Mutations in the tail domain of *DYNC1H1* cause dominant spinal muscular atrophy

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ABSTRACT

Objective: To identify the gene responsible for 14q32-linked dominant spinal muscular atrophy with lower extremity predominance (SMA-LED, OMIM 158600).

Methods: Target exon capture and next generation sequencing was used to analyze the 73 genes in the 14q32 linkage interval in 3 SMA-LED family members. Candidate gene sequencing in additional dominant SMA families used PCR and pooled target capture methods. Patient fibroblasts were biochemically analyzed.

Results: Regional exome sequencing of all candidate genes in the 14q32 interval in the original SMA-LED family identified only one missense mutation that segregated with disease state—a mutation in the tail domain of *DYNC1H1* (I584L). Sequencing of *DYNC1H1* in 32 additional probands with lower extremity predominant SMA found 2 additional heterozygous tail domain mutations (K671E and Y970C), confirming that multiple different mutations in the same domain can cause a similar phenotype. Biochemical analysis of dynein purified from patient-derived fibroblasts demonstrated that the I584L mutation dominantly disrupted dynein complex stability and function.

Conclusions: We demonstrate that mutations in the tail domain of the heavy chain of cytoplasmic dynein (*DYNC1H1*) cause spinal muscular atrophy and provide experimental evidence that a human DYNC1H1 mutation disrupts dynein complex assembly and function. DYNC1H1 mutations were recently found in a family with Charcot-Marie-Tooth disease (type 20) and in a child with mental retardation. Both of these phenotypes show partial overlap with the spinal muscular atrophy patients described here, indicating that dynein dysfunction is associated with a range of phenotypes in humans involving neuronal development and maintenance. ***Neurology*® 2012;78:1714–1720**

GLOSSARY

CMT = Charcot-Marie-Tooth; **gDNA** = genomic DNA; **indels** = insertions/deletions; **SDS-PAGE** = sodium dodecyl sulfate polyacrylamide gel electrophoresis; **SMA** = spinal muscular atrophy; **SMA-LED** = spinal muscular atrophy with lower extremity predominance; **SNP** = single nucleotide polymorphism.

Developmental and degenerative disorders affecting motor neurons or their axons produce a broad range of inherited human diseases, including spinal muscular atrophy (SMA), hereditary motor neuropathy, and amyotrophic lateral sclerosis. Many hypotheses regarding the pathophysiology of motor neuron loss (e.g., impaired axonal transport, aberrant protein aggregation, disrupted protein homeostasis, altered RNA metabolism[1,2]) were first suggested by the identification of new genes producing hereditary motor neuron disease. To identify additional genes required for motor neuron survival, we studied a large pedigree with a rare form of dominantly inherited SMA with early childhood onset of weakness and disproportionate involvement of

Editorial, page 1706

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the legs (SMA-LED, OMIM 158600), and demonstrated linkage to 14q32.[3] In this report, we describe the identification of a tail domain mutation in *DYNC1H1* as the cause of SMA-LED, and identify 2 additional families with similar lower extremity predominant SMA and missense mutations in the *DYNC1H1* tail domain. Finally, we demonstrate that purified dynein complexes from patients with the I584L mutation have altered stability and function, providing strong evidence of the pathogenicity of *DYNC1H1* mutations in disease.

METHODS To simultaneously resequence all 73 known or predicted genes in the 14q32 interval, gene annotations (RefSeq annotation release 37) were used to design a custom SureSelect (Agilent Technologies) target capture bait library using the eArray software. Capture baits were designed for all exons (coding and untranslated) plus 25 bp of flanking sequence. A total of 18,907 unique baits were generated, targeting 453,141 bp for capture. Genomic targets and designed baits are available on request. Genomic DNA (gDNA) libraries were prepared from 3 individuals from the SMA-LED pedigree and target captured using our custom bait set according to the SureSelect manufacturer's instructions. Each individual's captured gDNA was sequenced with a single lane of 36-bp single-end reads on an Illumina GAIIx (Cofactor Genomics, St. Louis, MO). Raw sequence reads were aligned to the human reference genome (hg18 assembly) using Novoalign version 2.07.05 (Novocraft Technologies) with default settings. SAMtools version 0.1.9[4] was used to remove potential PCR duplicates and call single nucleotide polymorphisms (SNPs) and insertions/deletions (indels). SNPs and indels from each individual were annotated using SeattleSeq (http://gvs.gs.washington.edu) and then overlapped to find novel (i.e., not in dbSNP or 1000 Genome Project databases) heterozygous variants shared by the affected siblings but absent from their unaffected parent.

To sequence the tail domain exons of *DYNC1H1* in 32 probands with lower extremity predominant SMA, Primer3Plus was used to design PCR primers to amplify exons 5–15 with at least 50 bp of flanking sequence (table e-1 on the *Neurology*® Web site at www.neurology.org). PCR amplicons were sequenced in forward and reverse directions using an Applied Biosystems 3730 DNA Sequencer and tracings were analyzed using LaserGene SeqMan Pro version 8.0.2 (DNAStar). Neurologically normal controls (Coriell plates NDPT020, NDPT079, NDPT082, NDPT095, NDPT096, and participants in the Washington University Neuromuscular Genetics Project) were screened for the identified mutations using a multiplexed MassARRAY assay (Sequenom).

We had sufficient DNA from 22 SMA probands to screen the entire *DYNC1H1* gene using pooled-sample target capture and next-generation sequencing. To ensure equal representation of each proband within a pool of 4 or 5 individuals, gDNA was carefully quantified with a Qubit fluorometer (Invitrogen) and equal amounts of gDNA were combined. gDNA library preparation, target capture, and sequencing were performed as above except that each pool underwent a single lane of 100 bp single-end sequencing on an Illumina HiSeq (Genome Technology Access Center, St. Louis, MO). This produced an average sequencing depth across *DYNC1H1* exons of >900×, equivalent to 180–225× coverage per individual in the pool. Raw sequencing reads were aligned as detailed above except that potential PCR duplicates were not removed. The resulting BAM alignment file for each pool was displayed using Partek Genomics Suite v6.09.0602 (Partek, St. Louis, MO). The reads covering every base of *DYNC1H1* (78 exons, plus 25 bp of flanking sequences) were visually inspected for the presence of sequencing reads with nonreference calls. Pooled-capture and next-generation sequencing has recently been shown to introduce minimal allelic bias, even in pools 5× larger than ours.[5] We also assessed the sensitivity of our method for the detection of polymorphisms known to be present on only 1 allele in each pool (from the prior Sanger sequencing of exons 5–15). We were able to detect 17 of 18 variants present as a single allele (sensitivity of 94%). Pooled-capture and sequencing detected known polymorphisms throughout the *DYNC1H1* gene but did not identify any additional putative disease-causing mutations.

Fibroblasts derived from skin biopsy were obtained from an affected SMA-LED individual and a neurologically normal control. For microtubule binding experiments, lysate from control and sample cells was incubated with a 2.5 μM final concentration of Taxol-stabilized microtubules at 37°C for 15 minutes, then centrifuged. Supernatant and microtubule pellet were either recovered for analysis, or the pellet was resuspended in Tris-KCl buffer containing 10 mM ATP, incubated at 37°C for 15 minutes, and centrifuged. Supernatants and pellets were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot probing for dynein intermediate chain (clone 74.1, K. Pfister, University of Virginia) and tubulin (Sigma, St. Louis, MO).

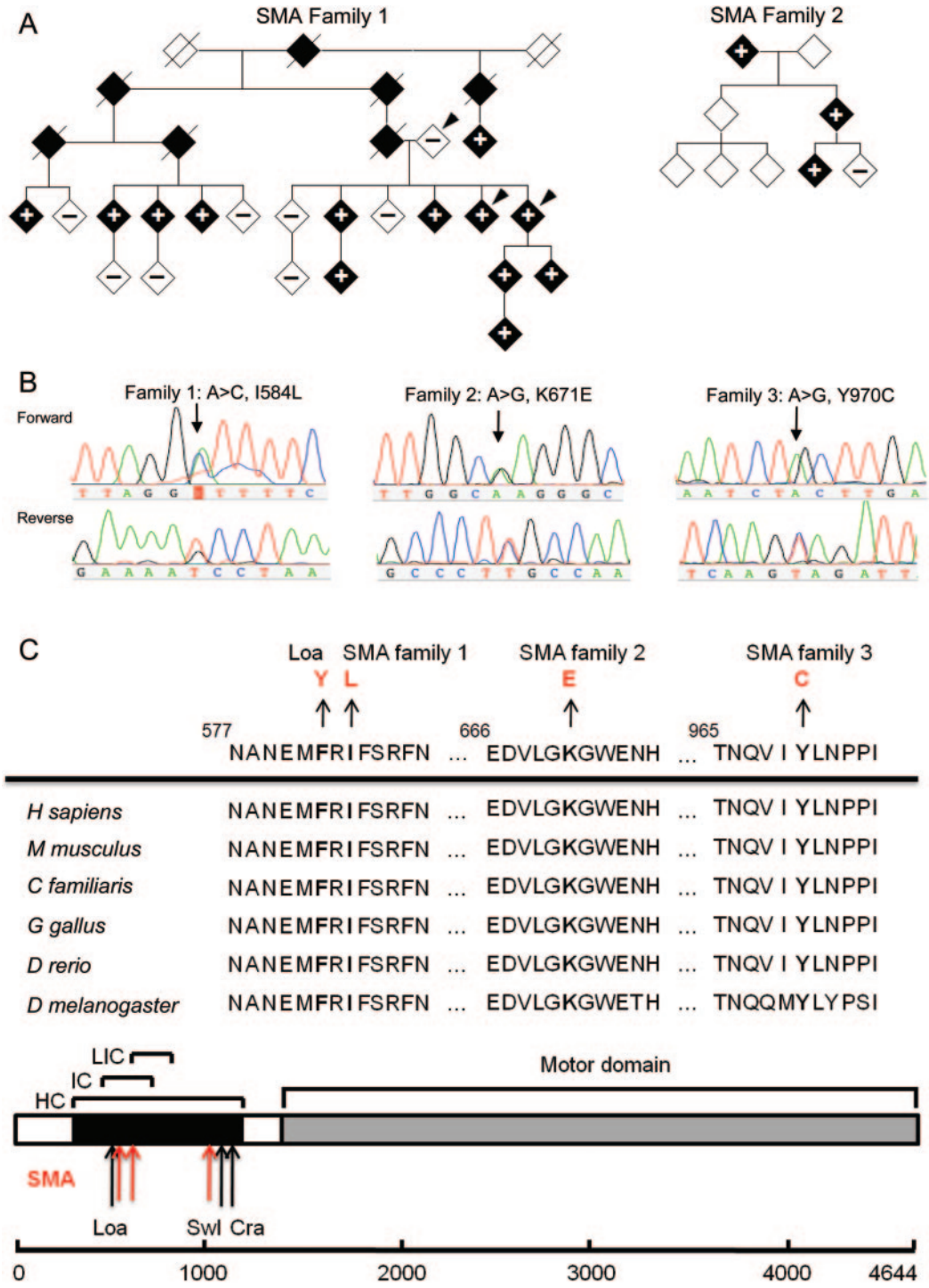
For sucrose gradient analysis, whole cell lysate from control and sample cells was fractionated on a 5%–20% Tris-KCl sucrose gradient (20 mM Tris-HCl, pH 7.6, 50 mM KCl, 5 mM $MgSO_4$, 0.5 mM EDTA). Fractions were analyzed by SDS-PAGE and Western blot probing for dynein intermediate chain (antibody as above).

Numbering of *DYNC1H1* nucleotides refers to NM_001376.4, while amino acid numbering references NP_001367.2.

Standard protocol approvals, registrations, and patient consents. This study received approval from the Washington University Human Studies Committee Institutional Review Board for experiments using human subjects. We obtained written informed consent from all subjects (or guardians of the subjects) participating in the study.

RESULTS **Identification of *DYNC1H1* mutations in SMA-LED and SMA families.** To identify the gene defect causing SMA-LED,[3] we used custom target capture followed by next generation sequencing to simultaneously sequence all exons for the 73 annotated genes in the 14q32 linkage interval. We sequenced 3 individuals from the original SMA-LED pedigree (figure 1A): an unaffected parent and 2 affected children, each of whom had inherited a different 14q32 haplotype from this parent. Sequencing coverage of targeted bases averaged 70× per individual and identified an average of 301 SNPs or small indels within exons or splice sites. We then used a filtering pipeline (table 1) to identify SNPs and indels that were 1) heterozygous, 2) predicted to

Figure 1 *DYNC1H1* mutations in pedigrees with dominant spinal muscular atrophy (SMA)



(A) Pedigrees for dominant SMA families 1 and 2, with filled symbols for affected individuals. Subjects from dominant SMA family 1 used for target enrichment sequencing are indicated by an arrowhead. Individuals where direct sequencing was performed for segregation analysis are indicated by a "+" (mutation carrier) or "−" (noncarrier). (B) Sanger sequencing traces are shown for each of the mutations identified in *DYNC1H1*. (C) Alignment of the DYNC1H1 protein showing complete conservation of the mutated residues through *D melanogaster*, and the relationship to the nearby residue mutated in the *Loa* mouse (F580Y in mouse). Domain diagram of the DYNC1H1 protein showing the position of the dominant SMA mutations relative to the known mouse mutants (*Loa*, *Swl*, *Cra*) and the mapped interaction sites for dynein intermediate chain (IC), light intermediate chain (LIC), and dynein heavy chain dimerization (HC).

Table 1 Analysis of 14q32 variants identified in spinal muscular atrophy with lower extremity predominance (family 1)[a]

	Unaffected parent	Affected sib 1	Affected sib 2
Heterozygous variants within interval	1,603	1,728	1,782
Within exons or at splice sites	241	307	356
Changes amino acid sequence or splice site	55	61	76
Not in 1000 Genomes Project or dbSNP132	17	10	16
Shared by affected siblings, absent from unaffected parent		1	

[a] Raw variants identified by target capture and next generation sequencing of genes at 14q32 were filtered as shown. The only novel, heterozygous, coding variant present in both affected subjects, but absent from the unaffected parent, was c.1750A>C in exon 8 of *DYNC1H1* producing the missense mutation p.I584L.

change coding sequence or disrupt splice sites, 3) absent from dbSNP/1000 Genomes Project, and 4) segregated with disease. Only 1 variant met these criteria, a c.1750A>C mutation in exon 8 of *DYNC1H1*, encoding the heavy chain of cytoplasmic dynein. This transversion produces a p.I584L substitution and demonstrated complete segregation with affected status within the larger pedigree (figure 1A).

The I584L mutation lies in the tail domain of the dynein heavy chain, a highly conserved region critical for organizing multiple dynein subunits into a complex and for binding to membranous cargo.[6] Notably, this mutation is only 2 amino acid residues away from the mutation responsible for the Legs at Odd Angles (*Loa*) phenotype in mice (figure 1C). *Loa* mice, along with 2 other *Dync1h1* tail domain mutant strains (Cramping [*Cra*], Sprawling [*Swl*]) show developmental and degenerative abnormalities of sensory, spinal motor, and cortical neurons.[7–9]

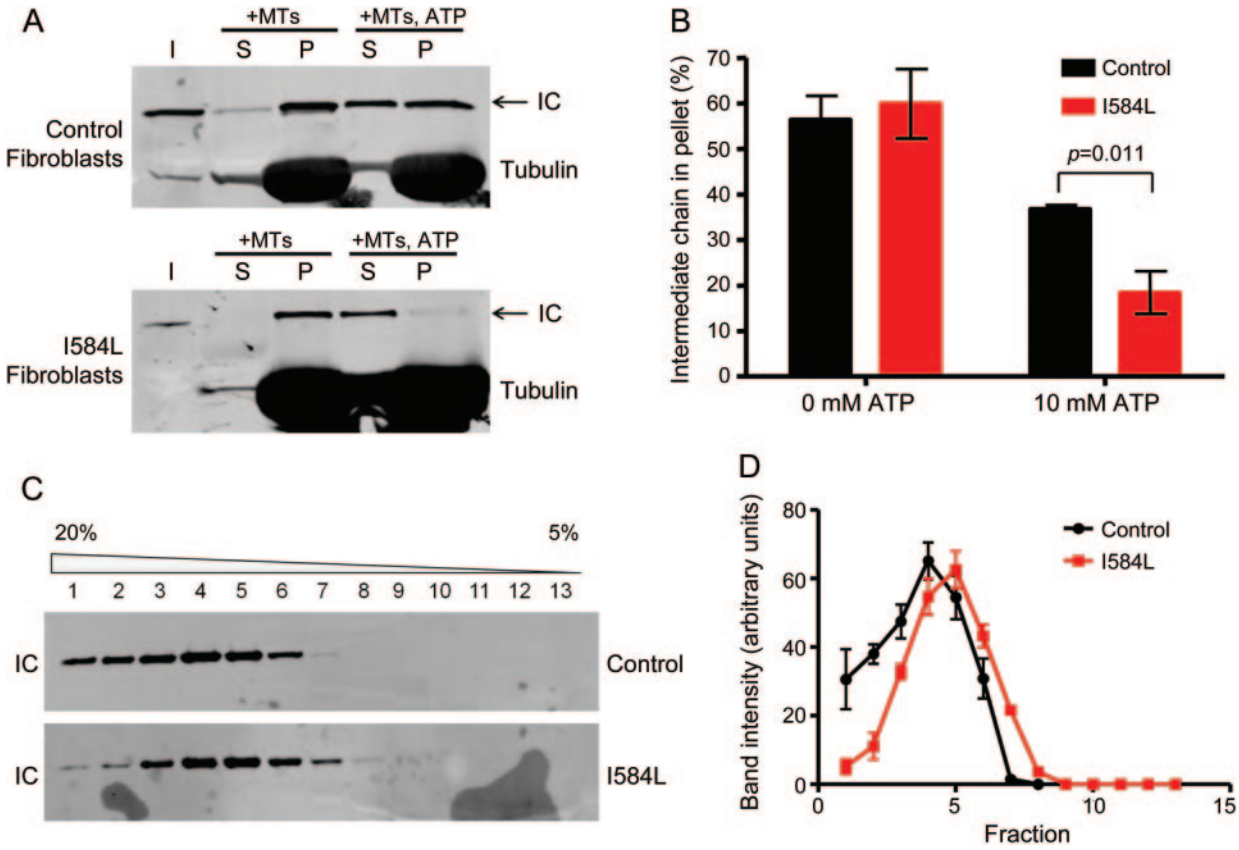
Although the phenotypic similarity between SMA-LED patients and mice with mutant *Dync1h1* strongly suggested that mutant *DYNC1H1* could produce human disease, we sought to confirm the association by screening for mutations in additional patients with SMA. We sequenced the tail domain-encoding exons[5–15] in 32 additional probands with either dominant or de novo SMA variants, and found 2 additional heterozygous missense mutations. In family 2 we identified another exon 8 mutation, p.K671E (c.2011A>G). In family 3, we found p.Y970C (c.3170A>G), located in exon 11. The K671E mutation was shown to segregate with disease in a dominant fashion in a 3-generation family (figure 1A), while DNA from additional family 3 members was not available.

To look for mutations outside of the tail domain, we also screened all 78 exons of *DYNC1H1* in 22 of our SMA cohort using a pooled-sample capture and sequencing strategy. No additional putative mutations were identified, confirming that dominant SMA is genetically heterogenous, and suggesting that the tail domain may be a hot spot for mutations in this disorder. None of the 3 mutations we identified were present in 500 neurologically normal control subjects, or in the most current 1000 Genomes Project low-coverage variant database (20101123 release). Furthermore, I584L and K671E were not found in a prior study that sequenced exon 8 in a cohort of 165 patients with familial motor neuron disorders and 100 Caucasian controls.[10]

Clinical characterization of patients with *DYNC1H1* mutations. The phenotype of the additional SMA pedigrees with *DYNC1H1* mutations is remarkably similar to those carrying the *I584L* mutation (family 1),[3] including early childhood onset of proximal leg weakness with muscle atrophy, and clinical findings supporting non–length-dependent motor neuron disease without sensory involvement.

Family 2 (K671E). The proband of family 2 (the eldest individual, figure 1A) was born without obvious deficits and walked on time, but always with a "waddling" gait. Running was awkward secondary to leg weakness but upper extremity function and strength were always normal. Because the muscle atrophy and weakness were largely stable throughout childhood, confined to the legs, and without sensory loss, the subject was first diagnosed with polio. Later, when the pattern of proximal-predominant leg weakness was appreciated, the diagnosis of a muscular dystrophy was considered. Over 6 decades, the subject noticed only minimal progression of weakness. At 74 years of age, neurologic examination abnormalities were restricted to the motor domain. Atrophy of the quadriceps, lower legs, and small hand muscles was evident, but there were no fasciculations or contractures. As in the original SMA-LED family, there was a notable strength discrepancy between knee extension and flexion—the quadriceps were only antigravity while the hamstrings showed mild weakness. Ankle dorsiflexion and plantar flexion were moderately weak. Hip extension, hip flexion, and the upper extremities showed only trace weakness. Deep tendon reflexes were reduced at the knees but normal elsewhere. The subject could not rise from a chair without pushing with the arms, showed excessive lumbar lordosis while standing, and walked with a waddling gait. Nerve conduction studies showed small motor response amplitudes in the feet and normal sensory response amplitudes. EMG of clinically weak muscles showed chronic denervation without evidence of active denervation, and giant motor unit action potentials (>10 mV). Histories, examinations, and EMGs of the proband's affected child and grandchild were nearly identical in pattern and pro-

Figure 2 Effect of disease mutations on stability and function of the dynein complex



(A) Dynein from a control patient (top) or DYNC1H1 I584L patient (bottom) fibroblasts were incubated with microtubules (MTs) in the absence or presence of ATP. After centrifugation, binding of dynein was assessed by immunoblotting for intermediate chain (IC) and tubulin in the supernatant (S) and microtubule-containing pellet (P) fractions. Input (I) is 10% of total protein. (B) Quantification using densitometry of intermediate chain binding to dynein complexes from replicate experiments (control n = 4, I584L n = 5; $p = 0.011$, control vs I584L). (C) Sucrose density gradient centrifugation analyzed by immunoblotting for dynein intermediate chain from dermal fibroblasts of control subject and a patient with an I584L mutation. (D) Band intensity plot of sucrose gradient fractions from replicate experiments. The peak position is shifted to lower s-values in the I584L patient fibroblasts, indicating altered stability of the dynein complex.

gression with 2 exceptions. First, the proband's child was born with heel cord contractures and in-turning feet that required treatment with leg braces/orthotics until the age of 5. Second, at age 22, the proband's grandchild notes fasciculations of the calves but EMG showed no ongoing denervation.

Family 3 (Y970C). The affected girl (pedigree not shown) showed significant motor delay in infancy, required serial casting for a calcaneo-valgus foot deformity at 18 months, and did not walk until 30 months. On examination at age 3½ years she was unable to run, climbed stairs on all fours, walked unsteadily, and fell frequently. In contrast, her arms showed age-appropriate strength and no sensory loss could be appreciated. In addition to lower extremity weakness, mild cognitive delay was present and she required an individual educational plan in nursery school. EMG of the tibialis anterior and genioglossus muscles showed findings of denervation and reinnervation, consistent with non–length-dependent motor neuron disease. SMN genetic testing showed a normal *SMN1* copy number. Her parents did not have overt motor complaints, but reported that the proband's older sister had been diagnosed with cerebral palsy based on a similar degree of motor delay, an abnormal gait, and MRI showing polymicrogyria. The parents and sibling were unavailable for evaluation.

Functional characterization of mutant *DYNC1H1*. To assess the consequence of mutations in the *DYNC1H1* tail domain, we examined the stability and function of dynein complexes purified from dermal fibroblasts derived from patients heterozygous for the I584L *DYNC1H1* mutation. We observed that dynein complexes from patient cells showed normal binding to microtubules in the absence of ATP, but markedly decreased binding to microtubules in the presence of ATP (figure 2, A and B). Additionally, sucrose gradient fractionation of the dynein complex showed a shift in the peak position to lower s-values in I584L

patient cells compared to control cells (figure 2C). These findings indicate that the I584L mutant dynein heavy chain dominantly disrupts the stability of the dynein complex and markedly decreases the affinity of the dynein complex for microtubules during ATP hydrolysis. This is the identical biochemical signature recently observed for *Loa* (F580Y) mutant dynein complexes purified from mouse brain,[11] and further supports *DYNC1H1* tail domain mutations as a cause of neurodegeneration in both mice and humans.

DISCUSSION Dynein is multi-subunit molecular motor important for basic cellular processes that include retrograde axonal transport,[12] Golgi localization,[13] and autophagy.[14] Pairs of the dynein heavy chain protein, encoded by *DYNC1H1,* form the core of the dynein complex, homodimerizing via their N-terminal tail domains. Based on the phenotype of mouse lines with dynein tail domain mutations, it has been theorized that mutations in *DYNC1H1* might cause motor neuron disease in humans.[10] By identifying *DYNC1H1* mutations as a genetic cause of dominant spinal muscular atrophy, this study provides confirmation of that hypothesis.

We also demonstrate that dynein complexes from the I584L patient fibroblasts act similarly to those from the *Loa* mouse, with decreased binding to microtubules in the presence of ATP, and altered stability on a sucrose gradient.[11] In the *Loa* mutant, these defects were shown to impair dynein run-lengths on microtubules, possibly due to disrupted coordination between the dimerized dynein heavy chains.[11] Impaired run-lengths may in turn disrupt dynein's role in axonal transport, the maintenance of protein homeostasis, and neuronal migration. Each of these cellular processes has been implicated in human motor neuron degeneration, and each has proven disrupted in the *Loa* mouse.[9,15,16] It is quite striking that mouse and human mutant dynein heavy chain demonstrate identical biochemical defects and both cause motor neuron disease in their hosts. Indeed, the vulnerability of motor neurons to mutations in *DYNC1H1* and the dynein interacting subunit *DCTN1*[17] may be due to a combined dependency of motor neurons on dynein for proper migration during development, axonal transport for trophic support, and maintenance of protein homeostasis over the course of their lifetime. This fact emphasizes the need for further work to determine how defective dynein function impairs the cellular processes required for motor neuron survival.

The core clinical features of dominant SMA with *DYNC1H1* mutations include congenital or very early onset, a pattern of weakness that is most severe in the proximal legs, and a static or minimally progressive course. The identification of 3 missense variants manifesting with similar phenotypes strongly supports tail domain mutations in *DYNC1H1* as a cause of human spinal muscular atrophy, including the SMA-LED phenotype. It is also notable that the affected individual in family 3 also showed cognitive impairment.

While this article was in preparation, a different missense mutation (H306R) in the tail domain of *DYNC1H1* was described in a single family with Charcot-Marie-Tooth disease (CMT 2O, OMIM 614228).[18] Although the CMT 2O family shares early childhood onset of symptoms with the families in the present study, their phenotype was that of length-dependent weakness and sensory loss. In contrast, the patients reported here (carrying I584L, K671E, and Y970C) uniformly showed proximal predominant weakness without sensory abnormalities on examination or electrophysiology, a combination that led to their classification as SMA rather than CMT.[3] Interestingly, 1 of 13 patients with CMT 2O was noted to have proximal predominant weakness similar to our SMA subjects.[18] This range of phenotypes is reminiscent of those found in patients with *TRPV4* mutations, which cause congenital distal SMA, scapuloperoneal SMA, and CMT 2C.[19–21]

A de novo mutation in the motor domain of *DYNC1H1* (H3822P) was also recently reported in a child with moderately severe mental retardation but without obvious SMA.[22] Interestingly, 5 patients with tail domain mutations (1 from this study and 4 in the CMT 2O family) showed learning difficulties or delays in speech development. The cognitive phenotype of these individuals warrants closer investigation but suggests that tail domain mutations also affect CNS development. This would not be surprising given the numerous functions of the dynein complex and the widespread neuronal deficits observed in the *Loa* mouse.[7–9] It may be that motor and tail domain mutations differentially impair DYNC1H1 function, in turn leading to variable involvement of the peripheral or central nervous systems.

Together, these observations suggest that a range of *DYNC1H1*-related disease exists in humans, ranging from a widespread neurodevelopmental abnormality involving the CNS, to more selective involvement of subsets of motor neurons as seen in our SMA pedigrees, to the combined involvement of sensory and motor nerves as in CMT 2O. The future identification of additional patients with *DYNC1H1* mutations will help clarify whether this phenotypic spectrum results from distinct molecular consequences of mutations in different regions of the tail domain or reflects genetic modifiers present in different families.

Finally, the absence of *DYNC1H1* mutations in other pedigrees with autosomal dominant SMA with

lower extremity predominance confirms the genetic heterogeneity of this disease. Whole-exome or whole-genome sequencing in these pedigrees (all of which are too small for traditional linkage-based approaches) will be useful to identify additional genes required for motor neuron survival and open up novel biological pathways for therapeutic development.

AUTHOR CONTRIBUTIONS

M.B. Harms: designed the study, recruited cases and collected clinical information, performed alignments, variant detection, bioinformatic analysis, and wrote the paper. K.M. Ori-McKenney performed biochemical analysis. M. Scoto recruited cases and collected clinical information. E.P. Tuck performed variant validation. S. Bell performed variant validation and sequencing. D. Ma performed alignments, variant detection, and bioinformatic analysis. S. Masi performed variant validation. P. Allred recruited cases, collected clinical information, and prepared figures. M. Al-Lozi recruited cases and collected clinical information. M.M. Reilly recruited cases and collected clinical information. L.J. Miller recruited cases and collected clinical information. A. Jani-Acsadi recruited cases and collected clinical information. A. Pestronk recruited cases and collected clinical information. M.E. Shy recruited cases and collected clinical information. F. Muntoni recruited cases and collected clinical information. R.B. Vallee performed biochemical analysis. R.H. Baloh designed the study, recruited cases, collected clinical information, and wrote the paper.

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DISCLOSURE

Dr. Harms, Dr. Ori-McKenney, Dr. Scoto, Ms. Tuck, Mr. Bell, Dr. Ma, Ms. Masi, Dr. Allred, Dr. Al-Lozi, Dr. Reilly, Ms. Miller, and Dr. Jani-Acsadi report no disclosures. Dr. Pestronk serves on the scientific advisory board of the Myositis Association; receives revenue related to patents and speaker honoraria from Athena; owns stock in Johnson & Johnson; is director of the Washington University Neuromuscular Clinical Laboratory which performs antibody testing and muscle and nerve pathology analysis (The Washington University Neurology Department bills for these procedures); and receives research support from NIH, the Muscular Dystrophy Association, Genzyme, Insmed, Knopp, Prosensa, ISIS and Sanofi. Dr. Muntoni, Dr. Vallee, and Dr. Baloh report no disclosures. **Go to Neurology.org for full disclosures.**

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