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The tumour suppressor gene *WWOX* is mutated in autosomal recessive cerebellar ataxia with epilepsy and mental retardation

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We previously localized a new form of recessive ataxia with generalized tonic-clonic epilepsy and mental retardation to a 19 Mb interval in 16q21-q23 by homozygosity mapping of a large consanguineous Saudi Arabian family. We now report the identification by whole exome sequencing of the missense mutation changing proline 47 into threonine in the first WW domain of the WW domain containing oxidoreductase gene, *WWOX*, located in the linkage interval. Proline 47 is a highly conserved residue that is part of the WW motif consensus sequence and is part of the hydrophobic core that stabilizes the WW fold. We demonstrate that proline 47 is a key amino acid essential for maintaining the WWOX protein fully functional, with its mutation into a threonine resulting in a loss of peptide interaction for the first WW domain. We also identified another highly conserved

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homozygous WWOX mutation changing glycine 372 to arginine in a second consanguineous family. The phenotype closely resembled the index family, presenting with generalized tonic-clonic epilepsy, mental retardation and ataxia, but also included prominent upper motor neuron disease. Moreover, we observed that the short-lived *Wwox* knock-out mouse display spontaneous and audiogenic seizures, a phenotype previously observed in the spontaneous *Wwox* mutant rat presenting with ataxia and epilepsy, indicating that homozygous WWOX mutations in different species causes cerebellar ataxia associated with epilepsy.

Keywords: ataxia; tonic-clonic epilepsy; WWOX; WW domain; hereditary spastic paraplegia

Introduction

Autosomal recessive cerebellar ataxias are a clinically and genetically heterogeneous group of inherited neurodegenerative disorders that affect the cerebellum, the spinocerebellar long tracts and often the peripheral nerves. Recessive ataxias may present as a pure cerebellar syndrome or are associated with neurological symptoms such as peripheral neuropathy, cognitive impairment, optic atrophy, hearing loss, seizures or extra neurological symptoms such as cardiomyopathy and diabetes mellitus in Friedreich's ataxia (MIM# 229300; Anheim *et al.*, 2012). Main clinical features are uncoordinated gait with frequent falls, upper limb coordination problems, impairment of speech, swallowing and eye movements.

Recessive ataxias associated with myoclonus, epilepsy and cognitive deterioration are classified within the progressive myoclonic epilepsies, and include Unverricht-Lundborg disease, Lafora disease, neuronal ceroid lipofuscinoses, sialidoses, action myoclonus-renal failure syndrome and Gaucher's disease, suggesting a global CNS involvement. Mitochondrial inheritance in myoclonic epilepsy with ragged red fibres (MERRF, MIM #545000) or autosomal dominant inheritance in dentate-rubro-pallido-luysian atrophy (DRPLA, MIM# 125370) may also be responsible for a progressive myoclonic epilepsy phenotype. Other recessive ataxias with a frequent occurrence of seizures, such as infantile onset spino-cerebellar ataxia (IOSCA, MIM# 271245) or recessive ataxia with coenzyme Q10 deficiency (ARCA2, MIM# 612016) are usually excluded from the group of progressive myoclonic epilepsies.

We reported a new form of childhood onset autosomal recessive cerebellar ataxia with generalized tonic-clonic epilepsy and mental retardation in a large consanguineous family from Saudi Arabia with four affected children (Gribaa *et al.*, 2007; SCAR12, MIM# 614322). MRI of one patient revealed posterior white matter hyperintensities, whereas muscle biopsy showed vacuolization of the sarco-tubular system. The gene was localized by homozygosity mapping to a 19-Mb interval in 16q21-q23 between markers D16S3091 and D16S3050. We report here the identification by whole exome sequencing of a homozygous missense mutation in the WW oxido-reductase gene (*WWOX*, MIM# 605131) located in the linkage interval. It affects the first WW domain of the mutant protein, which interacts with other proteins by specific binding with proline-proline-X-tyrosine motifs (Ludes-Meyers *et al.*, 2004). The causative involvement of the mutation was confirmed by functional analysis of the mutant domain and phenotypic studies of conditional knock-out murine model, and secondarily strengthened by a second homozygous missense mutation in a consanguineous Israeli Palestinian family with the same phenotypic key features.

Materials and methods

Genetic studies

To obtain patient's DNA and primary fibroblasts, written informed consents were obtained, as defined by the Local Ethical Committee of the College of Medicine, King Saud University, Riyadh. Whole exome sequencing was performed by DNAVision SA by exon capture with the Agilent SureSelect kit and high throughput sequencing with an Illumina HiSeq2000 sequencer. Approximately 102 million reads were obtained and mapped to the human reference genome (hg18). The median depth of reads over exons was 48.7 with 77.3% of total exons covered by 10 reads or more. Exon coverage with at least one sequence was 97.6%. dbSNP 130 served as a reference to exclude known single nucleotide polymorphisms, with additional search in Ensembl, NCBI and exome variant server (NHLBI GO Exome Sequencing Project) single nucleotide polymorphism databases when a variant was unknown. A second family was identified by whole exome sequencing performed on the same sequencer model and with the same kit, but analysed with help of the GEnomes Management Application (GEM.app; Gonzalez *et al.*, 2013). Search for mutations in genes known to be responsible for neurological phenotype including recessive cerebellar ataxia was performed and no mutation was found. Genomic DNA of 189 patients from 182 families with sporadic or recessive ataxias was screened by direct sequencing of the protein coding exons of *WWOX* (nine coding exons and an alternative exon 6).

WW domain three-dimensional modelling and western blot analysis

Protein sequence conservation was studied with the PipeAlign online software (http://bips.u-strasbg.fr/PipeAlign). Three-dimensional homology models of WW domains were generated with the online SWISS-MODEL software (http://swissmodel.expasy.org; Arnold *et al.*, 2006), based on sequence alignment with the closest WW homologues found in the protein database (pdb), which were used as reference. Homology models and nuclear magnetic resonance model of WW domains were then superimposed using PyMol (http://pymol.org). Western blot analysis was performed with polyclonal IgG antibodies directed against the first 19 amino acids of WWOX (Santa Cruz biotechnology, dilution 1:500). Human skin fibroblasts were cultured from a skin biopsy from Patient II-3 and from four different controls in

Dulbecco modified Eagle's medium with glucose 1 g/l, 10% foetal calf serum and gentamicine, at 37°C with 5% CO2.

Construction of wild-type and mutant WW domain fusion proteins and peptide pull-down assays

To study WWOX protein–protein interaction *in vitro*, we used synthetic constructs with glutathione-S-transferase (GST) tags for affinity purification. GST fusion proteins containing WWOX first and second WW domains (WWOX1–2), and only the first WW domain (WWOX1) were constructed by insertion of PCR amplified fragments of the WWOX complementary DNA into the BamHI and EcoRI sites of the bacterial expression vector pGEX-2TK (Pharmacia). The resulting fusion proteins contain the WWOX amino acids 16 to 94 (GST-WWOX1–2) and amino acids 16 to 53 (GST-WWOX1) as previously described (Ludes-Meyers *et al.*, 2004).

Site-directed mutagenesis was performed using the GeneArt Site-Directed Mutagenesis PLUS kit (Invitrogen). The target for mutation was p.Pro47Thr (c.139C>A) in the first WW domain of fusion proteins WWOX1–2 and WWOX1. All sequences were verified by DNA sequencing.

GST fusion proteins were purified from bacterial lysates through the use of glutathione sepharose beads (GE Healthcare). *In vitro* peptide pull-down assays were conducted by overnight pre-binding of 15 μg of the biotinylated WBP1 peptide (SGSGGTP**PPPY**TVG) with 1 μg of GST fusion protein in 500 μl of binding buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% NP-40, 5 mM EDTA, 5 mM EGTA, 15 mM MgCl2) and rocking at 4°C. After pre-washing with binding buffer, 25 μl of streptavidin agarose beads (Millipore) were incubated with the peptide-protein mix and rocked for 1 h at 4°C. Agarose beads were then washed three times with binding buffer. 30 μl of 2× protein loading buffer was added to the agarose beads and boiled. Protein separation was performed by SDS-PAGE and analysed by western blot using a rabbit anti GST-specific primary antibody developed in M.B. lab (Espejo *et al.*, 2002; 1:2000 dilution).

Animal experiments

All animal research was conducted in facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International at the University of Texas, M.D. Anderson Cancer Centre, following international guidelines and all research was specifically approved by the University of Texas M. D. Anderson Cancer Centre Institutional Animal Care and Use Committee (Animal Welfare Assurance Number A3343-01).

For the generation of *Wwox* knock-out mice, we crossed male $Wwox^{flox/flox}$ mice (129SV/C57Bl/6 background) (Ludes-Meyers *et al.*, 2009) with female BK5-Cre mice (129SV/C57Bl/6 background). Cre recombinase in these females is activated in oocytes with Cre protein persisting in the embryo leading to constitutive recombination and producing full knock-out progeny (Ramirez *et al.*, 2004).

Wwox knock-out mice and wild-type counterparts (16–20 days of age) were exposed to sound stimulation while in conventional polycarbonate cages. Exposure to digital 11 and 14 kHz tones (5–10-min exposure) was conducted using speakers adjacent to three sides of the cages. Mice behaviour was monitored and recorded using a video camera.

Results

Mutation analysis of Family 1

Exome sequencing of Individual II-1 revealed 11 unknown homozygous variations in the linkage region. Three coding variants appeared to be present in more recent single nucleotide polymorphism databases as dbSNP132 (rs74344827, rs76382044, rs77067228). Seven variants were intronic. Three of the intronic variants were subsequently found in an update of Ensembl single nucleotide polymorphism database (rs117678211, rs74647029 and rs74980679). The fourth intronic variant, in the gene encoding for the carboxyl esterase 3 (CES3) protein (position chr16: 65564056C>A), was not present in Ensembl but was present in Exome Variant Server (EVS) with a frequency of 34 A alleles for 10724 C alleles. The fifth intronic variant, in the gene encoding for the splicing factor 3b subunit 3 (SF3B3) protein (position chr16: 69155457), is located in the donor splice site of exon 18 (+3; A>G) and was also found in EVS at a low frequency (three A alleles for 10755 G alleles). The last two intronic variants, in the genes encoding for the neurexin CASPR4 (position chr16:75126896A>G) and for the carboxyl esterase 4A (CES4A) protein (position chr16: 65596532C>T), respectively, were present neither in Ensembl nor in EVS. None of the intronic variants are likely pathogenic since they are not predicted by the SplicePort progam to affect the adjacent splice sites or to activate cryptic splice sites.

The only unknown variant affecting a coding sequence (position chr16: 76699852 C>A [hg18], 99 reads coverage) was located in the gene encoding for WWOX, a protein with two WW domains and an oxidoreductase domain. As expected, the mutation (c.139C>A, exon 2) is homozygous in the patient and segregates with the disease (Fig. 1). The mutation affects the first WW domain (WW1) of WWOX (p.Pro47Thr) and causes a radical change from a hydrophobic amino acid, proline, to a polar one, threonine. The mutated proline is conserved in all metazoans (including distant ones such as *Trichoplax* and sea anemone, Fig. 1) which are the only species having a WWOX homologue (i.e. a protein with WW and oxidoreductase domains). Moreover, this highly conserved proline 47 is the last amino acid of the WW motif consensus and is one of the few aromatic residues (together with tryptophan 22 and tyrosine 34) that build up the hydrophobic core of WW domains (Macias *et al.*, 1996; Petrovich *et al.*, 2006).

Three-dimensional modelling of the first WW domain

We modelled the structure of the WW1 domain of WWOX according to the structure of its closest homologue rNEDD4 WWIII (pdb code 1I5H) and compared it to the WW structures of transcription elongation regulator 1/TCERG1 (previously known as FBP28, pdb code 1E0L) and polyglutamine binding protein 1/PQBP1 (modelled according to the structure of PIN1, pdb code 3KAB.pdb) for which mutation of the equivalent proline or of the equivalent interacting tyrosine within the core, respectively,

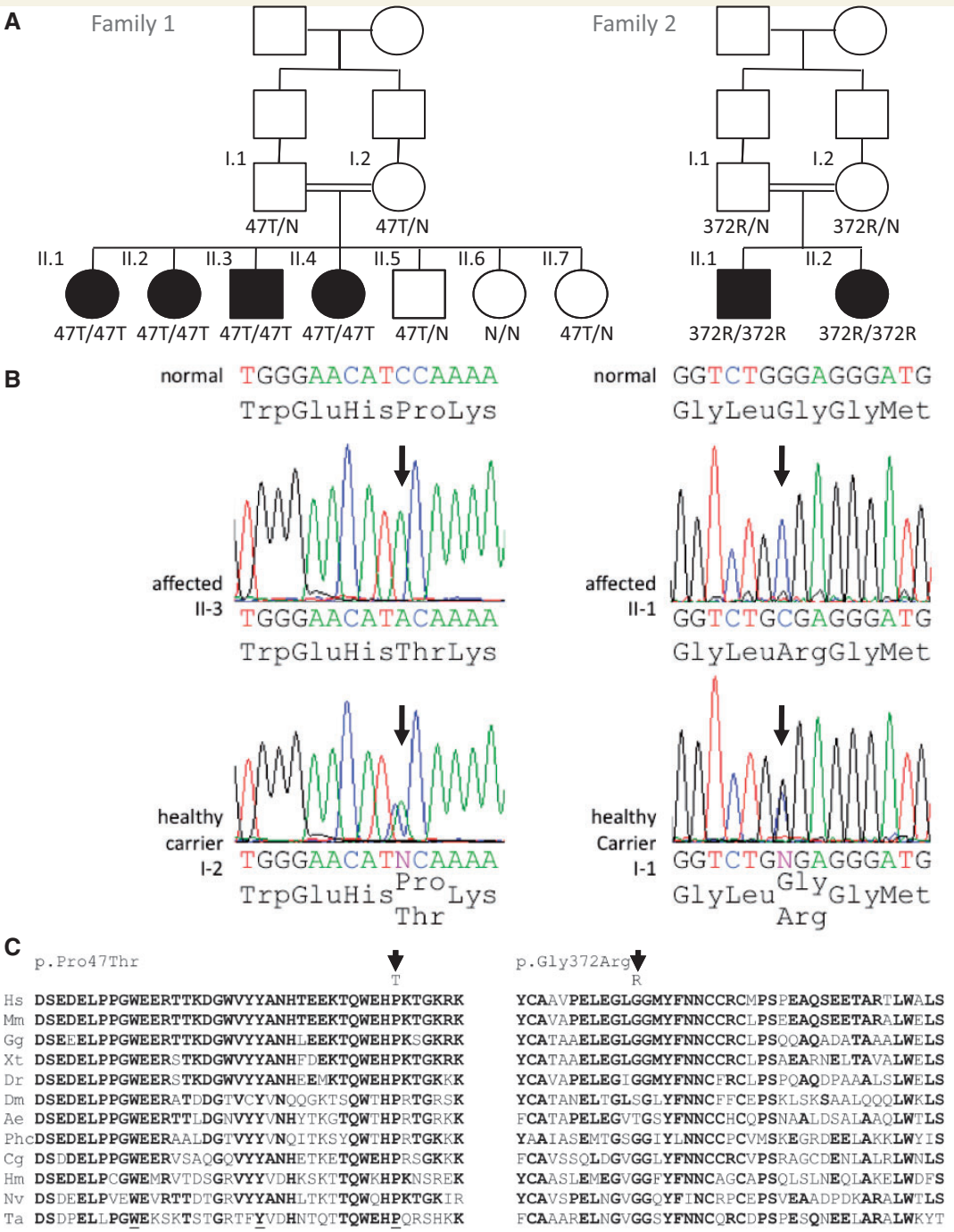

Figure 1 Identification of WWOX mutations in the Saudi and Israeli Palestinian families. (**A**) Family trees showing consanguinity and segregation of the disease with mutations c.139C>A, causing the p.Pro47Thr missense change (47T) and c.1114G>C causing the p.Gly372Arg missense change (372R). (**B**) Sanger sequencing of the mutations c.139C>A and c.1114G>C: index patients are homozygous for the substitution and carrier relatives (mother I-2 in Family 1 and father I-1 in Family 2) are heterozygous for the same substitution. (**C**) Sequence comparison of the WW1 domain (*left*) and of the C-terminal part of the dehydrogenase/reductase domain (*right*) of WWOX from different species. Amino acids that are identical to the human sequence are shown in bold. The highly conserved aromatic residues that are part of the hydrophobic core of the WW1 domain are underlined. The mutant amino acids (threonine, T, and arginine, R), on top of the mutated amino acids (proline, P, and glycine, G, respectively), are indicated by an arrow. The mutated amino acids are conserved in all metazoan species analysed, except glycine 372 in some insects (endopterygota branch). Hs = human; Mm = opossum; Gg = chicken; Xt = frog; Dr = bony fish; Dm = fly; Ae = ant; Phc = louse; Cg = oyster; Hm = hydra; Nv = sea anemone; Ta = trichoplax (multi-cellular amoeba).

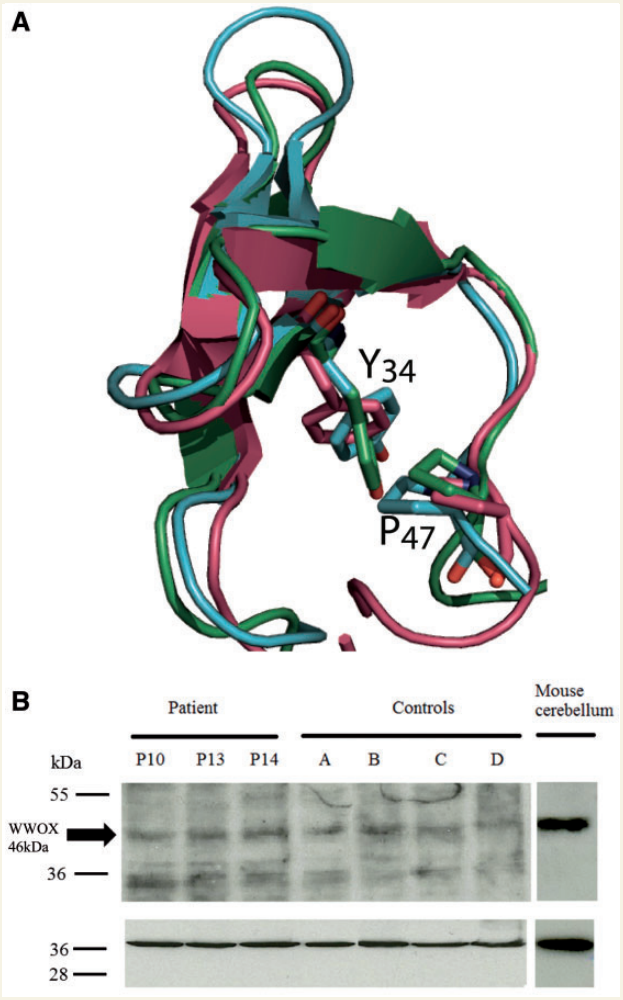

Figure 2 Analysis of normal and p.Pro47Thr mutant WWOX protein. (**A**) Superimposition of the well structurally characterized WW domain of TCERG1 (purple, pdb code 1E0L; Macias *et al*., 2000) and the 3D homology models of WWOX (green) and PQBP1 (blue) WW domains. Two amino acids belonging to the hydrophobic core of WW domains are highlighted and annotated according to the WWOX sequence: tyrosine 34 (Y34) and proline 47 (P47). In TCERG1, mutation of the proline into alanine results in a WW domain that is largely unfolded (Petrovich *et al*., 2006). In PQBP1, the mutation of the tyrosine into a cysteine is causing Golabi-Ito-Hall mental retardation syndrome: the mutation affects the WW domain folding, reduces its binding activity, and alters the function of PQBP1 (Tapia *et al*., 2010). These two residues are in direct hydrophobic contact within WW domains. (**B**) Western blot analysis of WWOX expression in cultured human skin fibroblasts and in mouse cerebellar tissue. *Upper panel*: WWOX expression in cultured skin fibroblasts from Patient II-3 of Family 1 at three passages 10, 13 and 14 (P10, P13, P14) and from fibroblasts from four different controls (A to D). Based on visual inspection, similar amounts of the mutant and wild-type WWOX protein (arrow) are observed. *Right lane*: cerebellar mouse extracts were analysed in a similar western blot with the same primary antibody, in order to show the position of the WWOX band and high expression of WWOX in this tissue. The nature of the 35 kDa band seen on the human fibroblast lanes is not known and is presumably non-specific cross-reaction since it is not seen in the mouse tissues (tissues other than cerebellum were also tested, not shown). *Lower panel*: the same western blot was analysed with anti-actin antibodies, to check for equal loading of protein extracts.

are known to dramatically destabilize the fold (Petrovich *et al*., 2006; Tapia *et al*., 2010). The position of WWOX proline 47 and tyrosine 34 precisely match the position of TCERG1 and PQBP1 equivalents, which are direct hydrophobic core neighbours and are crucial for proper folding of the WW domain (Fig. 2). Thus, the change of proline 47 into a threonine most likely destabilizes the WW1 domain.

Interaction studies of the mutant WW domain

To test this hypothesis, we constructed GST-WWOX fusion proteins containing the first WW domain (WWOX1) as well as both WW domains (WWOX1–2). We generated both wild-type and mutant versions [p.Pro47Thr (c.139C>A)] of these fusion proteins. As previously demonstrated, the first WW domain of WWOX represents a typical Group I WW domain with predilection for binding proline-proline-X-tyrosine motifs, where X stands for any amino acid, whereas no ligand was identified for the second WW domain (Ludes-Meyers *et al*., 2004). We performed peptide pull-down assays using as ligand a biotinylated oligopeptide from the WW domain-binding protein 1 (WBP1), a typical WW group I binder, containing a proline-proline-proline-tyrosine motif (PPPY). As can be observed in Fig. 3, the wild-type WWOX1 and WWOX1–2 fusion proteins were successfully pulled down whereas the p.Pro47Thr mutant versions failed to interact with the PPPY containing oligopeptide.

The high conservation of the proline 47 residue and the above experiments demonstrate the crucial role of this residue in WW domains, indicating that the observed mutation likely plays a key structural role leading to unfolding and dysfunction of WWOX1. This evidence suggests that p.Pro47Thr is the disease causing mutation in the ataxia/epilepsy family from Saudi Arabia.

Furthermore, western blot analysis from patient fibroblasts showed a similar level of WWOX protein compared to four different controls (Fig. 2) suggesting that the mutation does not alter global protein levels. Globally unchanged protein levels were also reported for the equivalent Tyr34 mutation of polyglutamine tract binding-protein 1 (PQBP1) involved in X-linked mental retardation disorders such as Golabi-Ito-Hall syndrome, showing that a disease causing mutation that affects the fold and the function of a WW domain does not necessarily result in enhanced mutant protein degradation (Tapia *et al*., 2010).

Mutation analysis of Family 2

After the identification and characterization of the first disease-associated WWOX mutation, a second mutation was identified by exome sequencing of a consanguineous Palestinian family. The two patients from this Israeli Palestinian family carried a highly conserved homozygous WWOX mutation (p.Gly372Arg;

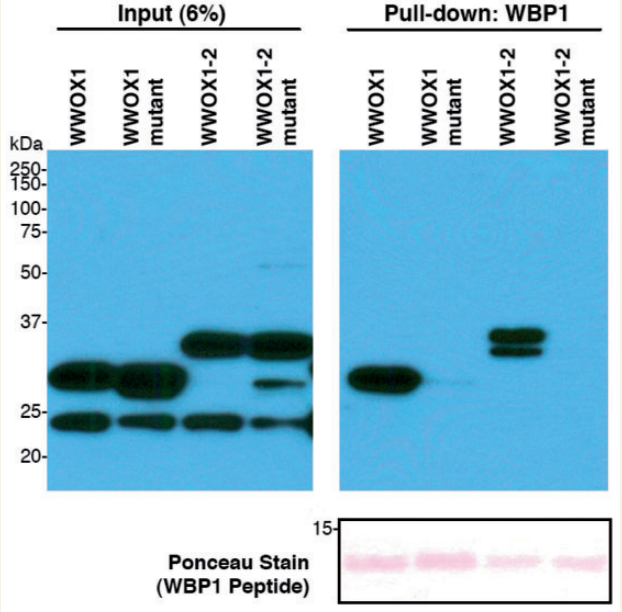

Figure 3 Wild-type and mutant WWOX WW domain pull-down assays. Purified GST-WWOX WW domain fusion proteins were incubated with biotinylated WBP1 peptide (SGSGGTP**PPPY**TVG) in pull-down assays. WWOX1, first WWOX WW domain only; WWOX1–2, WWOX WW1 and WW2 domains. Mutant indicates the fusion peptides with the p.Pro47Thr change. Western blot of 6% input of GST fusion proteins to demonstrate equal amounts of input material in the pull-down experiments (*left*). *Right*: only the wild-type and not the mutant fusion peptides were efficiently pulled down with the PPPY containing oligopeptide, demonstrating that the change of proline 47 to threonine is sufficient to abrogate the affinity of WWOX for PPXY motif containing interacting partners, presumably by destabilizing the WW1 fold. *Bottom*: red ponceau stain of the pull-down blot (oligopeptide range), to demonstrate incubation with equal amounts of biotinylated WBP1 peptide.

c.1114G>C in exon 9) (for conservation data and pedigree, see Fig. 1) which was not observed in dbSNP 137 or in the NHLBI 6500 exome variant server. The homozygous variant segregated with disease in the affected siblings and testing of the parents demonstrated localization in *trans*. The phenotype in this second family closely resembled the index family: both affected siblings showed an early disease onset in the first 2 years of life with generalized tonic-clonic epilepsy, mental retardation, and ataxia. In addition, prominent upper motor neuron affection with leg spasticity and positive bilateral extensor plantar response was present, thus resembling spastic ataxia or complicated hereditary spastic paraplegia. The mutated glycine 372 is located in the C-terminal part of the dehydrogenase/reductase domain of WWOX. The closest homologue of this domain with known 3D structure is 3RD5, a bacterial small dehydrogenase/reductase (pdb code 3RD5). Moderate conservation of the amino acid sequence between the human and bacterial homologues prevented reliable modelling of the sequence surrounding glycine 372 of WWOX. The extensive screening of *WWOX* in 189 unrelated ataxic patients with no molecular diagnosis (seven patients with childhood onset ataxia associated with epilepsy, 67 patients with onset of ataxia before 3 years, and 135 patients with onset of ataxia before 20 years) revealed no additional mutation.

Conditional knock-out mouse model

As it seems that ataxia/epilepsy associated with *WWOX* mutation is very rare in humans, we sought to confirm the involvement of *WWOX* in neurodegeneration from the study of our mice *Wwox* knock-out model (Ludes-Meyers *et al.*, 2009). These knock-out mice are characterized by a short lifespan of only 3 to 4 weeks maximum (Aqeilan *et al.*, 2008; Ludes-Meyers *et al.*, 2009). Interestingly, we observed that the *Wwox* knock-out mice start having a few spontaneous seizures at ~2 weeks of age. To investigate susceptibility to epilepsy, 16-day-old knock-out mice were exposed to sustained sound (11–14 kHz tone) for 5–10 min. A few knock-out mice (three of eight) presented with audiogenic tonic-clonic seizures in the first minutes after sound exposure. Seizures began with wild running and jumping, then progressed to tonic contraction of limbs and tail and often presented with limb clonic movements, followed by lethargy. At 20 days, the four surviving mice were exposed to a 14 kHz tone. All knock-out mice presented at different times with seizures, consisting as before of wild running followed by tonic contractions and clonic movements, and had uncontrolled sphincter relaxation (Fig. 4 and Supplementary Video 1). At the end of seizures, the mice presented with lethargy. Other stimuli such as animal handling also induced seizures on some occasions. At this point, all knock-out mice presented with balance disturbances when challenged to walk on the edge of the cage but they showed no pathological clasping reflexes. They eventually died before 4 weeks of age from failure to thrive. No wild-type mice of matched age and background ($n = 8$) presented with seizures upon 11 or 14 kHz sound exposure.

Discussion

We describe the first germline mutations in *WWOX* causing a recessive form of early onset neurodegenerative disease in man including epilepsy, mental retardation, cerebellar ataxia, and—at least in the second family—prominent spasticity. *WWOX* was identified by Bednarek *et al.* (2000) and codes for a 414 amino acid protein containing two WW domains and a short-chain dehydrogenase/reductase (SDR) domain (suggesting a role in steroid metabolism). WWOX is an ubiquitous protein with high expression in prostate, gonads, breast, lung, endocrine tissues, cerebellum and brain (Nunez *et al.*, 2006; Aqeilan *et al.*, 2007). *Wwox* is expressed in mouse developing nervous system including cerebral cortex and cerebellum from Day 12–14 *in utero* to adulthood (Chen *et al.*, 2004). We demonstrate that the *WWOX* mutation, p.Pro47Thr, which could be modelled by our *in silico* model, renders WWOX unable to bind protein partners through the WW1 domain, and that the complete loss of function of WWOX in mice results in epilepsy and balance disturbances, phenotypes reminiscent of the clinical presentation of all our affected patients with *WWOX* mutations. The *Wwox* knock-out mice show a progressive

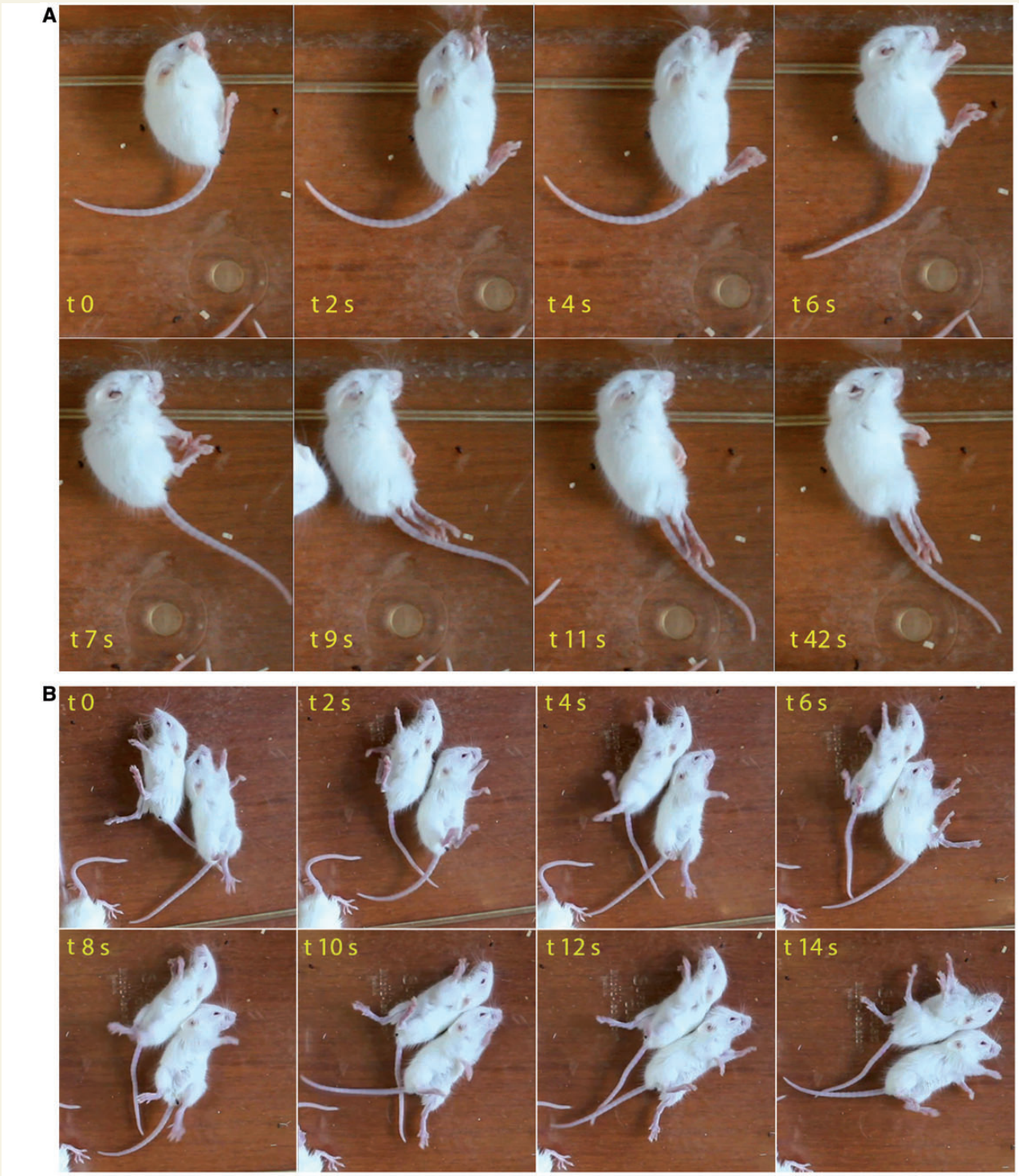

Figure 4 Still frames of audiogenic tonic-clonic seizures of *Wwox* knock-out mouse. Twenty-day-old *Wwox* knock-out mice were exposed for 10 min to a digital 14 kHz tone. After 30 s sound exposure, the first mouse presented a wild-running phase (see Supplementary Video 1), followed by tonic contractions that lasted over 42 s (**A**). After 4 min sound exposure, two other mice also experienced clonic movements (**B** and Supplementary Video 1).

susceptibility to spontaneous and audiogenic tonic-clonic epilepsy, suggesting a neurodegenerative process. However, the severe condition and early death of the *Wwox* knock-out mice prevented us from testing whether the balance problems were directly related to cerebellar dysfunction or not. Interestingly, a spontaneous rat mutation of *Wwox*, *Ide* (lethal dwarfism and epilepsy) results in a condition similar to the presentation of the mutant *WWOX* patients and the *Wwox* knock-out mice, as homozygous

Ide rats present with ataxic gait and audiogenic seizures (Suzuki *et al.*, 2007, 2009). The *Ide* mutation is a 13 bp deletion in exon 9 leading to a frameshift of the last 44 codons of *Wwox* and replacement with a novel open reading frame of 54 codons. Mutated WWOX is not detected in western blots of *Ide* rat tissues. The milder presentation of the human disease compared with the mouse and rat models is presumably because of a partial loss of function mutation as the mutant p.Pro47Thr WWOX protein is still present, at least in human skin fibroblasts, and the dehydrogenase/reductase domain of the mutant protein is presumably still functional in Family 1 and partially functional in Family 2, unlike the dehydrogenase/reductase domain of the WWOX mouse and rat models. Homozygous complete loss of function of WWOX in humans may also cause early death or embryonic lethality, suggesting that *WWOX* related ataxia cases are rare, in line with our failure to identify additional *WWOX* mutation in a large cohort of patients with ataxia.

WWOX is a cytoplasmic protein that participates in a number of cellular processes including growth, differentiation and tumour suppression. *WWOX* somatic mutations, including homozygous deletions, have been involved in different human cancer cell types (Paige *et al.*, 2001). WW domains are protein-protein interaction domains that recognize proline-rich sequences. Numerous interactors of the WW1 domain of WWOX have been identified, including p73, RUNX2, ERBB4, Dvl2, SIMPLE/LITAF among others (Aqeilan and Croce, 2007). Alteration of WW1 is responsible for a loss of interaction with Dvl2 (Bouteille *et al.*, 2009). Direct mutagenesis of amino acids 44 to 47 of the WW1 domain of WWOX leads to a loss of interaction with SIMPLE/LITAF (Ludes-Meyers *et al.*, 2004), a protein mutated in a dominant form of Charcot–Marie–Tooth disease, CMT1C (Street *et al.*, 2003). Heterozygous deletion of exons 6 to 8 (amino acids 173 to 352 of WWOX) has recently been described in a single patient with ambiguous genitalia having a 46,XY disorder of sex development (White *et al.*, 2012). The deletion is not causing a frame-shift but leads to a smaller protein missing part of the SDR domain. It is possible that the heterozygous deletion of exons 6 to 8 of *WWOX* is coincidental in the patient with ambiguous genitalia, as no such phenotype is present in heterozygous animal models or in the parents and carrier siblings of the Saudi or Palestinian patients. Future research should investigate the presence of *WWOX* variants in patients with epilepsy, mental retardation, and/or complicated hereditary spastic paraplegia (cHSP), as the phenotype of our affected subjects indicates that these might also be presenting features of *WWOX* patients. Analysis of larger paediatric cohorts of early onset ataxia and/or generalized epilepsy and mental retardation should allow us to evaluate the prevalence of *WWOX* mutations and to perform genotype–phenotype correlation studies.

Disease mechanisms resulting from *WWOX* mutations are difficult to infer because the WWOX protein has numerous functions and protein partners. Moreover, the potential substrates of the WWOX dehydrogenase domain are not known. *WWOX* has been described as a tumour suppressor gene. Interestingly, neither the patients (current age ranging from 17 to 26 years in Family 1, and 5 and 10 years in Family 2) nor the heterozygous carriers presented with tumour development, but we cannot exclude that the patients may present an increased risk of developing cancer at a later stage. Further studies are needed to establish the full spectrum of diseases resulting from *WWOX* alterations.

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Supplementary material

Supplementary material is available at *Brain* online.

Web Resources

UCSC Genome Browser: http://genome.ucsc.edu/index.html
Ensembl Genome Browser: http://www.ensembl.org/index.html
Exome Variant Server, NHLBI GO Exome Sequencing Project (ESP), Seattle, WA(http://evs.gs.washington.edu/EVS) [June, 2013]
SplicePort software: http://spliceport.cbcb.umd.edu
PipeAlign software: http://bips.u-strasbg.fr/pipealign
Swiss-Model Workspace: http://swissmodel.expasy.org
PyMol software: http://pymol.org

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