ORIGINAL CONTRIBUTION

# Autosomal Dominant Familial Dyskinesia and Facial Myokymia

## *Single Exome Sequencing Identifies a Mutation in Adenylyl Cyclase 5*

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**Background:** Familial dyskinesia with facial myokymia (FDFM) is an autosomal dominant disorder that is exacerbated by anxiety. In a 5-generation family of German ancestry, we previously mapped FDFM to chromosome band 3p21-3q21. The 72.5-Mb linkage region was too large for traditional positional mutation identification.

**Objective:** To identify the gene responsible for FDFM by exome resequencing of a single affected individual.

**Participants:** We performed whole exome sequencing in 1 affected individual and used a series of bioinformatic filters, including functional significance and presence in dbSNP or the 1000 Genomes Project, to reduce the number of candidate variants. Co-segregation analysis was performed in 15 additional individuals in 3 generations.

**Main Outcome Measures:** Unique DNA variants in the linkage region that co-segregate with FDFM.

**Results:** The exome contained 23 428 single-nucleotide variants, of which 9391 were missense, nonsense, or splice site alterations. The critical region contained 323 variants, 5 of which were not present in 1 of the sequence databases. Adenylyl cyclase 5 (*ADCY5*) was the only gene in which the variant (c.2176G>A) was co-transmitted perfectly with disease status and was not present in 3510 control white exomes. This residue is highly conserved, and the change is nonconservative and predicted to be damaging.

**Conclusions:** *ADCY5* is highly expressed in striatum. Mice deficient in *Adcy5* develop a movement disorder that is worsened by stress. We conclude that FDFM likely results from a missense mutation in *ADCY5*. This study demonstrates the power of a single exome sequence combined with linkage information to identify causative genes for rare autosomal dominant mendelian diseases.

*Arch Neurol. 2012;69(5):630-635*

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In 2001, Fernandez et al[1] described a white family of German ancestry manifesting a disorder characterized by predominantly perioral and periorbital myokymia and face, neck, and upper limb dystonic/choreic movements (familial dyskinesia with facial myokymia [FDFM]; OMIM 606703). Initially, the disorder had been described as familial essential (benign) chorea.[2] The onset of symptoms ranged from 2 ½ to 19 years. Initially paroxysmal and precipitated or worsened by stress, the dyskinetic episodes become nearly constant by the end of the third decade of life, but in some individuals, the dyskinetic episodes may diminish in frequency and severity at older ages. Autosomal dominant transmission of FDFM is supported by the presence of disease in all 5 generations, apparently complete penetrance in males and females, and instances of male-to-male transmission.

By targeted genotyping, we excluded 10 candidate genes chosen for their association with myokymia or chorea and 2 regions that contain single or clustered ion channel genes.[1] We mapped the disorder to a 71.73-cM region, corresponding to 72.5 Mb, on chromosome band 3p21-3q21.[3] Interrogation of such a large region, deemed by National Center for Biotechnology Information Entrez Gene Build 37 to contain 253 annotated genes, became feasible by the recent development of "next-generation" massively parallel sequencing technologies. We provide evidence that adenylyl cyclase 5 (*ADCY5*) is a strong candidate gene for FDFM and demonstrate the power of exome sequencing to identify causative genes for autosomal dominant disorders.

## METHODS

### HUMAN SUBJECTS

An updated pedigree is shown in **Figure 1**. Patients were evaluated and blood samples were collected from 16 individuals in 3 generations

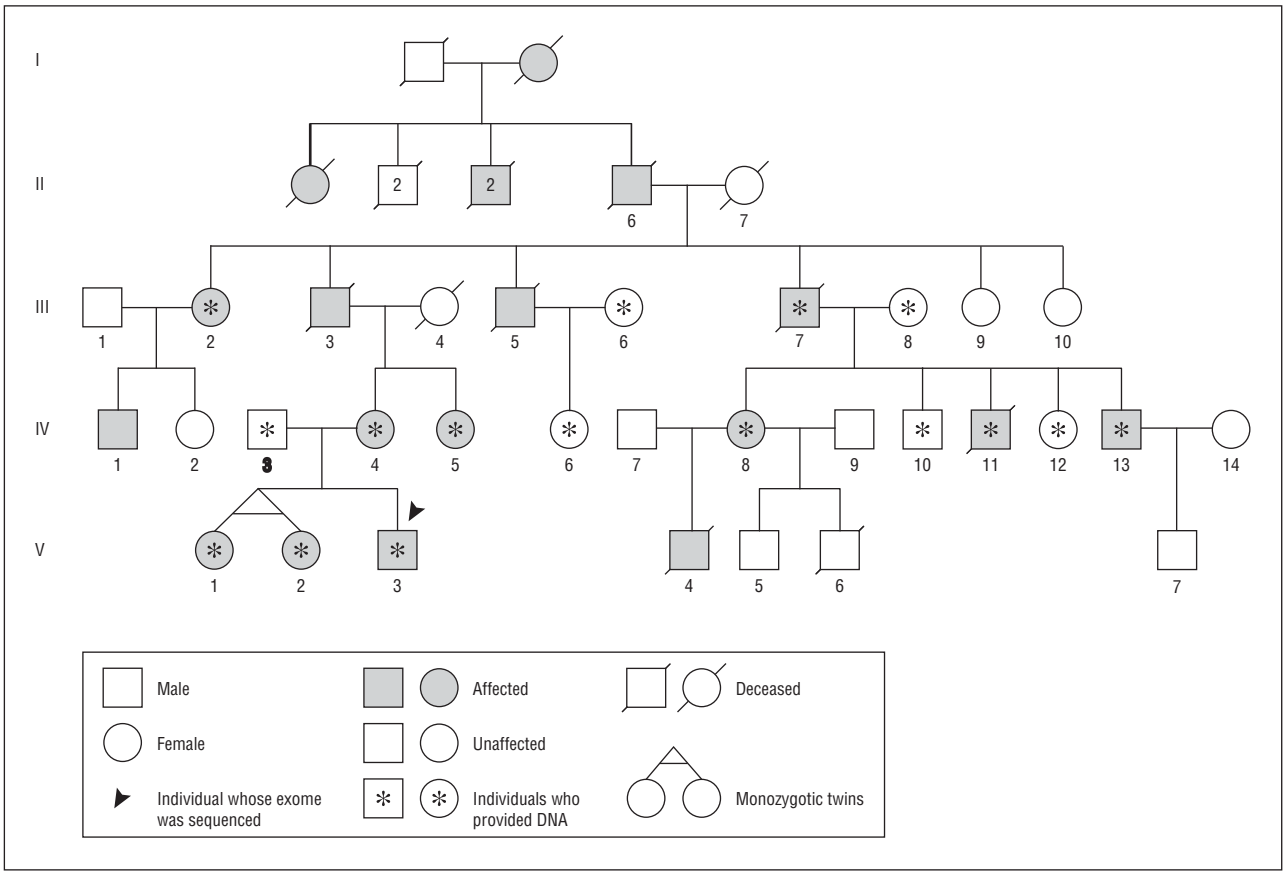


**Figure 1.** Family with autosomal dominant familial dyskinesia with facial myokymia.

under a protocol approved by the Institutional Review Board of the University of Washington.

## TARGETED CAPTURE AND EXOME SEQUENCING

Genomic DNA was extracted from peripheral blood using standard procedures. Five micrograms of DNA from an affected member of the FDFM family (V-3 in Figure 1) was used to construct a shotgun sequencing library as described previously.[4] The shotgun library was hybridized to NimbleGen microarrays (Nimblegen_solution_V2refseq2010.HG19) for target enrichment, followed by washing, elution, and additional amplification. We targeted all protein-coding regions as defined by RefSeq 36.3. Exome sequencing was performed using the Illumina GAIIx platform (Illumina, Inc) with paired-end 76–base pair (bp) reads. Sequence alignment and variant calling were performed against the reference human genome (National Center for Biotechnology Information 37/hg19). For single-nucleotide polymorphisms (SNPs) and short insertions or deletions (indels), calling and genotyping were performed using the GATK Unified Genotyper. Annotations of variants were based on the National Center for Biotechnology Information and University of California Santa Cruz (http://genome.cse.ucsc.edu/cgi-bin/hgGateway) databases using an in-house server (SeattleSeq Annotation 131; http://gvs.gs.washington.edu/SeattleSeqAnnotation/).

## RANKING OF CANDIDATE GENES/VARIANTS

Candidate genes/variants were ranked on the basis of the following: (1) Coding region sequence effect. Missense, nonsense, coding indels, and splice acceptor and donor sites were selected for further evaluation. (2) Variant frequency. Unique variants were selected based on nonoverlap with variants present in dbSNP131 (http://www.ncbi.nlm.nih.gov/SNP), the 1000 Genomes Project (http://www.1000genomes.org/page.php), and 3510 control exomes of European ancestry from the UW Genome Sciences Genomic Resource Center Exome Sequencing Projects. The UW Exome Variant Server (http://evs.gs.washington.edu/EVS/) catalogs variants identified through exome sequencing primarily of individuals with heart, lung, or blood disorders. (3) Location in the linkage region. Variants in the 72.5-Mb region on chromosome band 3p21-3q21 bounded by D3S1582 and D3S3606 were further considered. (4) Conservation scores as estimated by University of California Santa Cruz phastCons and Genomic Evolutionary Rate Profiling. phastCons scores are based on sequence conservation in 17 vertebrate species.[5] The score from 0 to 1 denotes the probability that each nucleotide belongs to a conserved element. Genomic Evolutionary Rate Profiling scores range from –11.6 to 5.86 and are based on sequence conservation in 46 mammals.[6] (5) Potential effect of an amino acid substitution on protein structure and function as predicted by SIFT (Sorting Intolerant From Tolerant; http://sift.jcvi.org/www/SIFT_intersect_coding_submit.html)[7] and PolyPhen-2 (Polymorphism Phenotyping v2; (http://genetics.bwh.harvard.edu/pph2/).[8,9]

## VARIANT VALIDATION BY SANGER SEQUENCING

Capillary sequencing using customized primers was performed to confirm 5 unique coding variants in the linkage region and to investigate co-transmission with the FDFM phenotype in all family members. Primer sequences are given in eTable 1 (http://www.archneurol.com). Genomic DNA was polymerase chain reaction amplified in a thermal cycler (DNA Engine Tetrad 2; MJ Research). Sequencing was performed using

**Table 1. Variants Detected in the Exome of Individual V-3**

| Variant | Whole Exome | 72.5-Mb Chromosome 3 Linkage Region |
|---|---|---|
| Single-nucleotide variants, No. | **23 428** | **323** |
| Synonymous | 10 500 | 130 |
| Coding-notMod3[a] | 240 | 0 |
| Intergenic | 837 | 13 |
| Intron | 1963 | 40 |
| Near gene (5′ and 3′) | 98 | 3 |
| 5′and 3′ UTR | 399 | 4 |
| Nonsense | 88 | 2 |
| Splice site (5′ and 3′) | 29 | 1 |
| Missense | 9274 | 130 |
| Indels, No.[b] | 414 | 9 |

Abbreviations: indels, short insertions or deletions; UTR, untranslated region.

[a] Variant within an exon and translated, but number of coding bases is not a multiple of 3, and no attempt is made to rate as synonymous or not.

[b] Seventy-nine percent of coding indels are 1– to 3–base pair long.

an ABI 3130xl sequencer and ABI BigDye dye terminator cycle-sequencing kits (Applied Biosystems). Further analysis was performed using SeqScape Software 2.1 (Applied Biosystems) or Lasergene 8.1 (DNASTAR Inc).

### EVALUATION FOR COPY NUMBER VARIATION BY ARRAY COMPARATIVE GENOMIC HYBRIDIZATION

To detect deletions and duplications, we used 2 array designs: (1) exon-focused 720×3 whole-exome NimbleGen microarray and (2) custom chromosome 3 tiling NimbleGen microarray with median probe spacing of 275 bp. All microarray hybridization experiments were performed as described previously[10,11] using a single unaffected male (GM15724 from Coriell Institute for Medical Research) as a reference. Copy number variation (CNV) calls were made using a hidden Markov model–based algorithm[12] and were compared with CNVs from 5674 control individuals as previously reported.[13]

## RESULTS

### CLINICAL HISTORY

Since description of the family and the disorder in 2001,[1] 2 affected males have died (individuals III-7 and IV-11). Reexamination of individual IV-13 at age 50 years demonstrated some improvement of the neurologic manifestations since he was first evaluated. He still exhibited subtle choreic hand and arm movements and facial myokymia. At age 46 years, he was diagnosed as having a severe dilated cardiomyopathy (left ventricular ejection fraction of 10% at diagnosis and 15%-20% with therapy). Cardiac catheterization detected nonocclusive atherosclerosis that was believed to be insufficient to cause the global cardiac dysfunction. A computed tomographic angiogram was negative for pulmonary emboli. Sleep apnea was diagnosed, but it could not be interpreted given the concurrent acute congestive heart failure (CHF). One year later, rest and Regadenoson–stress sestamibi myocardial imaging showed normal perfusion except for a questionable inferior wall defect. The participant provided information that 4 of his neurologically affected relatives had also experienced CHF. The age at onset of CHF and the age at and cause of death of his grandfather (II-6) were unknown. His father (III-7) had undergone coronary artery bypass grafting and died of CHF at age 83 years, but 2 uncles developed CHF at much earlier ages, 1 dying at age 63 years (III-3) and the other in his 50s (III-5). Individual IV-11 died of cancer at age 62 years. Records on these individuals were unavailable for review.

### ARRAY COMPARATIVE GENOMIC HYBRIDIZATION

To evaluate the possibility of a large CNV, array comparative genomic hybridization was performed on DNA from individuals IV-8 and V-2. Two strategies for CNV detection were pursued. First, we used an exon-focused 720×3 whole-exome NimbleGen microarray to interrogate large CNVs (>150 kbp).[10-12] Second, we targeted the entire chromosome 3 on a custom tiling NimbleGen microarray with median probe spacing of 275 bp. After filtering for variants identified in a large collection (approximately 6300) of population controls,[13] we assessed for rare, potentially pathogenic variants within and outside chromosome 3. No potentially pathogenic CNVs were detected individually or shared between the affected individuals. These results suggest that variants potentially below the resolution of the array or SNPs contribute to the observed phenotypes.

### MULTIPLY PARALLEL SEQUENCING

We generated 70 Mb of unpaired 76-bp sequencing reads from affected individual V-3. Of this total, 83.0% (58 Mb) were paired-end, 76-bp reads that passed the quality assessment and aligned to the human reference sequence, and 64.4% mapped to the targeted bases with mean coverage of 68-fold. At this depth of coverage, 94.8% of the targeted bases were sufficiently covered to pass the thresholds for variant calling.

The exome of individual V-3 contained 23 428 SNPs, of which 9391 alter the coding sequence (**Table 1**). These coding SNPs consist of 9274 missense, 88 nonsense, and 29 splice acceptor- or donor-site variants. In addition, 414 short indels were detected. Based on the hypothesis that the mutation underlying this rare familial disease would be of sufficiently low prevalence in the general population that it would not be present in databases of variations, SNPs identified in the 1000 Genomes Project or in dbSNP131 were removed. Application of this filter reduced the candidate gene pool to 715.

Within the 72 504 463-bp region (University of California Santa Cruz, hg19) on chromosome 3 bounded by D3S1582 (54 696 005) and D3S3606 (127 200 468), there were 133 missense, nonsense, and splice site variants (Table 1). Of these variants, only 5 (*SLC25A26*, *LMOD3*, *FILIP1L*, *WDR52*, and *ADCY5*), all of which were heterozygous, were not annotated in either dbSNP131 or the 1000 Genomes Project data set. In addition, there were 9 indels in the linkage region, but all were present in dbSNP131. Details of the 5 novel missense variants, in-

**Table 2. Novel Missense Variants in the Familial Dyskinesia With Facial Myokymia Linkage Region Detected in Individual V-3**

| Gene | hg19[a] | Reference[a] | Variant | Read Depth[b] | Residue | phastCons[c] | GERP[c] | PolyPhen-2[d] | SIFT[d] |
|---|---|---|---|---|---|---|---|---|---|
| *SLC25A26* | 66,287,056 | G | A | 61 | S41N | 0.717 | Not given | Benign | Benign |
| *LMOD3* | 69,168,628 | C | T | 88 | G293D | 0.997 | 5.26 | Damaging | Benign |
| *FILIP1L* | 99,569,603 | T | C | 79 | Q306R | 0.999 | 4.22 | Damaging | Benign |
| *WDR52* | 113,119,440 | C | T | 86 | V476M | 0.002 | −7.71 | Benign | Benign |
| *ADCY5* | 123,038,601 | C | T | 118 | A726T | 1.0 | 4.68 | Damaging | Damaging |

Abbreviations: GERP, Genomic Evolutionary Rate Profiling; PolyPhen-2, Polymorphism Phenotyping v2; SIFT, Sorting Intolerant From Tolerant.
[a] hg19 location (base pair) and reference allele according to Human Genome build 37 (GRCh37/hg19); in all but *SLC25A26*, the reference allele is from the reverse strand.
[b] Total number of reads for that base pair.
[c] phastCons[5] and GERP[6] scores are based on sequence conservation in 17 vertebrate species and 46 mammalian species, respectively.
[d] Predicted effect of the substitution on protein function per PolyPhen-2[8,9] or SIFT.[7]

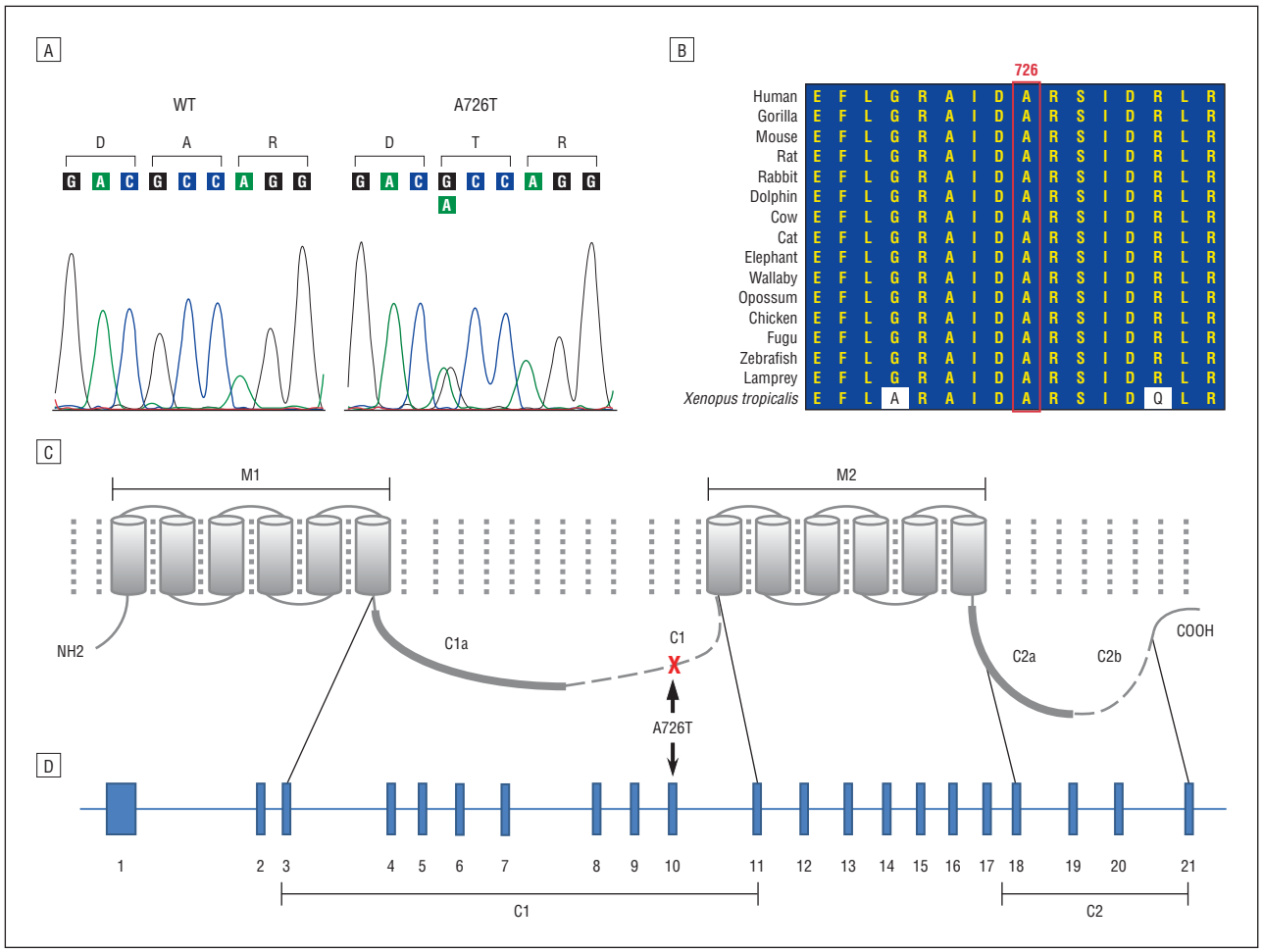


**Figure 2.** Missense mutation in adenylyl cyclase 5 (*ADCY5*) identified in familial dyskinesia with facial myokymia. A, Heterozygous c.2176G>A predicts p.A726T. B, Amino acid sequence alignment showing striking evolutionary conservation of *ADCY5* at and around residue 726. C, Exonic organization of *ADCY5* and its relationship to the primary structure of the protein. D, Secondary structure of *ADCY5* showing domains.

cluding conservation scores and predictions of effect on the protein, are given in **Table 2**.

## CO-TRANSMISSION ANALYSIS

The 5 variants not present in either dbSNP or the 1000 Genomes Project were sequenced in all available family members. Only the variants in *ADCY5* and *WDR52* associated completely with disease status (eTable 2). Because the variant in *WDR52* was detected in 17 of 3493 white exomes in the UW Genome Sciences Genomic Resource Center Exome Sequencing Projects and is neither conserved nor predicted to be damaging to the protein, it is deemed a rare polymorphism. The c.2176G>A transition (A726T) in *ADCY5* was predicted to alter protein function by PolyPhen-2 and SIFT analysis and remains the sole candidate variant for FDFM (**Figure 2**A). Amino acid A726 is conserved in all species (Figure 2B), with a Genomic Evolutionary Rate Profiling score of 4.68 and a phastCons score of 1.0.

## COMMENT

The combination of linkage analysis and the exome sequence of a single affected individual revealed a strong candidate gene, *ADCY5*, for FDFM. Adenylyl cyclases (ACs) convert adenosine triphosphate to cyclic adenosine monophosphate, the second messenger in a broad range of cellular activities. At least 1 AC isoform seems to participate in a large signaling complex with a $\beta_2$-adrenergic receptor and a class C L-type $Ca^{2+}$ channel.[14] There are 6 sequence-dissimilar classes of ACs, but most bacterial and all eukaryotic ACs belong to class III.[15] Nine of 10 AC class III isoforms are membrane bound, and the 10th is soluble, structurally quite distinct, and testis specific.[15,16] The membrane-bound ACs are further divided into 4 subgroups on the basis of regulatory properties. Group III consists of AC5 and AC6, highly homologous forms that are activated by stimulatory guanosine triphosphate–binding proteins (G proteins) and inhibited by inhibitory G proteins and calcium.[15,17-19]

The 21 exons of *ADCY5* code for a 1261–amino acid protein that contains an intracellular N-terminus, two 6-U membrane-spanning helices (M1 and M2) that flank the first of 2 intracellular cyclase homology domains (C1 and C2), and a short intracellular loop between the catalytic domains (Figure 2). Adenylyl cyclase activity is modulated through dopamine signaling.[20] Dopamine receptors are metabotropic G protein–coupled receptors. Dopamine D1 receptors are stimulatory, and dopamine D2 receptors are inhibitory. For activation, the catalytic domains of ACs must form a heterodimer that creates an adenosine triphosphate–binding pocket.[21] G proteins activate the enzyme by promoting the interaction between C1 and C2.[19] Inhibitory G proteins bind to C1 to impair this interaction. The AC5 variant we identified lies between C1 and M2. Although the Grantham score of 58 for this change places it in the moderately conservative class,[22,23] the substitution of polar hydrophilic threonine for nonpolar hydrophobic alanine might alter the structure of the adenosine triphosphate–binding pocket. It is possible that the change in conformation caused by the larger threonine residue for smaller alanine has an effect on the flexibility of the cytoplasmic portion and, thus, alters enzyme activation or inactivation. Perhaps the mutation alters the enzyme's affinity for inhibitory or stimulatory G proteins. Another possibility is that the mutant threonine residue could be an anomalous target for phosphorylation.

Expression data and animal models provide cogent support for the pathogenicity of the *ADCY5* mutation for FDFM. The AC5 is the major isoform in brain and heart and is especially predominant in the nucleus accumbens and striatum, where it contributes more than 80% of AC activity.[24-26] The striatum and nucleus accumbens are part of the mesolimbic dopaminergic system that is activated in response to stress.[27] Dopamine receptors are abundant in the striatum and nucleus accumbens, and these brain regions are major coordinators of movement. Mice deficient in AC5 develop a parkinsonian movement disorder that is worsened by stress.[26] Homozygous *Adcy5* knockout mice manifest abnormal coordination by the rotarod test, impaired locomotion in an open-field test, decreased rearing actions in the home cage, and bradykinesia on a pole test. Heterozygous *Adcy5*$^{+/-}$ mice performed slightly worse than did wild-type mice on these tests, but the impairment reached significance at the 5% level only for rearing frequency. The observation that D1 or D2 dopaminergic stimulation improved some but not all the motor deficits in the *Adcy5* knockout mice suggests that other ACs present in striatum, including the highly homologous AC6, cannot fully replace the functions of AC5.[26]

It is unclear what causes myokymia in human diseases. Abnormal firing of motor neurons and malfunction of peripheral nerves are possible explanations. The presence and activity of *ADCY5* in motor neurons or the peripheral nervous system remains unknown.

Behavioral stress increases cyclic adenosine monophosphate levels in the brain.[28] Mice deficient in AC5 displayed poor coping responses to restraint-induced stress, as evidenced by poor feeding and grooming, concomitant with overactivation of the hippocampal-pituitary-adrenal axis, as indicated by higher corticosterone levels.[29] This impaired stress tolerance was not affected by glucocorticoid receptor antagonists but was abrogated by diazepam, a dopamine D1 receptor antagonist. The motor phenotype of the knockout mice is in some ways opposite that seen in FDFM, where there is increased adventitious movement. Perhaps this reflects a gain of function effect of the mutation with respect to motor function. Similarly, exaggeration of the defect through stimulation of the cyclase pathway may account for the precipitating effect of stress in FDFM; alternatively, it may reflect a general phenomenon not specifically related to the *ADCY5* mutation.

In the family we present, the multigenerational history of CHF, in some cases at relatively young ages and in 1 individual without a clear etiology despite extensive workup, leads us to speculate that heart disease may be a component of FDFM. As with the neurologic phenotype, mouse models of cardiac disease suggest that the *ADCY5* mutation in FDFM may have a gain-of-function effect. In the mouse models, disruption of *Adcy5* is associated with longevity and protection against age-, stress-, and catecholamine-related cardiomyopathy,[30-32] whereas overexpression results in cardiomyopathy.[33,34]

In summary, we present strong evidence that FDFM results from a missense mutation in *ADCY5*. By targeted linkage studies, we had previously excluded 10 candidate genes/loci scattered in the genome, and in the present study, we detected no pathogenic CNV. In the exome sequence, the FDFM linkage region contained only 2 novel coding variants that co-segregated with disease, and one of these was shown to be a rare polymorphism. The variant in *ADCY5* affects a highly conserved residue, and the alteration is predicted to be damaging to protein function. The phenotypes of *Adcy5*-null and overexpressing mice make this enzyme a credible candidate for FDFM. This study demonstrates the power of a single exome sequence combined with linkage information to identify causative genes for rare autosomal dominant mendelian diseases. Detection of mutations in additional families and functional studies of the variant may further support this conclusion.

**Accepted for Publication**: January 12, 2012.
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**Author Contributions:** All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. *Study concept and design*: Chen, Girirajan, Nickerson, and Raskind. *Acquisition of data*: Chen, Matsushita, Rieder, Girirajan, Antonacci, Lipe, Eichler, Nickerson, and Bird. *Analysis and interpretation of data*: Chen, Robertson, Rieder, Girirajan, Nickerson, and Raskind. *Drafting of the manuscript*: Robertson, Girirajan, Lipe, Nickerson, and Raskind. *Critical revision of the manuscript for important intellectual content*: Chen, Matsushita, Rieder, Girirajan, Antonacci, Eichler, Nickerson, Bird, and Raskind. *Statistical analysis:* Robertson and Girirajan. *Obtained funding*: Eichler, Nickerson, Bird, and Raskind. *Administrative, technical, and material support*: Chen, Matsushita, Rieder, Girirajan, Antonacci, Lipe, Nickerson, and Raskind. *Study supervision*: Rieder, Eichler, and Raskind.

**Financial Disclosure:** Drs Bird and Raskind receive licensing fees from Athena Diagnostics.

**Funding/Support:** This research was supported by resources from the Veterans Integrated Service Network 20 Mental Illness Research, Education, and Clinical Center, VA Puget Sound Health Care System (Drs Bird and Raskind), and by American Recovery and Reinvestment Act funds through National Human Genome Research Institute grant RC2HG005608 (Drs Nickerson and Raskind) and National Institute of Neurological Disorders and Stroke grant R01NS069719 (Dr Raskind), National Institutes of Health. Dr Eichler is an investigator for the Howard Hughes Medical Institute.

**Role of the Sponsors:** The sponsors had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Online-Only Material:** The eTables are available at http://www.archneurol.com.

**Additional Contributions:** We are grateful to the family for their participation in this study. John Wolff, BS, provided expert technical support. Guy Chan, PhD, and Ning Zheng, PhD, provided helpful information and advice about AC biochemistry. We thank and recognize the following ongoing studies that produced and provided exome variant calls for comparison: the National Heart, Lung, and Blood Institute (NHLBI) Lung Cohort Sequencing Project (HL 1029230), the NHLBI Women's Health Initiative Sequencing Project (HL 102924), and the Northwest Genomics Center (HL 102926). The University of Washington is a Huntington Disease (Huntington's Disease Society of America) Center of Excellence.

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