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## ORIGINAL ARTICLE

# Biallelic mutation of *UNC50*, encoding a protein involved in AChR trafficking, is responsible for arthrogryposis

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## Abstract

Arthrogryposis multiplex congenita (AMC) is a developmental condition characterized by multiple joint contractures resulting from reduced or absent fetal movements. Homozygosity mapping of disease loci combined with whole exome sequencing in a consanguineous family presenting with lethal AMC allowed the identification of a homozygous frameshift deletion in *UNC50* gene (c.750\_751del:p.Cys251Phefs\*4) in the index case. To assess the effect of the mutation, an equivalent mutation in the *Caenorhabditis elegans* orthologous gene was created using CRISPR/Cas9. We demonstrated that *unc-50(kr331)* modification caused the loss of acetylcholine receptor (AChR) expression in *C. elegans* muscle. *unc-50(kr331)* animals were as resistant to the cholinergic agonist levamisole as *unc-50* null mutants suggesting that AChRs were no longer expressed in this animal model. This was confirmed by using a knock-in strain in which a red fluorescent protein was inserted into the AChR locus: no signal was detected in *unc-50(kr331)* background, suggesting that *UNC-50*, a protein known to be involved in AChR trafficking, was no longer functional. These data indicate that biallelic mutation in the *UNC50* gene underlies AMC through a probable loss of AChR expression at the neuromuscular junction which is essential for the cholinergic transmission during human muscle development.

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## Introduction

Arthrogryposis multiplex congenita (AMC) has an overall incidence of 1 in 3000 (1,2). Non-syndromic or isolated AMC are the direct consequence of reduced fetal movements which may lead, in addition to AMC, to pterygia, pulmonary hypoplasia, diaphragmatic defect or cleft palate. Some non-genetic factors such as extrinsic limitation of fetal movements or maternal autoimmune myasthenia may cause AMC. Otherwise, non-syndromic AMCs have been documented to primarily arise from mutations of genes encoding components required for the formation or the function of the neuromuscular junctions (NMJ), the skeletal muscle, the survival of motor neurons or the myelination of peripheral nerve (3). However, many affected individuals still remain without a genetic diagnosis suggesting the involvement of other pathogenic mechanisms. In addition, the identification of new disease genes contributes to a better information of families on the nature, inheritance of disease and evaluation of at risk relatives.

## Results

### Clinical case

Patient's parents provided written informed consent for genetic analysis of their children and themselves in accordance with the ethical standards of our institutional review boards. In the family, the first child born to consanguineous healthy parents was affected. The two subsequent children were non-affected (Fig. 1A). Based on ultrasound examination at 12 weeks of gestation (w.g.), the fetus displayed increased nuchal translucency (3.2 mm). At 28 w.g., ultrasound examination revealed bilateral club foot, bilateral flexion of elbows, hips and knees, bilateral extended wrists, cervical hyperextension and diffuse subcutaneous edema associated with muscle atrophy of limbs. The mother spontaneously delivered a stillborn male infant at 28 w.g. Postmortem examination confirmed AMC associated with pulmonary hypoplasia. Pathological examination of brain and spinal cord was normal. Histological examination of skeletal muscle revealed some atrophic muscle fibers and areas of myofibrillar disorganization. Western blot analysis revealed abnormal expression of calpain 3, but Sanger sequencing of all exons of CAPN3 remained negative.

### Identification of a homozygous frameshift deletion in UNC50

To identify the disease locus, we conducted genome-wide single nucleotide polymorphism (SNP) genotyping using 250K NspI microarrays in the family. We performed homozygosity mapping of SNP data and found 18 candidate loci of at least 500 kb in size with a maximum logarithm of odds (lod) score ( $Z_{max}$ ) of 1.2. The size of total candidate disease loci was 137 Mb (4.5% of the whole genome). Whole exome sequencing (WES) was performed from DNA of the index case. The median coverage of the WES was 74. Variants were filtered based on several criteria (see Materials and Methods). No mutation in genes known to be associated with AMC was found whatever the applied mode of inheritance suggesting that the disease was caused by a mutation of a novel gene. Variants in genes mapping to the candidate loci as determined by homozygosity by descent were selected (listed in Supplementary Material). A homozygous frameshift deletion was identified in UNC50 in the affected individual (NM\_014044.6:c.750\_751del:p.Cys251Phefs\*4, Fig. 1B and D). This

variant was annotated in dbSNP146 (rs764893954) with a very low minor allele frequency (MAF) in ExAC database (0.00002529). The homozygous mutation was confirmed by Sanger sequencing using primers flanking the mutations in the affected fetus (Fig. 1B). Both parents were heterozygous for the mutation indicating that the mutations were allelic (Fig. 1B). The non-affected siblings were either homozygous for the wild-type allele or heterozygous for the mutation (Fig. 1B). The mutation in UNC50 introduces a frameshift in the last coding exon causing the truncation of the last nine amino acids, which are replaced by the three residues Phe–Phe–Leu (Fig. 1D). These last nine amino acids are highly conserved across species (Fig. 1C).

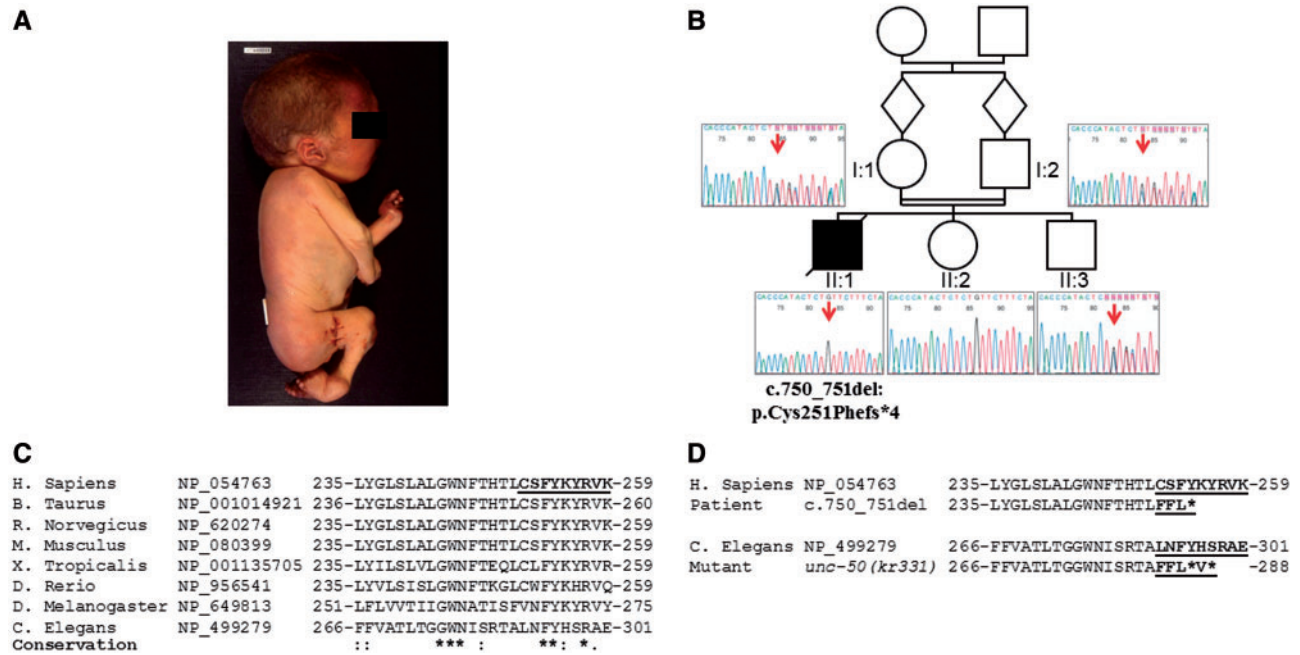
### Generation and characterization of a UNC50 knock-in *Caenorhabditis elegans* model using CRISPR/Cas9

The UNC50 mutation found in the affected individual causes the truncation of the last nine amino acids. These residues are located after the last transmembrane domain of UNC50 and are predicted to face the luminal compartment (Supplementary Material, Fig. S1). To determine whether the mutation found in the affected individual had deleterious effects, it was introduced into the orthologous position in the *C. elegans* gene using CRISPR/Cas9 to generate *unc-50(kr331)* knock-in animals (Fig. 1D). The resulting knock-in animals displayed an uncoordinated phenotype as observed for *unc-50* null mutants (4); therefore, suggesting that the modified UNC-50 protein was no longer functional.

To test this hypothesis more directly, we analyzed the expression of AChRs at the NMJ. In *C. elegans*, two types of AChRs are present at NMJs. Heteromeric L-AChRs resemble vertebrate AChRs at mammalian NMJs and are sensitive to the nematode-specific cholinergic agonist levamisole. Homomeric N-AChRs that resemble  $\alpha 7$  nicotinic receptors are activated by nicotine and are insensitive to levamisole (5,6). UNC-50 was shown to be specifically required for the biogenesis of L-AChRs. To test if *unc-50(kr331)* animals have functional L-AChRs at the NMJ, the animals were assayed for sensitivity to levamisole, which causes hypercontraction and paralysis of the worms. *unc-50(kr331)* animals were as resistant to levamisole (Fig. 2A and B) as *unc-50(e306)* null mutants (4). This suggested that L-AChRs were no longer expressed in *unc-50(kr331)* mutants. To confirm this hypothesis, we used a knock-in strain in which the red fluorescent protein TagRFP was inserted into the genomic locus of the essential L-AChR subunit *unc-29* (*unc-29(kr208::rfp)*). In *unc-29(kr208::rfp)* animals, clustered L-AChR are detected at the NMJs along the ventral and dorsal nerve cords (7). In contrast, no signal was detected when the *unc-50(kr331)* mutation was introduced in the background, suggesting that the UNC-50 protein was no longer functional (Fig. 2C). To exclude that the manipulation of the *unc-50* locus might have impaired the expression or the stability of the *unc-50* mRNA, we analyzed *unc-50* mRNA by quantitative RT-PCR and found no difference between wild-type and *kr331* mutant backgrounds (Fig. 2D). Altogether, these data indicate that the C-terminal truncation of UNC-50 impairs its function and causes a loss of AChR expression in *C. elegans* muscle, establishing the loss of function effect of the mutation found in the affected individual.

## Discussion

Congenital myasthenic syndromes (CMS) are caused by abnormal signal transmission at the NMJ leading to muscle weakness with variable age of onset from embryo/fetal development to



**Figure 1.** Characterization of the *UNC50* mutation in the AMC family. (A) AMC phenotype of the affected individual (II:1): note the bilateral club foot, bilateral flexion of elbows, hips and knees and bilateral extended wrists. (B) Pedigree of the AMC family and Sanger sequencing. Arrows indicate mutant nucleotide position. The affected individual carry homozygous mutation (II:1, c.750\_751del:p.Cys251Phefs\*4). The nucleotide and amino acid changes are indicated with respect to the reference sequences (Genbank: NM\_014044.6 and Genbank: NP\_054763, respectively). Open symbols: unaffected; filled symbols: affected. (C) Phylogenetic conservation of the C-terminus of *UNC50* homologs using ClustalO alignment. Accession numbers are from NCBI reference sequence. \* represents fully conserved residue, : represents residues with strongly similar properties, . represents residues with weakly similar properties. The last nine amino acids truncated in the patient is underlined. (D) Human wild-type and mutant amino acid sequence: the modified residues are underlined. *C. elegans*: amino acid sequence in wild type and *unc-50(kr331)* mutant.

early childhood. In early onset CMS, AMC may also be present due to the reduction or lack of fetal movements in utero. Neuromuscular transmission depends on the release of acetylcholine (ACh) from the presynaptic nerve terminal and its interaction with AChRs on the postsynaptic membrane. *CHAT*, *CHRNE*, *COLQ*, *DOK7*, *GFPT* and *RAPSN* are the genes most commonly found to be mutated in CMS (8). Other genes have been found to be mutated in CMS including *CHRNA1*, *CHRNA1*, *CHRNA1* encoding, with *CHRNE*, the subunits of AChR (8).

Here we found homozygous frameshift deletion in *UNC50* in an affected individual with AMC. This mutation, when inserted in *C. elegans*, leads to a strong decrease of AChR surface expression. *UNC50* is the ortholog of *GMH1*, a family of transmembrane proteins conserved from yeast to human, which localizes to the Golgi apparatus. It interacts with a guanine nucleotide exchange factor of the small G-protein Arf and potentially regulates COPI-dependent trafficking events (9,10). In *C. elegans*, *unc-50* mutant animals display slow and abnormal locomotion, called an 'uncoordinated phenotype', on which the gene was originally named (11). Inactivation of the *unc-50* gene eliminates the expression of heteromeric acetylcholine receptors (AChR) at the NMJ (11). *UNC-50* is cell-autonomously required in body-wall muscles to promote the targeting of AChRs to the plasma membrane by preventing routing and degradation in lysosomes (4). *UNC-50* has therefore an essential role in L-AChR trafficking and consequently in cholinergic transmission at the NMJ of *C. elegans*. In *C. elegans*, *UNC-50* is expressed from early embryogenesis to adulthood and both the uncoordinated movement and the levamisole resistance phenotype are already observed

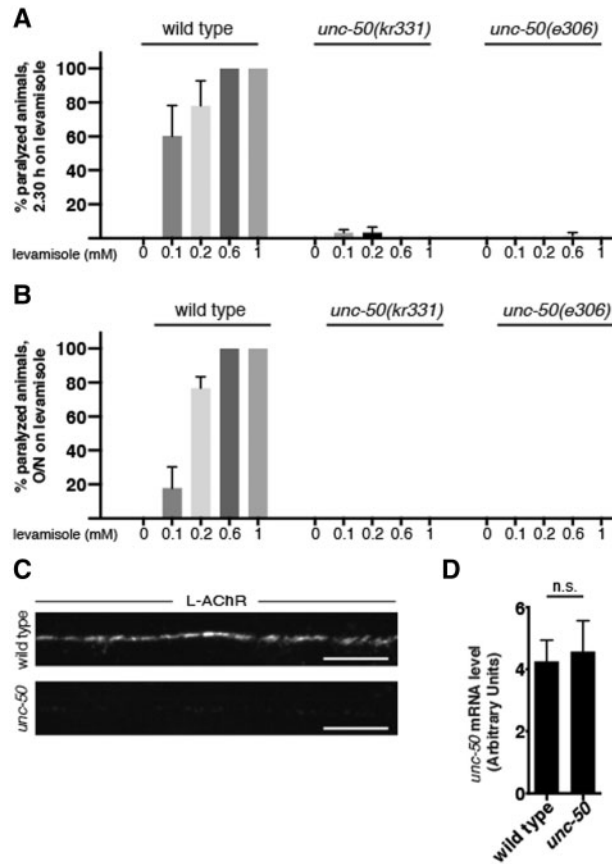
after hatching in *unc-50* mutant larvae (4). It is therefore reasonable to propose that *UNC50* mutation would impair AChR early during development. *UNC50* is widely expressed in various human tissues including skeletal muscle (NCBI; GEO Profiles ID: 3936010). During embryonic development, expression profiling by array revealed an expression of *UNC50* from development stage E9.5 in *Mus musculus* skeletal muscle (NCBI; GEO Profiles ID: 87743396). In mammals, refined analysis of *UNC50* and AChR expression at the NMJ during development should provide critical information about the role of *UNC50* in AChR trafficking at the NMJ. This gene may be regarded as strong candidate in undiagnosed AMC and CMS.

## Materials and Methods

Homozygosity by descent of disease loci was carried out using Affymetrix GeneChip Human Mapping 250K SNP microarray and bioinformatics analysis was performed using the ALOHOMORA (12) and Merlin softwares (13).

WES was performed from DNA of the index case by using the Exome Capture Agilent SureSelect XT V5 kit for library preparation and exome enrichment as previously described (14). Sequencing was performed on a Genome Analyzer Iix Illumina instrument in paired-end mode with a read length of 2 × 100 bp. Reads were aligned to the human reference genome sequence (UCSC hg19, NCBI build 37.3) via the BWA program (15). Variants were selected using the SAMtools (16) then annotated using Annovar softwares (17). Variants in coding regions (including non-synonymous and non-sense mutations), intron-exon





**Figure 2.** The *unc-50* disease mutation impairs L-AChR expression in *C. elegans*. Percentage of paralyzed animals after 2.5 h (A) and overnight (B) exposure to increasing concentrations of the AChR agonist levamisole in wild-type and *unc-50* mutant alleles. e306 is a reference loss of function allele of *unc-50*. Data are displayed as mean  $\pm$  SEM, experiment was repeated three times,  $n \geq 50$  for each genotype. L-AChR are not detectable at NMJs in *unc-50(kr331)* mutants (C) based on the expression of the RFP-tagged UNC-29L-AChR subunit from the *unc-29(kr208::rfp)* knock-in locus. Scale bars 10  $\mu$ m. *unc-50* mRNA levels are not altered by the *kr331* mutation (D). RNA samples were collected from synchronized L4 larvae and *unc-50* mRNA levels were quantified by RT-qPCR. Fold-change mean  $\pm$  SEM in *unc-50(kr331)* is shown in four independent experiments. n.s. (not significant),  $P = 0.9714$ , Mann-Whitney-Wilcoxon test.

junctions or short coding insertions or deletions were selected when the MAF was  $<0.0030$ . Prediction of pathogenicity of non-synonymous or splice mutations was performed using the Polyphen-2 (18) and Human splicing finder respectively (19).

### Sanger sequencing

PCR primer pair was designed from genomic DNA to amplify and sequence UNC50 variant (5'-CTTTTCCAGCATTGCCATTT-3' and 5'-TTTTAAGGAATGTACCTGACCACA-3'). PCR amplification was carried out using 1.5 mM  $MgCl_2$ , 0.6 U DNA Polymerase, 0.25  $\mu$ M each primer and 50 ng DNA. After an initial cycle of denaturation at 94°C for 5 min, 30 cycles were performed consisting in denaturation at 95°C for 30 s, annealing at 60°C for 1 min. and extension at 72°C for 1 min., and final extension 7 min at 72°C, on an ABI9700 Thermal Cycler. PCR products were then purified then sequenced using the forward or reverse primers (Eurofins Genomics). The obtained DNA sequences were compared with published sequences (BLAST, NCBI). Sanger sequencing was also performed to establish the genotype of each

family member, and to analyze the segregation of the variant within each family. A cohort of 95 ethnically matched controls was analyzed for the selected variant.

### *Caenorhabditis elegans* strains and genetics

*Caenorhabditis elegans* strains were cultured as described previously (20) and kept at 20°C. The following mutations were used in this study: LG I: *unc-29(kr208::tagRFP)*, LG III: *unc-50(e306)*, *unc-50(kr331)*.

### Generation of deletion and single-copy insertion alleles by a modified Co-CRISPR protocol

The final *unc-50(kr331)* knock in containing the Y284\* mutation was generated using a modified version of the Co-CRISPR technique; this protocol consists of two steps.

In the first step, wild-type worms were injected with sgRNAs against the conserved tyrosine in position 284 (Y284). The pMD14 vector was injected as a rescue template. In pMD14, a 1.5-kilobase (kb) left *unc-50* homology sequence and a 2.2-kb right homology sequence (containing a premature stop codon in place of Y284) flank a HyHis-Cl cassette. The HyHis-Cl cassette is inserted in the fourth exon of *unc-50* (63 nucleotides before the stop codon). The HyHis-Cl cassette contains both positive (hygromycin B) and negative (Histamine) selection markers flanked by two *LoxP* sites. *unc-50(kr325)* knock-in allele was identified using positive selection of worms containing the HyHis-Cl cassette, therefore selecting worms resistant to hygromycin B. In the second step, the *loxP*-flanked HyHis-Cl cassette was excised by using the Cre recombinase system. *unc-50(kr331)* knock-in animals were identified based on their resistance to Histamine, followed by PCR analysis.

The following plasmids were used for generation of deletion and single-copy insertion alleles:

- pDD104: *Peft-3::Cre::tbb-2* 3'UTR (Addgene), Cre recombinase expression plasmid.
- pHT76: *Prps-0::HygR::gpd-2* 3'UTR::*HisCl1::unc-54* 3'UTR. This plasmid contains Hygromycin B resistance and Histamine selection markers (21,22). The hygromycin B resistance gene (hygromycin B phosphotransferase, HygR) and the Histamine-gated chloride channel were driven under the control of a small ribosomal subunit promoter (*rps-0*). Histamine is not synthesized or used as a neurotransmitter by *C. elegans*; however, expression of Histamine-gated channel renders worms sensitive to exogenously applied histamine. The HyHis-Cl cassette was flanked by two *LoxP* sites.
- pMD14: *unc-50::HyHis-Cl::unc-50*. A PCR fragment of 1583 nucleotides containing part of exon2, exon3 and exon4 from *unc-50* was inserted in pHT76 vector before the left *LoxP* site (between *PmeI* and *MfeI* sites). A PCR fragment of 2228 nucleotides, encompassing the last 35 nucleotides of exon4 and the 3'UTR from *unc-50* gene, was inserted in pHT76 vector after the right *loxP* site (between *Eco105I* and *PCiI* sites). In this fragment, a premature stop was introduced in place of Y284 (specifically N282, F283 and Y284 were mutated into, respectively, F282, L283 and \*284). Different fragments were assembled using isothermal assembly (23).
- pMD12 (Tyr287 sgRNA): This vector was created on the basis of pPT02. The pPT02 vector contains a *C. elegans* U6 promoter and 3'UTR (24) and two restriction sites (*PmeI* and *SexAI*) to linearize the vector, followed by the invariant sgRNA scaffold

sequence (25). To generate the sgRNA expression vector pMD12, the protospacer sequence was inserted between the U6 promoter and the sgRNA scaffold, using PmeI and SexAI sites of pPT02. The protospacer was synthesized by Sigma: AATTGCAAATCTAAATGTTTCACAAATTTGCGCCCAACAGT TTTAGAGCTAGAAATAGC.

### Levamisole assay

Assays for levamisole sensitivity after overnight exposure were performed as previously described in Richard et al. (26).

### Quantitative real-time PCR assays

Quantitative Real-Time PCR assays were as described previously (26). In brief, total RNA was isolated from a synchronized population of worms using the RNeasy Kit (Qiagen) according to the manufacturer's instructions. All samples were treated with DNase (Fermentas). First-strand cDNA was synthesized from 200 ng of total RNA using an oligo(dT) primer and a Moloney murine leukemia virus reverse transcriptase (Fermentas) at 42°C for 1 h. Quantitative PCR was performed using LightCycler 480 SYBR Green I Master (Roche). A relative quantification model was used to evaluate the relative expression ratio of the target genes and RNA levels were normalized to the three housekeeping genes *tba-1*, *pmp-3* and *Y45F10D.4*.

### Microscopy

Animals were mounted on 2% agarose pads, immobilized using 2%(v/v) polybead microspheres (0.1 mm diameter, Polyscience, 00876-15) in M9 buffer. Pictures of dorsal cords were acquired with either an Olympus IX83 microscope equipped with a spinning disk CSU-X1 (Yokogawa) and a iXon Ultra 888 camera. Image analysis was performed with Image J.

### Supplementary Material

Supplementary Material is available at HMG online.

Conflict of Interest statement. None declared.

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