifically altering the localized levels of particular mRNAs, few of which have been annotated as axonally localized mitochondrial mRNAs (Willis et al., 2007).

A recent genome-wide association study found the strongest genetic association with bipolar disorder residing within the Prss35/Snap91 gene complex (Goes et al., 2012). Interestingly, Prss35 is one of the most highly enriched axonal mRNAs identified in our microarray analysis. The second highest axonally enriched nuclear-encoded mitochondrial mRNA in our mitochondrial mRNA population, Cryab, is a small heat shock protein shown to be upregulated in the substantia nigra of patient's with Parkinson's disease (Liu et al., 2015). Additionally, Alzheimer's disease has been associated with reduced expression of numerous nuclear-encoded mitochondrial genes (Liang et al., 2008). Consistent with these observations, the most significant Kegg pathways identified by gene ontology analysis of our microarray data include that for Huntington's disease, Alzheimer's disease, and Parkinson's disease. Taken together, these findings raise the possibility that dysregulation of the axonal trafficking and/or local translation of nuclear-encoded mitochondrial mRNAs may play an important role in the pathophysiology of a variety of neurodegenerative diseases.

5. CONCLUSIONS

In conclusion, our studies delineated the composition of population of nuclear-encoded mitochondrial mRNAs present in the axons of sympathetic neurons, and provide an important resource for future studies on the role played by these genes in the regulation of mitochondrial function in this important neuronal compartment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- The axonal mitochondrial mRNA population was annotated by microarray analysis
- These data were validated by qPCR methodology and *in situ* hybridization
- Axons contain > 100 different nuclear-encoded mitochondrial mRNAs
- Mitochondrial mRNAs are highly enriched in the axons of sympathetic neurons
- Proteins encoded by these mRNAs function in several diverse biological processes

Fig. 1.

RNA prepared from the distal axons of SCG neurons is free of neuronal cell body contamination. RT-PCR analysis was conducted on RNA obtained from the lateral and central compartments of the Campenot cell culture chambers using gene-specific primer sets for *Scn3b* and *Glrb*. PCR products were fractionated on 2.5% agarose gels and amplicons visualized by ethidium bromide staining. PCR reactions lacking cDNA served as negative (H_2O) controls.

Fig. 2.

The presence of nuclear-encoded mitochondrial mRNAs can be visualized in the somata and axons of primary SCG neurons. Visualization of nuclear-encoded mitochondrial mRNAs was achieved using RNAscope *in situ* hybridization. No signal was detected in axons hybridized with the DAPb control riboprobe. In axons hybridized with a COXIV riboprobe, mRNA appears as puncta of varying dimensions (arrows). In contrast, barely detectable fluorescent signals were observed using AADAT riboprobes.

Fig. 3. Subcellular distributions of nuclear-encoded mitochondrial mRNAs in the axons and cell body of SCG neurons as assessed by microarray analysis. (A) A scatter plot depicting the relative abundance of nuclear-encoded mitochondrial mRNA in the axons and cell bodies of SCG neurons. The distribution of the mRNAs levels in the axons and cell bodies is highly correlated (Pearson correlation coefficient of 0.967). (B) Comparison of axonal and cell body abundance ratios (fold-change) as a function of relative expression (intensity) for

nuclear-encoded mitochondrial mRNAs suggests that Prss35, Cryab and AT5i are highly enriched in SG axons.

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Fig. 4. qRT-PCR based quantification of Prss35, Cryab, ATP5i, Ndufa1 and Lym2 expression levels in the cell body and axons of SCG neurons. Data are shown as mean $\pm$ SEM; p-values are determined by Student's t-test. * $p < 0.05$, ** $p < 0.001$.

Table 1

Gene ontology analysis of axonally abundant nuclear-encoded mitochondrial mRNAs reveals association of these genes with multiple molecular and biological processes.

Category	GO_id	Term	# of molecules	P values
Molecular Processes	GO:0015078	hydrogen ion transmembrane transporter activity	9	2.21E-09
	GO:0004129	cytochrome-c oxidase activity	5	1.31E-05
	GO:0016675	oxidoreductase activity, acting on a heme group of donors	5	1.61E-05
	GO:0009055	electron carrier activity	6	3.47E-05
	GO:0016491	oxidoreductase activity	12	4.12E-05
	GO:0015077	monovalent inorganic cation transmembrane transporter activity	9	1.30E-04
	GO:0022890	inorganic cation transmembrane transporter activity	9	1.63E-03
	GO:0008137	NADH dehydrogenase (ubiquinone) activity	4	2.55E-03
	GO:0050136	NADH dehydrogenase (quinone) activity	4	2.55E-03
	GO:0003954	NADH dehydrogenase activity	4	2.94E-03
	GO:0008324	cation transmembrane transporter activity	9	5.63E-03
	GO:0016655	oxidoreductase activity, acting on NAD(P)H, quinone or similar compound as acceptor	4	6.26E-03
	GO:0015075	ion transmembrane transporter activity	9	4.00E-02
	GO:0044710	single-organism metabolic process	16	3.87E-01
	GO:0055114	oxidation-reduction process	13	3.91E-05
Biological Processes	GO:1902600	hydrogen ion transmembrane transport	8	2.68E-07
	GO:0015992	proton transport	8	8.92E-07
	GO:0006818	hydrogen transport	8	9.71E-07
	GO:0098662	inorganic cation transmembrane transport	8	1.45E-02
	GO:0015672	monovalent inorganic cation transport	8	2.16E-02
	GO:0006812	cation transport	10	2.56E-02
	GO:0098655	cation transmembrane transport	8	2.89E-02
	GO:0098660	inorganic ion transmembrane transport	8	3.47E-02
Kegg Pathway	190	Oxidative phosphorylation	22	1.74E-32
	5016	Huntington's disease	21	9.04E-28
	5010	Alzheimer's disease	20	1.79E-26
	5012	Parkinson's disease	19	6.29E-26
	4932	Non-alcoholic fatty liver disease (NAFLD)	17	1.29E-21
	1100	Metabolic pathways	27	7.11E-18
	4260	Cardiac muscle contraction	6	9.46E-06
	3010	Ribosome	5	5.20E-03