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## Identification and functional analysis of novel *THAP1* mutations

Katja Lohmann, PhD<sup>1,#</sup>, Nils Uflacker, BS<sup>1,#</sup>, Alev Erogullari, MS<sup>2</sup>, Thora Lohnau, BS<sup>1</sup>, Susen Winkler, BS<sup>1</sup>, Andreas Dendorfer, MD<sup>3,4</sup>, Susanne A. Schneider, MD, PhD<sup>1</sup>, Alma Osmanovic, BS<sup>2</sup>, Marina Svetel, MD<sup>5</sup>, Andreas Ferbert, MD<sup>6</sup>, Simone Zittel, MD<sup>7</sup>, Andrea A. Kühn, MD<sup>8</sup>, Alexander Schmidt, MD<sup>9</sup>, Eckart Altenmüller, MD<sup>9</sup>, Alexander Münchau, MD<sup>7</sup>, Christoph Kamm, MD<sup>10</sup>, Matthias Wittstock, MD<sup>10</sup>, Andreas Kupsch, MD<sup>7</sup>, Elena Moro, MD<sup>11</sup>, Jens Volkmann, MD<sup>12</sup>, Vladimir Kostic, MD<sup>5</sup>, Frank J. Kaiser, PhD<sup>2</sup>, Christine Klein, MD<sup>1</sup>, Norbert Brüggemann, MD<sup>1</sup>

<sup>1</sup>Section of Clinical and Molecular Neurogenetics at the Department of Neurology, <sup>2</sup>Institute of Human Genetics, and <sup>3</sup>Institute of Experimental and Clinical Pharmacology and Toxicology, University of Lübeck, Lübeck, Germany;

<sup>4</sup>Walter Brendel Centre of Experimental Medicine, Ludwig-Maximilians-University Munich, Munich, Germany

<sup>5</sup>Institute of Neurology, CCS, Belgrade, Serbia;

<sup>6</sup>Department of Neurology, Hospital Kassel, Kassel, Germany;

<sup>7</sup>Department of Neurology, University Medical Centre Hamburg-Eppendorf, Hamburg, Germany;

<sup>8</sup>Department of Neurology, Neurosurgery and Neuroradiology, Charité University Medicine Berlin, Berlin, Germany;

<sup>9</sup>Institute of Music Physiology and Musicians' Medicine, Hanover University of Music, Drama and Media, Hanover, Germany;

<sup>10</sup>Department of Neurology, University of Rostock, Rostock, Germany;

<sup>11</sup>Movement Disorders Center, Toronto Western Hospital, University of Toronto, University Health Network, Toronto, ON, Canada

<sup>12</sup>Department of Neurology, Christian Albrecht University Kiel, Kiel, Germany;

<sup>#</sup>These two authors have equally contributed to this study.

**Correspondence to:** Katja Lohmann, PhD

Department of Neurology, University of Lübeck; Ratzeburger Allee 160; 23538 Lübeck, Germany;

Tel: +49-451-2903-353; Fax: +49-451-2903-355; Email: [katja.lohmann@neuro.uni-luebeck.de](mailto:katja.lohmann@neuro.uni-luebeck.de)

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**Running head:** Functional analysis of novel THAP1 variants

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**Abstract**

Mutations in *THAP1* have been associated with dystonia 6 (DYT6). *THAP1* encodes a transcription factor that represses the expression of *DYT1*. To further evaluate the mutational spectrum of *THAP1* and its associated phenotype, we sequenced *THAP1* in 567 patients with focal (n=461), segmental (n=68), or generalized dystonia (n=38). We identified ten novel variants, including six missense substitutions within the DNA-binding THAP domain (Arg13His, Lys16Glu, His23Pro, Lys24Glu, Pro26Leu, Ile80Val), a 1bp-deletion downstream of the nuclear localization signal (Asp191Thrfs\*9), and three alterations in the untranslated regions. The effect of the missense variants was assessed using prediction tools and luciferase reporter gene assays. This indicated the Ile80Val substitution as a benign variant. The subcellular localization of Asp191Thrfs\*9 suggests a disturbed nuclear import for this mutation. Thus, we consider six of the ten novel variants as pathogenic mutations accounting for a mutation frequency of 1.1%. Mutation carriers presented mainly with early onset dystonia (<12 years in 5 of 6 patients). Symptoms started in an arm or neck and spread to become generalized in three patients or segmental in two patients. Speech was affected in four mutation carriers. In conclusion, *THAP1* mutations are rare in unselected dystonia patients and functional analysis is necessary to distinguish between benign variants and pathogenic mutations.

**Key words:** dystonia, THAP1, mutation, DNA binding, phenotype-genotype

## Introduction

Dystonia is a movement disorder, characterized clinically by involuntary twisting, repetitive movements, and abnormal postures (1). Mutations in the *THAP1* (*THAP domain containing, apoptosis-associated protein 1*) gene were identified to underlie dystonia 6 (DYT6), a form of primary torsion dystonia (2). *THAP1* encodes a 213-amino acid transcription factor featuring a specific DNA-binding THAP (Thanatos-associated protein) zinc finger domain at the N terminus and a nuclear localization signal (NLS) towards the C terminus (2). We and others recently demonstrated *in vitro* that the *DYT1* promoter is a target for the transcription factor activity of THAP1 (3, 4). Mutations in *DYT1* cause another form of primary torsion dystonia (5).

About 50 different *THAP1* mutations have been reported to date including missense, nonsense, and frameshift mutations (2, 6-17). In addition to the disease-causing mutations, two variants, c.-237\_236delinsTT and c.71+9C>A, in the non-coding region of *THAP1* may be associated with dystonia (9, 13).

DYT6 typically manifests as early-onset generalized or segmental dystonia, frequently with prominent laryngeal involvement and a rostrocaudal evolution of symptoms (6-15). The phenotype though is highly variable even within a single family ranging from unaffected carriers to generalized dystonia (8, 9, 13). Mutation frequency varies between 0.5% in unselected primary dystonia patients (13) and 25% in non-DYT1 multiplex families in whom at least one individual had non-focal involvement and onset of symptoms < 22 years (7).

The clinical relevance of some reported mutations in *THAP1* is equivocal. Recently, *in vitro* tests have become available to explore the functional consequences of different mutations (3, 4, 18). In the present study, we investigated 567 patients with primary

dystonia for mutations in the *THAP1* gene and performed an *in-vitro* assay to assess the putative functional consequences of the mutations. These findings were related to the clinical phenotype of mutation carriers.

## **Patients and Methods**

### *Patients*

The study was approved by the local ethics committee and all participants gave written informed consent. Since our initial study in 2009 (9), we collected another 498 unrelated patients and included 69 patients who were tested in 2009 for known mutations only (published as Group B (9) including patients with sporadic focal dystonia without facial or laryngeal involvement and onset >26 years). Patients were consecutively recruited at different movement disorders centers including the Section of Clinical and Molecular Neurogenetics Lübeck, Germany, the General Hospital Kassel, Germany, the University Hospitals of Hamburg-Eppendorf, Berlin (Charité), Rostock, and Kiel, the Institute of Music Physiology and Musicians' Medicine, University of Music, Drama and Media, Hanover, the University Hospital of Belgrade, Serbia, and the Toronto Western Hospital, Canada. All patients were examined by at least one movement disorder specialist. The diagnosis of primary dystonia was established based on the absence of a history of neuroleptic exposure, head trauma, and anoxia and of any clinical symptoms or signs suggestive of secondary dystonia. Further, secondary causes were excluded by brain MRI. Patients were of German (n=436), Serbian (n=119), or Canadian (n=12) origin. All patients tested negative for the GAG deletion in the *DYT1* gene. Patients presented mainly with focal dystonia (n=461; 81%).

### *Genotyping*

To test for mutations, we sequenced all three exons and exon-intron boundaries of the *THAP1* gene using a capillary sequencing machine. Sequences were aligned to the reference sequence (NC\_000008.10) using the Mutation Surveyor software. In addition, the frequency of two known polymorphisms and all novel missense variants were determined in 365 German controls by sequencing. To assess the effect of the missense mutations *in silico*, the prediction softwares PolyPhen2 (19) (HumVar- tool) and SIFT (20) were used.

### *Measurement of transcription factor activity*

We previously showed that THAP1 represses the expression of *DYTI* in a concentration-dependent manner and that DYT6-associated mutations result in decreased repression of *DYTI* in Luciferase reporter gene assays (4). Here, we used these reporter gene assays to characterize the novel missense mutations (Lys16Glu, Lys24Glu, Pro26Leu, Ile80Val) within the DNA binding THAP domain in addition to the two previously tested substitutions (Arg13His and His23Pro) (4). In brief, human endothelial cells (HeLa) were transfected with wildtype or mutated THAP1 constructs in the pcDNA3.1/myc-his plasmid (Invitrogen) and the *DYTI* core promoter fragment (4) in the pGL4.10 vector (Promega) using FuGENE-HD (Roche). Activity of firefly and renilla luciferase was measured after 24-48 hours incubation with the Dual Luciferase Reporter Assay System (Promega) in a Mithras-Luminometer (Bertholdt). All measurements were verified in a minimum of three independent experiments and as triplicates in each experiment.

### *Subcellular localization of truncated THAP1*

We investigated the subcellular localization of the novel frameshift mutation c.570delA [Asp191Thrfs\*9] that is downstream of the predicted NLS. The respective coding regions of wildtype and mutated *THAP1* were inserted into the pEGFP-N3 plasmid (Clontech) to generate green fluorescent protein (GFP)-labeled THAP1 fusion proteins. These GFP-labeled constructs were transiently expressed for 48 hours in OVCAR-3 cells and localization was determined using confocal laserscanning microscopy as described (18). OVCAR-3 cells are well suited for immunocytochemistry experiments due to their size and handling.

## **Results**

### *Patients and mutation screening*

Clinical details of the patients are presented in Table 1. Among the 567 patients with primary dystonia, we identified ten novel variants. These included six missense substitutions within the DNA-binding THAP domain (Arg13His, Lys16Glu, His23Pro, Lys24Glu, Pro26Leu, Ile80Val), a 1bp-deletion resulting in a frameshift downstream of the predicted NLS (Asp191Thrfs\*9), a base pair substitution in the 5' untranslated region (UTR; c.-32C>T), and two single base pair substitutions in a single patient in the 3'UTR (c.\*1A>G + c.\*10A>T). Clinical and genetic information on mutation carriers is given in Table 2. A detailed case report including description of available family members has been presented elsewhere for the patient with the Arg13His mutation (14). None of the novel substitutions was found among 730 German control chromosomes.

In our sample, we identified the c.-237\_236delinsTT polymorphism only in the heterozygous state. Frequencies were comparable in patients and controls with 4.5% (in

German patients), 5.0% (in Serbian patients), and 5.2% (in controls). Frequencies of the variant for different subgroups of dystonia are shown in Table 3.

The other polymorphism that was found more often among dystonia patients (8/1,210) compared to controls (1/400) (13), c.71+9G>A, was present in 1/567 patients and in 1/365 controls.

#### *Characterization of the coding variants*

In a first step, we performed an *in silico* analysis of the newly identified nonsynonymous variants. Using PolyPhen2, all but Lys24Glu and Ile80Val were predicted to have a possible (His23Pro) or probable (Arg13His, Lys16Glu, Pro26Leu) damaging effect. The SIFT software, predicted an effect on protein function for five of the missense variants but not for Ile80Val (Table 2).

Since THAP1 regulates the expression of the *DYT1* gene (4), we used reporter gene assays as a readout of mutant THAP1 function. We analyzed the six missense variants within the DNA binding THAP domain. THAP1 activity was most prominently reduced (80%) for His23Pro, around 50% for Lys16Glu, Lys24Glu, and Pro26Leu, and about 20% for Arg13His. In our assay, the Ile80Val variant did not have any effect on the THAP1 activity (Figure 1; Table 2).

To determine the effect of the Asp191Thrfs\*9 frameshift mutation, we assessed its subcellular localization by confocal laserscanning microscopy. While wild type THAP1-GFP fusion protein was exclusively located in the nuclei, truncated THAP1 Asp191Thrfs\*9 showed an impaired nuclear import and was also detected in the cytoplasm (Figure 2).

For none of the samples with rare variants in the non-coding regions, i.e. 5' or 3' UTR



or intron 1, RNA was available to test for a potential effect on RNA stability or splicing. Affected family members were not available to test for segregation of the variants. It was not possible to reveal whether the two substitutions in individual L4455 were located on the same chromosome.

## **Discussion**

We screened a group of 567 dystonia patients and identified ten novel variants (1.8%), including six missense (1.1%) and a frameshift variant (0.2%). Based on functional analysis, we consider six of them (Arg13His, Lys16Glu, His23Pro, Lys24Glu, Pro26Leu, Asp191Thrfs\*9) to represent mutations, i.e. to be pathogenic (1.1%). The detected noncoding variants are unlikely to be pathogenic since they don't affect the protein sequence. However, it is conceivable that they alter the expression efficiency but proof is lacking due to unavailability of the respective biological material such as RNA or affected family members to test for segregation. The missense variant Ile80Val seems to represent a benign alteration as indicated by the remaining full THAP1 activity in the *in-vitro* assay and as also calculated by both prediction tools. This finding is supported by three additional notions: First, the amino acids Isoleucine and Valine have a similar structure and belong both to the unpolar amino acids. Second, Lysine 70 was revealed as the most C-terminal amino acid residue responsible for DNA-binding by detailed structural determination of the THAP domain (21) Thus, Isoleucine 80 should not be involved in DNA-binding. Third, the patient has a rather late age at onset (41 years) and speech is not affected (see below). On the other hand, lack of an effect in the reporter gene assay might be specific to the tested target promoter or, alternatively, Isoleucine 80 may be involved in another function of THAP1, such as protein-protein interactions.

THAP1 contains a bipartite nuclear localization signal spanning 16 amino acids (aa 146-162) in the C-terminal part of THAP1 (18). Although the frame-shift mutation Asp191Thrfs\*9 does not affect the NLS itself, we demonstrated an impaired nuclear import of mutant THAP1 *in vitro*. This altered intracellular distribution of THAP1 may be explained by a protein misfolding affecting at least the C-terminal region of THAP1 which disturbs the formation of the bipartite NLS and results in a reduced interaction with nuclear importing proteins. Alternatively, it is also conceivable that there is remaining THAP1 nuclear import and function *in vivo* and the variant is benign. The DYT6-atypical phenotype in our patient with late onset (49 years) and focal dystonia without involving speech actually supports this possibility.

The results of prediction programs for functional effects of the mutations and the effect size measured *in vitro* clearly correspond for four of the six missense changes including the Ile80Val variant. For Arg13His however, an effect on function was predicted by both programs but experimental evidence suggests a rather mild effect. The results for the Lys24Glu mutation were more conflicting since experimental evidence indicates a medium effect but one of the prediction programs (PolyPhen2) predicted no functional change at all.

We detected a THAP1 mutation in 1.1% of our mainly focal dystonia patients. Mutation frequency in the literature ranges from about 0.5% to 1.8% for mainly unselected primary dystonia patients (9-13, 15). Thus, *THAP1* mutations are rare in dystonia patients. About 50 different *THAP1* mutations have been reported to date (2, 6-17, 22). A third of the mutations represents missense mutations located in the THAP domain and is thought to interrupt DNA binding. Another third of the mutations are considered to disturb the NLS such as nonsense mutations, small insertions/deletions or missense

mutations within the NLS. For these proteins, the nuclear import is disturbed resulting in impaired transcriptional activity. Thus, in about 70% of the reported mutations, the DNA binding of mutant THAP1 is considered to be affected. However, functional proof for most of the reported mutations is missing. In the present study, we show that the rare missense variant Ile80Val is probably not pathogenic.

Regarding, the noncoding variants c.-237\_236delinsTT, we confirm that there is no significant association with dystonia. Interestingly however, most studies demonstrate a higher (but not significantly higher) frequency of the variant in dystonia patients compared to controls (9, 10, 23, 24). The association may occur only in subtypes of dystonia. To date, the total number of carriers is too small to obtain significant differences for this rare variant. A differential effect in dystonia subtypes may also be the explanation for the lack of any trend in another study (11). In contrast to our study design, these authors included only about 20% patients with focal dystonia and 75% of the patients had an age of onset <30 years (11). These inclusion criteria are likely to preferentially select patients with monogenic causes of dystonia rather than those carrying polygenic risk factors, thereby masking a possible association. The c.71+9C>A was too rare in our sample to evaluate any trend of a possible association with dystonia. Among the about 100 described mutation carriers, most presented with an early-onset and cervical or arm dystonia at onset (6-15). In more than 80% of the patients dystonia spreads to other body parts. Speech was affected in about 70% of patients. Clinical phenomenology in mutation carriers reported here was similar. All had onset of symptoms in the neck or arm. All but one mutation carrier had an age at onset between 6 and 11 years and presented with generalized (n=3) or segmental (n=2) dystonia. Only the carrier of the frameshift mutation close to the end of THAP1 had a late-onset at the

age of 49 years and dystonia remained focal for 10 years after onset. It can be speculated that the effect of this mutation may be rather mild compared to the missense mutations since a considerable proportion of the protein can still enter the nucleus. We also verified the high frequency of laryngeal involvement (4/6) ranging from mild spasmodic dysphonia to aphonia.

Finally, we aimed to correlate the severity of missense changes in the DNA-binding domain with clinical features. Interestingly, the carriers of the benign Ile80Val variant had a rather mild, DYT6-atypical phenotype with a late age at onset, focal dystonia, normal speech and a negative family history. Further, the carriers of the two mutations with the highest effect on protein function were the only two with a positive family history (Table 2) suggesting that these mutations may exhibit a higher penetrance. To date, these correlations are highly speculative and investigations of larger samples will reveal if *in-vitro* functional analyses can be correlated to the phenotypic presentation.

Taken together, we report ten novel variants in *THAP1* and demonstrate a functional effect for six of them, underlining that functional analysis is necessary to distinguish between benign variants and pathogenic mutations.

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**Figure legends**

**Figure 1:** Effect of missense changes in the DNA-binding domain on THAP1 activity.

Activity of THAP1 was measured by repression of the *TOR1A* promoter in a luciferase reporter gene assay. Wildtype THAP1-mediated repression of the *TOR1A* core promoter activity was set as 100% (lane 1). The THAP1 activity of Ile80Val was comparable to the wildtype protein. All other missense changes resulted in lower THAP1 activity of 20 to 80%. Bars indicate standard error.

**Figure 2:** Subcellular localization of Asp191Thrfs\*9. Subcellular localization of GFP-labeled fusion constructs of THAP1 (green signal; left panel) transiently expressed in OVCAR-3 cells visualized by confocal laserscanning microscopy. Microscopic detection of THAP1-GFP demonstrates an exclusive nuclear distribution of THAP1 wildtype protein (upper panel), whereas Asp191Thrfs\*9 mutant is also present in the cytosol (lower panel). Corresponding cells are shown by transmitted-light microscopy (right panel).



**Table 1:** Characteristics of the study population

Type of dystonia	Patients (n)	Sex male (%)	Mean $\pm$ SD age at onset	Mean $\pm$ SD age	Family history (n)		
					Positive	Negative	Unknown
Generalized	38	61.9	19.1 $\pm$ 17.8	40.9 $\pm$ 15.1	5	26	7
Segmental	68	48.4	43.5 $\pm$ 19.0	59.1 $\pm$ 12.8	6	40	22
Focal	461						
Cervical	155	39.9	42.8 $\pm$ 13.1	54.9 $\pm$ 13.7	22	62	71
Blepharospasm	63	30.2	56.9 $\pm$ 10.7	68.6 $\pm$ 10.3	2	29	32
Writer's cramp	53	54.7	41.4 $\pm$ 10.9	55.4 $\pm$ 10.8	1	22	30
Musician's dystonia	168	73.2	34.8 $\pm$ 10.5	43.8 $\pm$ 12.0	13	135	20
Spasmodic dysphonia	18	33.3	50.9 $\pm$ 11.3	65.4 $\pm$ 11.2	0	3	15
Other	4	50.0	58.2 $\pm$ 10.9	61.5 $\pm$ 12.2	1	3	0

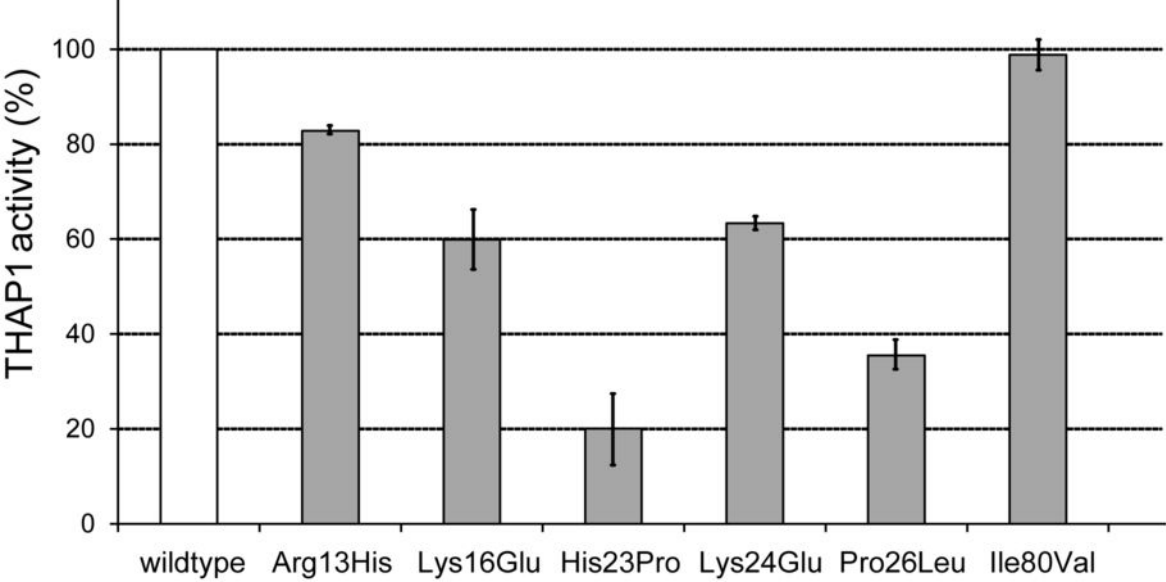
**Table 2.** Clinical and genetics information of mutation carriers

Ind. ID	Age	Sex	Family history	Age at onset	Site at onset	Dystonia at examination	Speech affected	Mutation	PolyPhen-2 prediction (19) (score)	SIFT prediction (score)	THAP1 activity (Luciferase assay)
L3641	59	f	Pos.	49	Neck	Focal	No	c.570delA p. Asp191Thrfs*9	n.a.	n.a.	n.a.
L3841	32	F	Neg.	8	Neck	Generalized	Yes (mild)	c.70A>G p.Lys24Glu	Benign (0.100)	Affects function (0.03)	60%
L3969	30	m	Neg.	6	Arm	Generalized	Yes	c.38G>A p.Arg13His	Probably damaging (0,989)	Affects function (0.02)	80%
L4071	46	f	Neg.	11	Neck	Generalized	Yes	c.46A>G p.Lys16Glu	Probably damaging (0,914)	Affects function (0.03)	60%
L4155	33	m	Pos.	9	Arm	Segmental	No	c.68A>C His23Pro	Possibly damaging (0.612)	Affects function (0.00)	20%
L4318	56	m	Pos.	10	Arm	Segmental	Yes	c.77C>T p.Pro26Leu	Probably damaging (0.994)	Affects function (0.00)	40%
L4325	35	m	Neg.	33	Musician's	Focal	No	c.-32C>T (5' UTR)	n.a.	n.a.	n.a.
L4455	33	m	Neg.	19	Arm	Generalized*	Yes (aphonia)	c.[*1A>G (+) *10A>T] (3' UTR)	n.a.	n.a.	n.a.
L4457	54	m	Neg.	41	Neck	Focal	No	c.238A>G p.Ile80Val	Benign (0.010)	Tolerated (0.79)	100%

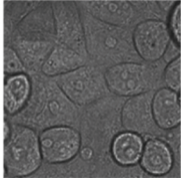
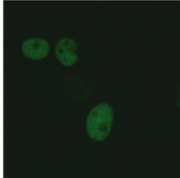
\* Improvement of 20% by Levodopa intake

**Table 3:** Frequency of the c.-237\_236delinsTT polymorphism in different samples.

	Djarmati et al. <sup>3</sup>	Replication Germans	Replication Serbians	All Patients	Replication Controls
<b>Total</b>	<b>19/320 (5.9%)</b>	<b>17/378 (4.5%)</b>	<b>6/120 (5.0%)</b>	<b>42/818 (5.1%)</b>	<b>19/365 (5.2%)</b>
Generalized	0/35	1/32	0/6	3/73 (4.1%)	
Segmental	3/60	2/55	0/12	5/127 (3.9%)	
Focal	16/225	14/291	6/102	36/618 (5.8%)	
Cervical	8/68	6/111	3/43	17/222 (7.7%)	
Musicians' dystonia	8/94	6/122	0	14/216 (6.5%)	
Writer's cramp	0/34	0/18	2/16	2/68 (2.9%)	
Blepharospasm	0/29	2/32	0/30	2/91 (2.2%)	
Spasmodic dysphonia	0	0/5	1/13	1/18 (5.6%)	
Focal, different region	0	0/4	0	0/4 (0.0%)	



wildtype



Asp191Thrfs\*9

