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Published in:
Human Molecular Genetics

DOI:
[10.1093/hmg/ddt102](https://doi.org/10.1093/hmg/ddt102)

2013

[Link to publication](#)

Citation for published version (APA):

Vemula, S. R., Puschmann, A., Xiao, J., Rudzinska, M., Frei, K. P., Truong, D. D., Wszolek, Z. K., & LeDoux, M. S. (2013). Role of Gα(olf) in Familial and Sporadic Adult-Onset Primary Dystonia. *Human Molecular Genetics*, 22(12), 2510-2519. <https://doi.org/10.1093/hmg/ddt102>

Total number of authors:
8

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Role of G α (olf) in Familial and Sporadic Adult-Onset Primary Dystonia

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Abstract

The vast majority of patients with primary dystonia are adults with focal or segmental distribution of involuntary movements. Although approximately 10% of probands have at least one first- or second-degree relative with dystonia, large families suited for linkage analysis are exceptional. After excluding mutations in known primary dystonia genes (*TOR1A*, *THAP1*, and *CIZ1*), whole-exome sequencing was used to identify a *GNAL* missense mutation (c.682G>T, p.V228F) in an African-American pedigree with clinical phenotypes that include cervical, laryngeal and hand-forearm dystonia. *GNAL* encodes guanine nucleotide-binding protein G(olf), subunit alpha [G α (olf)]. G α (olf) plays a role in olfaction, coupling D1 and A2a receptors to adenylyl cyclase, and histone H3 phosphorylation. Screening of 760 subjects with familial and sporadic primary dystonia identified three additional pedigrees with *GNAL* mutations (c.591dupA [p.R198Tfs*13]; c.733C>T [p.R245*]; and c.3G>A [p.M1_Q61del]). These mutations show incomplete penetrance. African-American subjects harboring the p.V228F mutation exhibited microsmia. Lymphoblastoid cell lines from subjects with the p.V228F mutation showed up-regulation of genes involved in cell cycle control and development. Consistent with known sites of network pathology in dystonia, immunocytochemical studies indicated that G α (olf) is highly expressed in the olfactory bulb, striatum and cerebellar Purkinje cells, and co-localized with corticotropin-releasing hormone receptors in the latter.

INTRODUCTION

Dystonia, defined as a syndrome of involuntary, sustained muscle contractions affecting one or more sites of the body, frequently causing twisting and repetitive movements or abnormal postures, is a genetically and clinically heterogeneous movement disorder (1). Dystonias are categorized by etiology (primary, secondary, dystonia-plus, and hereditary degenerative diseases with dystonia), age of onset (<20 or >20 years), and anatomical distribution (focal, segmental, multifocal, hemi-dystonia, or generalized) (1, 2). Most cases of primary dystonia begin in adults and primary adult-onset dystonia is more common in females (2, 3). Cervical dystonia (CD) or spasmodic torticollis is the most common form of focal dystonia, characterized by involuntary contractions of the neck muscles producing abnormal posturing of the head upon the trunk (4). In the United States, primary dystonia may be less common among African-Americans than Caucasians (5, 6). Genetic factors contribute to the pathogenesis of adult-onset primary dystonia since 10% of patients have one or more affected first- or second-degree relatives (2, 7). Familial and sporadic dystonia appear to share the same genetic underpinnings (8).

To date, four genes (*TOR1A*, *THAP1*, *CIZ1*, and *ANO3*) and several additional genetic loci (*DYT4*, *DYT7*, *DYT13*, and *DYT21*) have been associated with primary dystonia (9-16). Mutations in *TOR1A* are typically associated with early-onset generalized dystonia, whereas mutations in *THAP1* and *ANO3* most commonly cause segmental craniocervical dystonia. Mutations in *CIZ1* have only been reported in patients with adult-onset cervical dystonia (12). In aggregate, these four genes account for less than 5% of adult-onset cases of primary dystonia. Although adult-onset primary dystonia has a considerable heritable component, penetrance is reduced and the identification of genetic etiologies has been hampered by the availability of large pedigrees that are sufficiently powered for linkage analysis. With the advent of whole-exome sequencing, smaller pedigrees are proving suitable for the identification of sequence variants (SVs) causally associated with dystonia and other movement disorders.

In the present study, we demonstrate that familial adult-onset primary dystonia can result from mutations in *GNAL*, which encodes guanine nucleotide binding protein, alpha activating activity polypeptide, olfactory type (G α (olf), Golfalpha), a key player in signal transduction within olfactory neuroepithelium and the basal ganglia (17, 18).

RESULTS

Linkage analysis and exome sequencing

GNAL mutations were identified in the four independent pedigrees (Fig. 1). The largest family (A) was employed for linkage analysis and whole-exome sequencing. As previously described (6), the members of this pedigree reported ages of onset from 45 to 63 years (Table 1). All affected subjects in Families A, B, C and D had dystonia and varying degrees of microsmia with otherwise normal neurological examinations. In particular, no subject showed clinical evidence of ataxia, spasticity, oculomotor abnormalities, Parkinsonism, or neuropathy.

Eighteen subjects from Family A were genotyped with the HumanLinkage-24 Bead Chip (Illumina, San Diego, CA, USA). Call rates were over 99.6% for all 18 samples and reproducibility was 100% for 6 samples subjected to technical replication (Supplementary Material, Table S1). SNP genotypes were analyzed with Superlink-Online SNP version 1.0 (<http://cbl-hap.cs.technion.ac.il/superlink-snp/>) (19) using a dominant model with a mutant allele frequency of 0.0001 and penetrance of 0.5 (Supplementary Material, Table S2). The highest multi-point LOD score was 1.10. LOD scores of 1 or less were obtained within the DYT7, DYT13 and DYT21 loci.

In-solution whole-exome capture and massively parallel sequencing was performed on two definitely affected and one unaffected subject from Family A using the Agilent SureSelect^{XT} All Exon Kit 51 Mb (Santa Clara, CA, USA). After filtering and elimination of read errors with Sanger sequencing, three SVs were common to the two affected subjects and absent from the unaffected subject. However, only a single SV co-segregated with dystonia in Family A (*GNAL*; c.682G>T, p.V228F).

Post hoc linkage analysis of Family A using the c.682G>T *GNAL* genotype in place of clinical phenotypes with penetrance values of 0.5 and 0.99 yielded LOD scores of 2.90 and

4.03, respectively, at rs8795888 (Supplementary Material, Table S4; Fig. S1). This SNP is located near *GNAL* and within the previously published DYT7 locus on Chr 18p11.2 (14). Haplotype analysis of Chr 18p showed that SNPs near *GNAL* co-segregated with c.682G>T in subjects with dystonia (Fig. S2). *In silico* analyses with ClustalW2 (20), Polyphen-2 (21), SIFT (22) and MutationTaster (23) indicated that c.682G>T (p.V228F) altered a highly conserved amino acid and was disease causing (Supplementary Material, Table S5).

Mutation screening and *in silico* analysis

High-resolution melting (HRM) (7, 12) and Sanger sequencing were used for *GNAL* mutation screening in 760 subjects with mainly CD and 768 neurologically-normal controls (Supplementary Material, Tables S6-7). Five additional novel SVs were identified and *In silico* analyses predicted three of these variants to be pathogenic: c.591dupA (p.R198Tfs*13), c.733C>T (p.R245*), and c.3G>A (p.M1_Q61del). Two (c.591dupA and c.733C>T) of these three mutations are predicted to cause a frameshift and probably induce nonsense-mediated decay (NMD) while c.3G>A disrupts the start codon.

Role of G α (olf) in Olfaction

Given the established role of G α (olf) in olfaction and to investigate the potential utility of olfactory testing as a preclinical diagnostic tool in primary dystonia, Families A, B, C and D were examined with the 40-item University of Pennsylvania Smell Identification Test (24) (UPSIT; Sensonics, Inc., Haddon Heights, NJ) (Supplementary Material, Table S8). UPSIT data was analyzed with SAS 9.3 (SAS Institute Inc., Cary, NC). When data from all 4 families was grouped together, differences in mean UPSIT scores \pm standard error of the mean (SEM) among manifesting carriers ($n = 7$, 30.8 \pm 3.1), non-manifesting carriers ($n = 8$, 33.7 \pm 2.8) and non-carrier neurologically-normal family members ($n = 14$, 35.1 \pm 3.7) were not significant. However, an independent one-tailed *t*-test restricted to data from Family A indicated that

manifesting and non-manifesting mutation carriers ($n = 6$, 25.5 ± 2.9) had lower UPSIT scores than non-carrier neurologically-normal family members ($n = 5$, 33.0 ± 1.1 ; $p < 0.26$).

Relative expression of *GNAL* using qRT-PCR

GNAL has three isoforms. Isoform 1 (NM_182978.3) is the longest whereas Isoform 2 (NM_001142339.2) is the major isoform (Fig. 2). Relative expression of Isoforms 1 and 2 was examined in human brain and leukocytes using quantitative RT-PCR (Supplementary Material, Table S9). Adult human whole brain total RNA (FirstChoice® Human Brain Reference RNA [1 mg/ml]) was obtained from Ambion (Austin, TX, USA). Fetal human whole brain and adult human cerebral cortex, cerebellum, and substantia nigra total RNA were purchased from Clontech (Mountain View, CA, USA). Striatum total RNA was acquired from Agilent. Overall *GNAL* expression was highest in striatum and fetal whole brain whereas relative expression of Isoform 2 to Isoform 1 was highest in striatum and cerebral cortex. Suggestive of NMD, overall leukocyte expression of *GNAL* was reduced in one subject from Family B (c.591dupA). We were unable to detect Isoform 1 in leukocytes. The c.3G>A mutation in Family D had no apparent effect on leukocyte expression of *GNAL*.

Immunocytochemistry of G α (olf) in central nervous system

Since previous study of G α (olf) had focused on the olfactory bulb and striatum, we performed immunocytochemistry to obtain a more complete picture of G α (olf)'s distribution in the central nervous system. G α (olf)-immunoreactivity (IR) was present in olfactory bulb, striatum, thalamus, substantia nigra, and cerebellum at P14 and in adult rat brains (Fig. 3). In cerebellum, G α (olf)-IR was most prominent in Purkinje cells with weaker IR in granule cells. At P14, G α (olf)-IR was seen throughout the dendritic arbor of Purkinje cells. However, in adult cerebellum, G α (olf)-IR was largely restricted to the soma and proximal dendrites of Purkinje cells. In Purkinje cells, G α (olf) co-localized with CRH-RI/II, but not PMCA4. At P14, G α (olf)-IR

was concentrated in the somas and proximal dendrites of medium spiny and cholinergic neurons within the striatum. In adult brain, more diffuse striatal G α (olf)-IR was apparent. G α (olf)-IR was localized to ChAT-positive in striatal neurons at P14. However, in adult rat brain, G α (olf)-IR in striatal cholinergic neurons was weak. At P14, G α (olf)-IR was prominent in both dopaminergic and non-dopaminergic neurons of the substantia nigra. In adult rat brain, G α (olf)-IR remained robust in TH-positive neurons of the substantia nigra but was less conspicuous in TH-negative neurons.

Gene expression analysis

Given the diverse role of G-proteins in regulating myriad cellular processes, gene expression studies were performed to identify pathways possibly dysregulated by mutant G α (olf) [p.V228F]. These experiments employed RNA derived from lymphoblastoid cell lines established for 4 affected carriers and 4 non-carriers from Family A. Unpaired *t*-tests were used to filter significant probes. Probes that were significant at $p \leq 0.05$ with a mean fold change ≥ 1.5 were retained for downstream analyses.

In total, 82 genes were up-regulated and 29 were down-regulated (Supplementary Material, Tables S11 and S12; Fig. S3). Enrichment analysis of differentially expressed genes was performed with WebGestalt Gene Set Analysis Toolkit 2.0 (<http://bioinfo.vanderbilt.edu/webgestalt/>) (25). The differentially expressed gene set was compared to the human genome using the hypergeometric test followed by correction for multiple testing using the Benjamini & Hochberg (BH) method at a significance level of 0.05 (FDR <0.05) (26). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were accessed from WebGestalt (27, 28). Dysregulated cellular networks were examined with Ingenuity Pathway Analysis (IPA; Ingenuity, Redwood City, CA, USA).

Our gene set enrichment identified 15 significant KEGG pathways (Supplementary Material, Table S13). Up-regulated pathways included Wnt signaling (*LRP5*, *PLCB2* and

FZD3), cytokine-cytokine interactions (*IL17RB*, *TNFRSF14* and *CXCL10*), and arrhythmogenic right ventricular cardiomyopathy (*ACTN1*, *DMD* and *LMNA*). Top canonical pathways up-regulated, as indicated by IPA, included vitamin D receptor/retinoic acid X receptor (VDR/RXR) and G-protein receptor coupled signaling (*EMR2*, *FZD3*, *OXTR*, *PDE6G*, *PLCB2* and *PRKCE*) (Supplementary Material, Tables S14 and S15). The top dysregulated networks were involved in cell cycle control, development, cell death, and cellular proliferation (Table 2).

DISCUSSION

Previous work from several groups had suggested that a dystonia-associated gene resides on Chr 18p (14, 29-35). In 1996, Leube and colleagues reported a large German pedigree with CD and linkage to the region telomeric to D18S1153 on Chr 18p with a maximal LOD score of 3.17 (36). Follow-up studies narrowed this dystonia locus to a 30 cM region near D18S1098 but also presented evidence of locus heterogeneity in adult-onset CD (14, 37). More recent work has shed doubt on the DYT7 locus for CD on Chr 18p (38). Although CD and other forms of adult-onset dystonia are genetically heterogeneous, dystonia is a relatively common manifestation of 18p deletion syndrome (MIM 146390) (29, 31-35). Dystonia in 18p deletion syndrome can be focal, segmental or generalized. Some subjects with the 18p deletion syndrome also exhibit myoclonus and white matter abnormalities on magnetic resonance imaging of the brain. Nasir et al. identified a Chr 18p 15 Mb deletion that included *GNAL* in a mother and her son both affected with dystonia (33). Moreover, linkage to Chr 18p was reported in three brothers with late-onset hand-forearm dystonia (30). When integrated with previous work, the data reported herein indicates that (1) *GNAL* is the dystonia-associated gene within the DYT7 locus on Chr 18p, and (2) loss of function mutations in *GNAL* cause various anatomical distributions of dystonia.

G α (olf) belongs to a class of GTP-binding proteins (G proteins) which couple G-protein receptors to adenylyl cyclase. Heterotrimeric G protein complexes are composed of three

subunits (α , β , and γ). G proteins are categorized into four subfamilies according to their α -subunits (*Gas*, *Gai/o*, *Gaq*, and *Gα12*). *Gα(olf)* which has 88% amino acid homology to *Gas* is considered a member of the *Gas* family. Although *Gα(olf)* was originally discovered in the olfactory neuroepithelium and striatum, it has been identified in pancreatic β -cells, vestibular end organs, testis, spleen, lung and heart (39, 40). Our immunocytochemical work indicates that *Gα(olf)* is widely expressed in brain, especially in motor regions that have previously been associated with dystonia.

At the network level, dystonia has been considered a disorder of the (1) basal ganglia, (2) olivocerebellar pathways or (3) their interaction (41-43). In dystonic rats, dystonia has been associated with abnormal neurotransmission at the climbing fiber-Purkinje cell synapse (44). Climbing fiber activity leads to the release of CRH which facilitates induction of long-term depression at both parallel fiber and climbing fiber synapse (45). In the striatum, *Gα(olf)* is upregulated after lesioning the nigrostriatal dopaminergic pathway which contributes to levodopa-induced dyskinesias in models of Parkinson's disease (17).

Although mutations in *GNAL* had not previously been associated with olfactory dysfunction in humans, two Iranian families with isolated congenital anosmia showed linkage to 18p11.23-q12.2 (46). More importantly, mice deficient in *Gα(olf)* are anosmic (18). In our study, microsmia was most prominent in those members of African-American Family A also affected with dystonia. However, in the Caucasian families, microsmia was absent or variable in mutation carriers. These data suggest that, like dystonia, the penetrance of olfactory dysfunction may be reduced and dependent on genetic background. In addition, alternate olfactory signaling pathways may compensate for *Gα(olf)* deficiency given that, in contrast to the Caucasian families, Family A harbored a missense mutation in *GNAL*.

At the cellular level, some forms of primary dystonia have been characterized as neurodevelopmental disorders and several dystonia associated proteins (*THAP1*, *TAF1* and *CIZ1*) play important roles at the G₁-S checkpoint of the cell cycle (2, 10, 12, 47). Similarly, our

gene expression studies of Gα(olf) p.V228F lymphoblastoid cells lines identified dysregulated cellular networks involved in cell cycle, development and gene expression. Given that blockade of dopamine receptors promotes histone H3 phosphorylation through adenosine A2A receptor-mediated activation of Gα(olf), it is possible that loss Gα(olf) function may also disrupt G₁-S cell cycle control (48, 49).

MATERIALS AND METHODS

Human subjects

Human studies were conducted in accordance with the Declaration of Helsinki, with formal approval from the institutional review boards at each participating study site. All genetic and phenotypic analysis and publication of the results were approved by the University of Tennessee Health Science Center Institutional Review Board (#01-07346-XP). Enrollment of patients with primary dystonia and neurologically-normal controls is described in previous publications (7, 12). Genomic DNA was extracted from peripheral whole blood or saliva (Oragene DNA Self-Collection kit, DNA Genotek[®], Kanata, Ontario, Canada).

Exome sequencing and variant analysis

Three micrograms of genomic DNA from two affected and one control subject was sheared to yield 100-450 bp fragments. Sheared DNA was then subjected to Illumina paired-end library preparation followed by enrichment for target sequences (Agilent SureSelect^{XT} Automated Target Enrichment for Illumina Paired-End Multiplexed Sequencing). Enriched DNA fragments were sequenced on Illumina's HiSeq 2000 platform as paired-end 100 base reads (OtoGenetics Co., Norcross, GA USA). Over 99.5% of exons were covered at $\geq 2X$ and over 96.1% of exons were covered at $\geq 20X$ (Supplementary Material, Table S3). Percentage of exome coverage was based on exons targeted by the 51 Mb All Exon v4 Kit which incorporates Consensus Coding Sequence (CCDS), NCBI Reference Sequence (RefSeq) and GENCODE annotations.

Sequence reads (FASTQ) were mapped to the human reference genome (NCBI build 37.1) with NextGENe® (SoftGenetics, State College, PA). The average read length for all 3 samples was 97 nt. To maximize the probability of detecting the causal SV, all base changes occurring in ≥ 2 reads in any individual sample were classified as variants for downstream analyses. Variant comparison was done between the two affected and one unaffected subjects to filter down the number of SVs in order to identify the putative causal variant. With NextGENe® software, all filtering parameters were implemented simultaneously: 1) homozygous SVs, 2) intergenic SVs, 3) deep intronic SVs [≥ 12 nt from splice sites], 4) SVs reported in dbSNP, 5) synonymous SVs, and 6) non-pathogenic non-synonymous SVs (12, 50). In order to filter benign missense variants, the pathogenicity of non-synonymous single amino acid substitutions was interrogated with 5 *in silico* tools: PolyPhen-2, MutationTaster, SIFT_{new}, LRT_{new} (Likelihood Ratio Test) and PhyloP_{new} (50).

Immunocytochemistry

Perfusion-fixed (normal saline-4% paraformaldehyde in 0.1 M phosphate-buffered saline [PBS]) Postnatal Day 14 (P14) and adult Sprague-Dawley rat brains were sectioned in the mid-sagittal or coronal planes and processed for detection of G α (olf) with Purkinje cell marker (calbindin D-28K), tyrosine hydroxylase (TH), choline acetyltransferase (ChAT), a parallel fiber marker (PMCA4), and corticotropin releasing hormone receptors I and II (CRH-RI/II) (Supplementary Material, Table S10). After endogenous peroxidases were quenched, slides were blocked and exposed to the primary rabbit anti-GNAL antibody (1:100) overnight. After rinsing with PBS, slides were exposed to biotinylated goat anti-rabbit antibody (1:500) for 4 hrs, followed by rinsing and exposing to streptavidin for 1 hr. Labeling was visualized with nickel-intensified 3, 3'-diaminobenzidine (DAB) solution (Vector, Burlingame, CA, USA). For double-label fluorescent immunocytochemistry, mouse anti-calbindin monoclonal (1:1000; Sigma-aldrich), mouse anti-tyrosine hydroxylase (TH) polyclonal (1:1000; Chemicon International), or a goat

anti-choline acetyltransferase (ChAT) polyclonal (1:50; Chemicon International) antibodies were used in combination with rabbit anti-GNAL (1:100). The secondary antibodies were Cy2-tagged donkey anti-rabbit (1:250), and rhodamine red-X (RRX)-tagged donkey anti-mouse or anti-goat (1:250; Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Sections were incubated with secondary antibodies for 4 h and then rinsed, dehydrated, cleared and coverslipped with 1, 3 diethyl-8-phenylxanthine mounting compound (DPX; Sigma). For triple-label fluorescent immunocytochemistry, goat anti-CRH-RI/II polyclonal (1:100, Santa Cruz Biotechnology), mouse anti-PMCA4 monoclonal (1:100, Affinity BioReagents) antibodies were used in combination with rabbit anti-GNAL. The secondary antibodies were Cy2-tagged donkey anti-mouse (1:250), and rhodamine red-X (RRX)-tagged donkey anti-goat and Cy5-tagged donkey anti-rabbit (1:250). Sections were visualized with both epifluorescence (Leica DM 6000B; Wetzlar, Germany) and confocal laser-scanning (Zeiss LSM 710; Thornwood, NY, USA) microscopes.

Lymphoblastoid transformation and RNA extraction

Leukocytes from 4 affected carriers and 4 non-carriers from Family A were transformed to lymphoblastoid cell lines for downstream analysis. To establish lymphoblastoid cell lines, peripheral blood mononuclear cells were separated by centrifugation on a sodium diatrizoate polysucrose gradient and transformed with Epstein-Barr virus (Coriell). The lymphoblastoid cell lines were propagated in RPMI-1640 medium (Sigma-Aldrich) supplemented with 20% fetal bovine serum (Sigma-Aldrich) and 2 mM L-glutamine (Invitrogen) at 37°C in 5% CO₂. Confluent cells (1 × 10⁶ cells/ml) were harvested for RNA isolation. Ambion's TRI Reagent® was used to isolate RNA. The quality of total RNA derived from leukocytes and lymphoblastoid cell lines was assessed with a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies), and Agilent 2100 Bioanalyzer using the Agilent RNA 6000 Nano Chip kit.

Gene expression studies

Total RNA from lymphoblastoid cell lines of eight subjects were processed on Illumina® HumanHT-12 v.4 expression microarray platform (Illumina, San Diego, CA, USA), flexible for high throughput processing of 12 samples per beadchip, to assess the expression levels in each individual specimen. These arrays investigate whole-genome expression, providing coverage for more than 47,000 transcripts and known splice variants across the human transcriptome. Total RNA (200 ng) was processed with the Illumina® TotalPrep™ RNA Amplification Kit (Applied Biosystems) according to the company's protocol. The raw data was processed for errors and quality checks using Illumina's proprietary GenomeStudio® software. Data was normalized and summarized further with GeneSpring GX® 12.0 software (Agilent® Technologies, Santa Clara, CA).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

ACKNOWLEDGMENTS

The authors thank J. Searcy and A. Strongosky for their assistance with collecting clinical data.

Conflict of Interest statement. None declared

FUNDING

MSL was supported by the Bachmann-Strauss Dystonia & Parkinson Foundation, Dystonia Medical Research Foundation and NIH/NINDS R01NS069936. AP was supported by governmental funding of clinical research within the Swedish National Health Services (ALF-YF) and by the Swedish Parkinson Foundation (Parkinsonfonden). ZKW is partially supported by

the NIH/NINDS P50 NS072187, Mayo Clinic Florida (MCF) Research Committee, and Dystonia Medical Research Foundation.

REFERENCES

1. Fahn, S. (1988) Concept and classification of dystonia. *Advances in neurology*, 50, 1-8.
2. Ledoux, M.S. (2012) The genetics of dystonias. *Advances in genetics*, 79, 35-85.
3. LeDoux, M.S. (2012) Dystonia: phenomenology. *Parkinsonism & related disorders*, 18 Suppl 1, S162-164.
4. Chan, J., Brin, M.F. and Fahn, S. (1991) Idiopathic cervical dystonia: clinical characteristics. *Movement disorders : official journal of the Movement Disorder Society*, 6, 119-126.
5. Marras, C., Van den Eeden, S.K., Fross, R.D., Benedict-Albers, K.S., Klingman, J., Leimpeter, A.D., Nelson, L.M., Risch, N., Karter, A.J., Bernstein, A.L. *et al.* (2007) Minimum incidence of primary cervical dystonia in a multiethnic health care population. *Neurology*, 69, 676-680.
6. Puschmann, A., Xiao, J., Bastian, R.W., Searcy, J.A., LeDoux, M.S. and Wszolek, Z.K. (2011) An African-American family with dystonia. *Parkinsonism & related disorders*, 17, 547-550.
7. Xiao, J., Zhao, Y., Bastian, R.W., Perlmutter, J.S., Racette, B.A., Tabbal, S.D., Karimi, M., Paniello, R.C., Wszolek, Z.K., Uitti, R.J. *et al.* (2010) Novel THAP1 sequence variants in primary dystonia. *Neurology*, 74, 229-238.
8. Defazio, G., Abbruzzese, G., Girlanda, P., Liguori, R., Santoro, L., Tinazzi, M. and Berardelli, A. (2012) Phenotypic overlap in familial and sporadic primary adult-onset extracranial dystonia. *Journal of neurology*, 259, 2414-2418.
9. Ozelius, L.J., Hewett, J.W., Page, C.E., Bressman, S.B., Kramer, P.L., Shalish, C., de Leon, D., Brin, M.F., Raymond, D., Corey, D.P. *et al.* (1997) The early-onset torsion dystonia gene (DYT1) encodes an ATP-binding protein. *Nature genetics*, 17, 40-48.
10. Fuchs, T., Gavarini, S., Saunders-Pullman, R., Raymond, D., Ehrlich, M.E., Bressman, S.B. and Ozelius, L.J. (2009) Mutations in the THAP1 gene are responsible for DYT6 primary torsion dystonia. *Nature genetics*, 41, 286-288.
11. Charlesworth, G., Plagnol, V., Holmstrom, K.M., Bras, J., Sheerin, U.M., Preza, E., Rubio-Agusti, I., Ryten, M., Schneider, S.A., Stamelou, M. *et al.* (2012) Mutations in ANO3 Cause Dominant Craniocervical Dystonia: Ion Channel Implicated in Pathogenesis. *Am J Hum Genet*, 91, 1041-1050.
12. Xiao, J., Uitti, R.J., Zhao, Y., Vemula, S.R., Perlmutter, J.S., Wszolek, Z.K., Maraganore, D.M., Auburger, G., Leube, B., Lehnhoff, K. *et al.* (2012) Mutations in CIZ1 cause adult-onset primary cervical dystonia. *Annals of Neurology*, in press., n/a-n/a.
13. Ahmad, F., Davis, M.B., Waddy, H.M., Oley, C.A., Marsden, C.D. and Harding, A.E. (1993) Evidence for locus heterogeneity in autosomal dominant torsion dystonia. *Genomics*, 15, 9-12.
14. Leube, B., Hendgen, T., Kessler, K.R., Knapp, M., Benecke, R. and Auburger, G. (1997) Sporadic focal dystonia in Northwest Germany: Molecular basis on chromosome 18p. *Annals of Neurology*, 42, 111-114.
15. Norgren, N., Mattson, E., Forsgren, L. and Holmberg, M. (2011) A high-penetrance form of late-onset torsion dystonia maps to a novel locus (DYT21) on chromosome 2q14.3-q21.3. *Neurogenetics*, 12, 137-143.
16. Valente, E.M., Bentivoglio, A.R., Cassetta, E., Dixon, P.H., Davis, M.B., Ferraris, A., Ialongo, T., Frontali, M., Wood, N.W. and Albanese, A. (2001) DYT13, a novel primary torsion dystonia locus, maps to chromosome 1p36.13--36.32 in an Italian family with cranial-cervical or upper limb onset. *Ann Neurol*, 49, 362-366.
17. Alcacer, C., Santini, E., Valjent, E., Gaven, F., Girault, J.A. and Herve, D. (2012) Galpha(olf) mutation allows parsing the role of cAMP-dependent and extracellular signal-regulated kinase-

- dependent signaling in L-3,4-dihydroxyphenylalanine-induced dyskinesia. *J Neurosci*, 32, 5900-5910.
18. Belluscio, L., Gold, G.H., Nemes, A. and Axel, R. (1998) Mice deficient in G(olf) are anosmic. *Neuron*, 20, 69-81.
 19. Silberstein, M., Tzemach, A., Dovgolevsky, N., Fishelson, M., Schuster, A. and Geiger, D. (2006) Online system for faster multipoint linkage analysis via parallel execution on thousands of personal computers. *Am J Hum Genet*, 78, 922-935.
 20. Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R. *et al.* (2007) Clustal W and Clustal X version 2.0. *Bioinformatics*, 23, 2947-2948.
 21. Adzhubei, I.A., Schmidt, S., Peshkin, L., Ramensky, V.E., Gerasimova, A., Bork, P., Kondrashov, A.S. and Sunyaev, S.R. (2010) A method and server for predicting damaging missense mutations. *Nature methods*, 7, 248-249.
 22. Ng, P.C. and Henikoff, S. (2001) Predicting deleterious amino acid substitutions. *Genome research*, 11, 863-874.
 23. Schwarz, J.M., Rodelsperger, C., Schuelke, M. and Seelow, D. (2010) MutationTaster evaluates disease-causing potential of sequence alterations. *Nature methods*, 7, 575-576.
 24. Doty, R.L., Shaman, P. and Dann, M. (1984) Development of the University of Pennsylvania Smell Identification Test: a standardized microencapsulated test of olfactory function. *Physiology & behavior*, 32, 489-502.
 25. Zhang, B., Kirov, S. and Snoddy, J. (2005) WebGestalt: an integrated system for exploring gene sets in various biological contexts. *Nucleic acids research*, 33, W741-748.
 26. Benjamini, Y. and Hochberg, Y. (1995) Controlling the False Discovery Rate - a Practical and Powerful Approach to Multiple Testing. *J Roy Stat Soc B Met*, 57, 289-300.
 27. Ashburner, M., Ball, C.A., Blake, J.A., Botstein, D., Butler, H., Cherry, J.M., Davis, A.P., Dolinski, K., Dwight, S.S., Eppig, J.T. *et al.* (2000) Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. *Nature genetics*, 25, 25-29.
 28. Kanehisa, M. and Goto, S. (2000) KEGG: kyoto encyclopedia of genes and genomes. *Nucleic acids research*, 28, 27-30.
 29. Awaad, Y., Munoz, S. and Nigro, M. (1999) Progressive dystonia in a child with chromosome 18p deletion, treated with intrathecal baclofen. *Journal of child neurology*, 14, 75-77.
 30. Bhidayasiri, R., Jen, J.C. and Baloh, R.W. (2005) Three brothers with a very-late-onset writer's cramp. *Movement disorders : official journal of the Movement Disorder Society*, 20, 1375-1377.
 31. Graziadio, C., Rosa, R.F., Zen, P.R., Pinto, L.L., Barea, L.M. and Paskulin, G.A. (2009) Dystonia, autoimmune disease and cerebral white matter abnormalities in a patient with 18p deletion. *Arquivos de neuro-psiquiatria*, 67, 689-691.
 32. Kowarik, M.C., Langer, S., Keri, C., Hemmer, B., Oexle, K. and Winkelmann, J. (2011) Myoclonus-Dystonia in 18p Deletion Syndrome. *Movement Disord*, 26, 560-561.
 33. Nasir, J., Frima, N., Pickard, B., Malloy, M.P., Zhan, L.P. and Grunewald, R. (2006) Unbalanced whole arm translocation resulting in loss of 18p in dystonia. *Movement Disord*, 21, 859-863.
 34. Postma, A.G., Verschuuren-Bemelmans, C.C., Kok, K. and van Laar, T. (2009) Characteristics of dystonia in the 18p deletion syndrome, including a new case. *Clinical neurology and neurosurgery*, 111, 880-882.
 35. Tezzon, F., Zanoni, T., Passarin, M.G. and Ferrari, G. (1998) Dystonia in a patient with deletion of 18p. *Ital J Neurol Sci*, 19, 90-93.
 36. Leube, B., Rudnicki, D., Ratzlaff, T., Kessler, K.R., Benecke, R. and Auburger, G. (1996) Idiopathic torsion dystonia: Assignment of a gene to chromosome 18p in a German family with adult onset, autosomal dominant inheritance and purely focal distribution. *Hum Mol Genet*, 5, 1673-1677.

37. Klein, C., Ozelius, L.J., Hagenah, J., Breakefield, X.O., Risch, N.J. and Vieregge, P. (1998) Search for a founder mutation in idiopathic focal dystonia from northern Germany. *Am J Hum Genet*, 63, 1777-1782.
38. Winter, P., Kamm, C., Biskup, S., Kohler, A., Leube, B., Auburger, G., Gasser, T., Benecke, R. and Muller, U. (2012) DYT7 gene locus for cervical dystonia on chromosome 18p is questionable. *Movement disorders : official journal of the Movement Disorder Society*, in press.
39. Regnaud, K.L., Leteurtre, E., Gutkind, S.J., Gspach, C.P. and Emami, S. (2002) Activation of adenylyl cyclases, regulation of insulin status, and cell survival by G alpha olf in pancreatic beta-cells. *Am J Physiol-Reg I*, 282, R870-R880.
40. Wackym, P.A., Cioffi, J.A., Erbe, C.B. and Popper, P. (2005) G-protein Golfalpha (GNAL) is expressed in the vestibular end organs and primary afferent neurons of Rattus norvegicus. *J Vestibul Res-Equil*, 15, 11-15.
41. Neychev, V.K., Fan, X., Mitev, V.I., Hess, E.J. and Jinnah, H.A. (2008) The basal ganglia and cerebellum interact in the expression of dystonic movement. *Brain*, 131, 2499-2509.
42. Argyelan, M., Carbon, M., Niethammer, M., Ulug, A.M., Voss, H.U., Bressman, S.B., Dhawan, V. and Eidelberg, D. (2009) Cerebellothalamocortical connectivity regulates penetrance in dystonia. *J Neurosci*, 29, 9740-9747.
43. LeDoux, M.S. (2011) Animal models of dystonia: Lessons from a mutant rat. *Neurobiol Dis*, 42, 152-161.
44. LeDoux, M.S. and Lorden, J.F. (2002) Abnormal spontaneous and harmaline-stimulated Purkinje cell activity in the awake genetically dystonic rat. *Experimental brain research. Experimentelle Hirnforschung. Experimentation cerebrale*, 145, 457-467.
45. Ohtsuki, G., Piochon, C. and Hansel, C. (2009) Climbing fiber signaling and cerebellar gain control. *Frontiers in cellular neuroscience*, 3, 4.
46. Ghadami, M., Majidzadeh, A.K., Morovvati, S., Damavandi, E., Nishimura, G., Komatsu, K., Kinoshita, A., Najafi, M.T., Niikawa, N. and Yoshiura, K. (2004) Isolated congenital anosmia with morphologically normal olfactory bulb in two Iranian families: a new clinical entity? *American journal of medical genetics. Part A*, 127A, 307-309.
47. Makino, S., Kaji, R., Ando, S., Tomizawa, M., Yasuno, K., Goto, S., Matsumoto, S., Tabuena, M.D., Maranon, E., Dantes, M. *et al.* (2007) Reduced neuron-specific expression of the TAF1 gene is associated with X-linked dystonia-parkinsonism. *Am J Hum Genet*, 80, 393-406.
48. Bertran-Gonzalez, J., Hakansson, K., Borgkvist, A., Irinopoulou, T., Brami-Cherrier, K., Usiello, A., Greengard, P., Herve, D., Girault, J.A., Valjent, E. *et al.* (2009) Histone H3 phosphorylation is under the opposite tonic control of dopamine D2 and adenosine A2A receptors in striatopallidal neurons. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology*, 34, 1710-1720.
49. Rodriguez-Collazo, P., Snyder, S.K., Chiffer, R.C., Zlatanova, J., Leuba, S.H. and Smith, C.L. (2008) cAMP signaling induces rapid loss of histone H3 phosphorylation in mammary adenocarcinoma-derived cell lines. *Experimental cell research*, 314, 1-10.
50. Liu, X., Jian, X. and Boerwinkle, E. (2011) dbNSFP: a lightweight database of human nonsynonymous SNPs and their functional predictions. *Human mutation*, 32, 894-899.

ILLUSTRATIONS

Figure 1. Family pedigrees

Filled symbols, definitely affected. Half-filled symbols, probably affected. Symbols with central dots, unaffected carriers. Arrows, probands. *GNAL* genotypes: wild-type (+/+) and heterozygous mutant (+/-).

Figure 2. Organization of *GNAL* gene, transcripts and full-length protein

(A) Structure of *GNAL* on Chr 18p presented in the 5' to 3' direction showing the location of four identified mutations in probands with dystonia. (B) The long and major isoforms of *GNAL* differ at Exon 1. (C) Missense mutations in highly conserved regions of G α (olf) are shown in relationship to GTP binding domains (G1 – G5). (D) The three missense mutations in G α (olf) show conservation across species.

Figure 3. Immunolocalization of G α (olf) in P14 and adult rat brain.

(A) Para-sagittal rat brain section. OB, olfactory bulb. ST, striatum. TH, thalamus. B1-6, Double-label fluorescence immunocytochemistry for simultaneous detection of Purkinje cell marker calbindin (red), TH or ChAT (red) with G α (olf) (green) in P14 (B1-3) and adult (B4-6) rat brain. G α (olf)-IR was present in ChAT-positive cholinergic neurons in striatum (C1-6) and TH-positive dopaminergic neurons in substantia nigra (D1-6). E1-8, Triple-label fluorescent immunocytochemistry for simultaneous detection of PMCA4 (green), CRH-R1/II (red) and G α (olf) (blue) in P14 (E1-4) and adult (E5-8) rat brains. Scale bar, 2 mm for A and 100 μ m for the remaining images.

Table 1. *GNAL* mutant phenotypes

Subject	Age	Gender	Age of Onset	Anatomical Distribution	<i>GNAL</i> Mutation
A-II-03	80	F	50	segmental dystonia (cervical*, blepharospasm, oromandibular, spasmodic dysphonia), anosmia	c.682G>T (p.V228F)
A-II-05	78	M	NA	segmental dystonia (spasmodic dysphonia, hand-forearm)	c.682G>T (p.V228F)
A-III-02	67	F	63	generalized dystonia (cervical*, hand-forearm, lower limb)	c.682G>T (p.V228F)
A-III-05	55	F	50	segmental dystonia (cervical*, oromandibular, spasmodic dysphonia, hand-forearm), moderate microsmia	c.682G>T (p.V228F)
A-III-08	49	M	45	cervical dystonia*, severe microsmia	c.682G>T (p.V228F)
B-III-02	56	F	38	cervical dystonia*	c.591dupA (p.R198Tfs*13)
B-III-08	46	F	37	cervical dystonia*	c.591dupA (p.R198Tfs*13)
B-III-04	54	F	NA	cervical dystonia (probable)	c.591dupA (p.R198Tfs*13)
C-II-05	55	F	45	cervical dystonia, moderate microsmia	c.733C>T (p.R245*)
D-II-02	55	F	40	cervical dystonia	c.3G>A (p.M1_Q61del)
D-II-04	44	F	41	cervical dystonia*	c.3G>A (p.M1_Q61del)

*dystonic head tremor

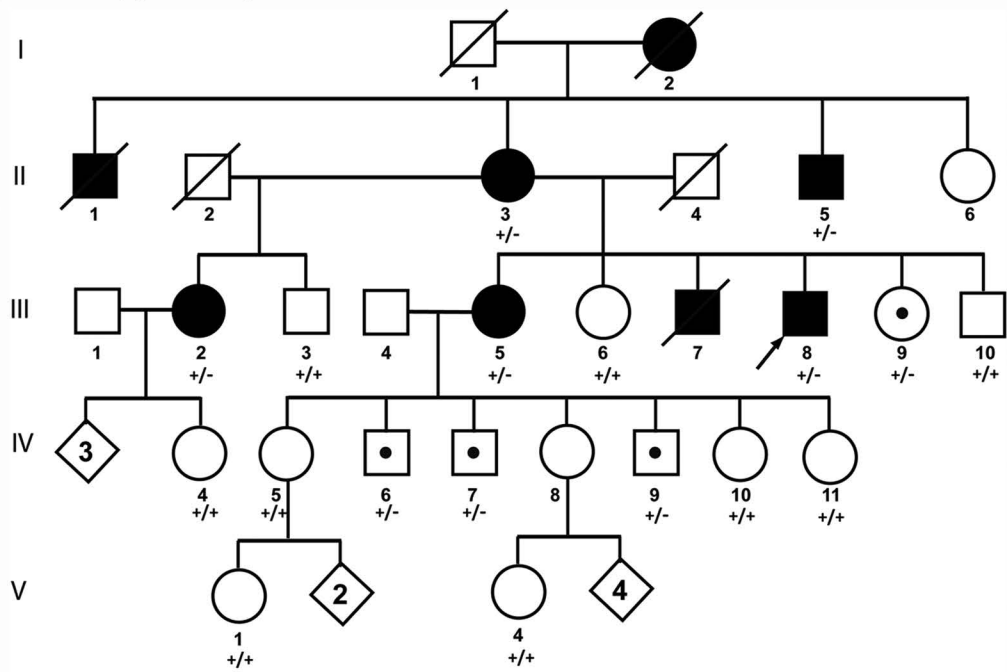
Table 2. Ingenuity Pathway Analysis - Top dysregulated networks

Network	Genes	Score ^a	Focus Genes ^b	Top Functions
1	<i>CSTB, DMD, F13A1, FHL3, FLOT2, FZD3, MIR155HG, MSX1, NFIC, NT5C3, PABPC3, PDE6G, SIK3, TSC22D1</i>	24	14	Cell Cycle, Cellular Development, Connective Tissue Development and Function
2	<i>EMR2, EXOC6, FXVD2, HES1, IFITM3, IKZF1, KRTAP17-1, LIN7A, RAB9A, SFMBT2, SP140, SYT11</i>	19	12	Cellular Development, Cellular Growth and Proliferation, Hematological System Development and Function
3	<i>AKAP7, CERS6, CKLF, CLINT1, HLA-DRB4, IL17RB, SLC2A8, TNFRSF14, TPST1, TRAF3IP3</i>	15	10	RNA Damage and Repair, Cell Death and Survival, Gene Expression

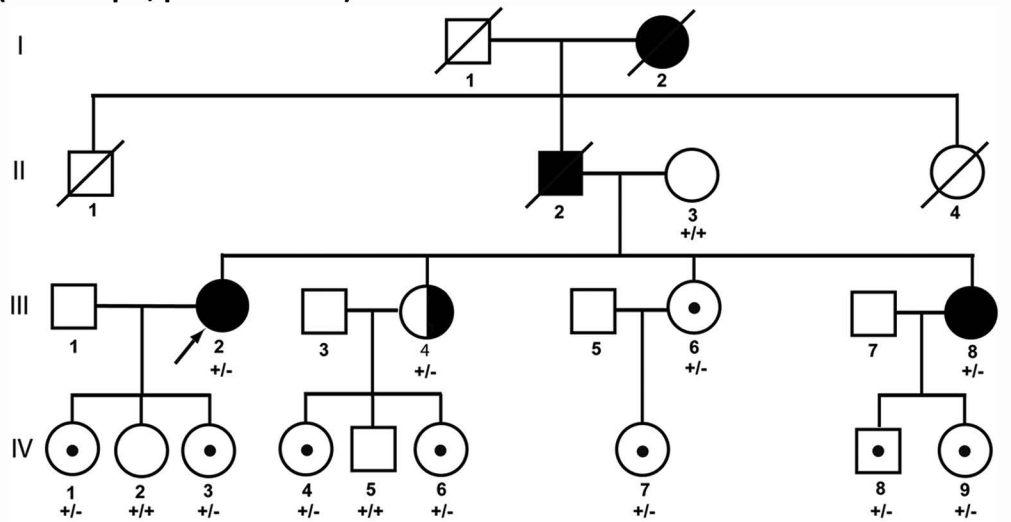
^aNetwork score is the negative log of the *p*-value for the likelihood that network molecules would be found together by chance. Higher scores indicate a greater statistical likelihood that molecules depicted in the network are interconnected. ^bNumber of significantly dysregulated genes associated with that pathway in subjects from Family A with the p.V228F mutation.

ABBREVIATIONS: None

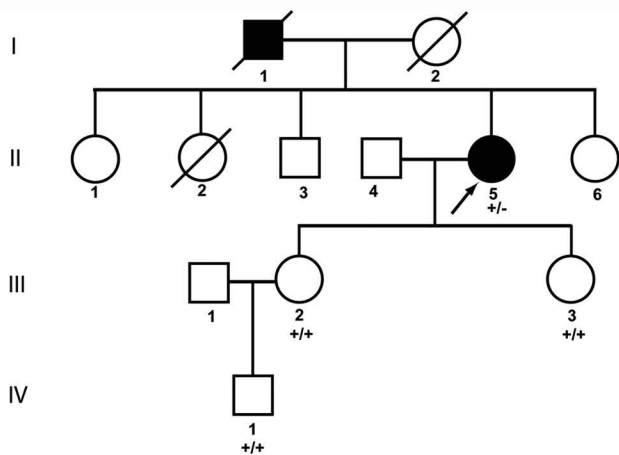
Family A (c.682G>T, p.V228F)



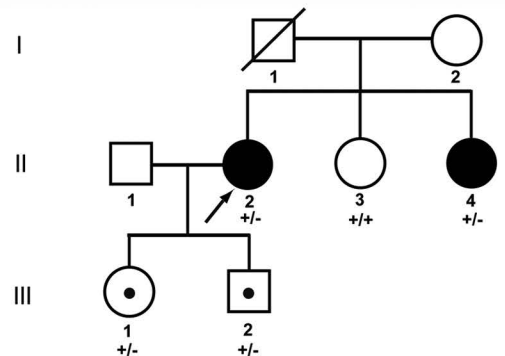
Family B (c.591dupA, p.R198Tfs*13)

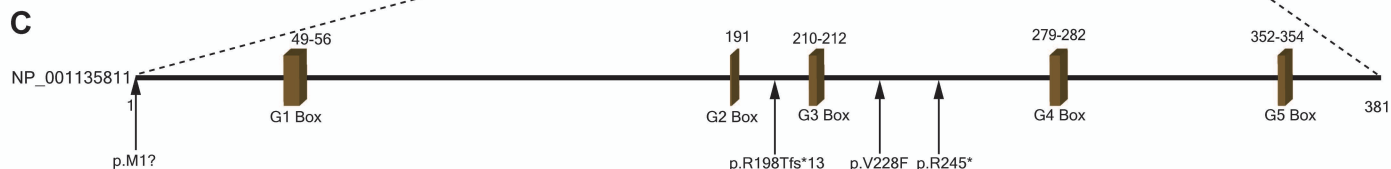
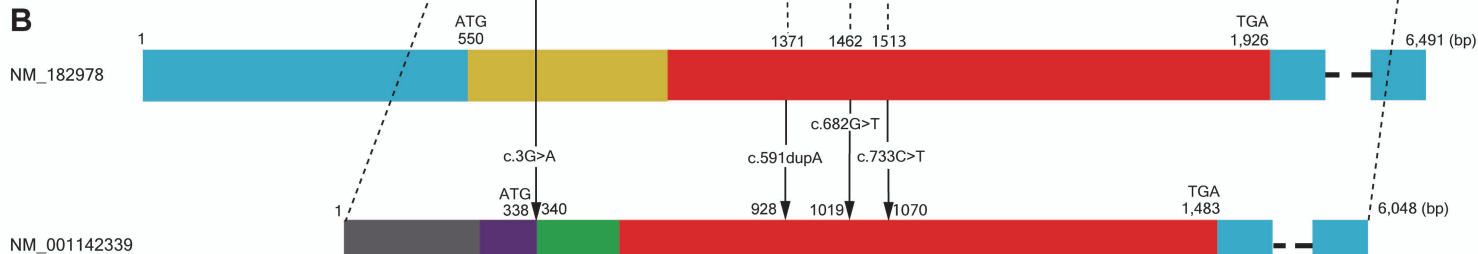


Family C (c.733C>T, p.R245*)



Family D (c.3G>A, p.M1?)





D

Homo sapiens	GCLGGNSKTTEDQGVDE-KERREANKKIEKQLQKERLAYKATHRLLLLL	49	QVDKVN FHMFDVGGQRDERRKWIQC FND TAI IYVAACSSYNMVI EDNN	249
Mus musculus	GCLGNSSKTAE DQGVDE-KERREANKKIEKQLQKERLAYKATHRLLLLL	49	QVDKVN FHMFDVGGQRDERRKWIQC FND TAI IYVAACSSYNMVI EDNN	249
Rattus norvegicus	GCLGNSSKTAE DQGVDE-KERREANKKIEKQLQKERLAYKATHRLLLLL	49	QVDKVN FHMFDVGGQRDERRKWIQC FND TAI IYVAACSSYNMVI EDNN	249
Callithrix jacchus	GCLGN-SKTTEDQGVDE-KERREANKKIEKQLQKERLAYKATHRLLLLL	48	QVDKVN FHMFDVGGQRDERRKWIQC FND TAI IYVAACSSYNMVI EDNN	248
Pan troglodytes	GCLGGNSKTTEDQGVDE-KERREANKKIEKQLQKERLAYKATHRLLLLL	49	QVDKVN FHMFDVGGQRDERRKWIQC FND TAI IYVAACSSYNMVI EDNN	249
Sus scrofa	GCLGN--SKTDQRNEE-KAQREANKKIEKQLQKDKQVYKATHRLLLLL	47	QVDKVN FHMFDVGGQRDERRKWIQC FND TAI IYVAACSSYNMVI EDNQ	247
Cricetulus griseus	GCLGNSSKTAE DQGVDE-KERREANKKIEKQLQKERLAYKATHRLLLLL	49	QVDKVN FHMFDVGGQRDERRKWIQC FND TAI IYVAACSSYNMVI EDNN	249
Oryctolagus cuniculus	GCLGN-SKTTEDQGVDE-KERREANKKIEKQLQKERLAYKATHRLLLLL	48	QVDKVN FHMFDVGGQRDERRKWIQC FND TAI IYVAACSSYNMVI EDNN	248
Ailuropoda melanoleuca	GCLGN-SKTTEDQGVDE-KERREANKKIEKQLQKERLAYKATHRLLLLL	48	QVDKVN FHMFDVGGQRDERRKWIQC FND TAI IYVAACSSYNMVI EDNN	248
Canis lupus familiaris	GCLGN-SKTAEDQGVDE-KERREANKKIEKQLQKERLAYKATHRLLLLL	48	QVDKVN FHMFDVGGQRDERRKWIQC FND TAI IYVAACSSYNMVI EDNN	248
Otolemur garnettii	GCLGN-SKTAEDQGVDE-KERREANKKIEKQLQKERLAYKATHRLLLLL	48	QVDKVN FHMFDVGGQRDERRKWIQC FND TAI IYVAACSSYNMVI EDNN	248
Loxodonta africana	GCLGN-NKTTEDQGVDE-KERREANKKIEKQLQKERLAYKATHRLLLLL	48	QVDKVN FHMFDVGGQRDERRKWIQC FND TAI IYVAACSSYNMVI EDNN	248
Bos taurus	GCLGN-SKTS EDQGVDE-KERREANKKIEKQLQKERLAYKATHRLLLLL	48	QVDKVN FHMFDVGGQRDERRKWIQC FND TAI IYVAACSSYNMVI EDNN	248
Monodelphis domestica	GCLGN--SKTDQRIDE-KERREANKKIEKQLQKERLAYKATHRLLLLL	47	QVDKVN FHMFDVGGQRDERRKWIQC FND TAI IYVAACSSYNMVI EDNN	247
Gallus gallus	GCLGN--SKTDQRIDE-KAQREANKKIEKQLQKERLAYKATHRLLLLL	47	QVDKVN FHMFDVGGQRDERRKWIQC FND TAI IYVAACSSYNMVI EDNN	247
Danio rerio	GCLGN--SKTDQRIDE-KAQREANKKIEKQLQKERLAYKATHRLLLLL	47	QVDKVN FHMFDVGGQRDERRKWIQC FND TAI IYVAACSSYNMVI EDNN	247
Xenopus laevis	GCLGN--SKTDQRIDE-KAQREANKKIEKQLQKERLAYKATHRLLLLL	47	QVDKVN FHMFDVGGQRDERRKWIQC FND TAI IYVAACSSYNMVI EDNN	247
Caenorhabditis elegans	GC V G A-----GADAE G-REARKVNKIEQLQKDKQVYKATHRLLLLL	43	EVDKVR FHMFDVGGQRDERRKWIQC FND TAI IYVAACSSYNMVI EDNT	243
Drosophila melanogaster	GC FGS P TSKQSDVNS EDSKSRKRSDAI SRQLQKDKQLYRATHRLLLLL	50	QVDKVN FHMFDVGGQRDERRKWIQC FND TAI IYVAACSSYNMVI EDPT	250

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A