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Upregulation of PKD1L2 provokes a complex neuromuscular disease in the mouse

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Following a screen for neuromuscular mouse mutants, we identified ostes, a novel N-ethyl N-nitrosourea-induced mouse mutant with muscle atrophy. Genetic and biochemical evidence shows that upregulation of the novel, uncharacterized transient receptor potential polycystic (TRPP) channel PKD1L2 (polycystic kidney disease gene 1-like 2) underlies this disease. Ostes mice suffer from chronic neuromuscular impairments including neuromuscular junction degeneration, polyneuronal innervation and myopathy. Ectopic expression of PKD1L2 in transgenic mice reproduced the ostes myopathic changes and, indeed, caused severe muscle atrophy in Tg(Pkd1l2)/Tg(Pkd1l2) mice. Moreover, double-heterozygous mice (ostes/+, Tg(Pkd1l2)/0) suffer from myopathic changes more profound than each heterozygote, indicating positive correlation between PKD1L2 levels and disease severity. We show that, in vivo, PKD1L2 primarily associates with endogenous fatty acid synthase in normal skeletal muscle, and these proteins co-localize to costameric regions of the muscle fibre. In diseased ostes/ostes muscle, both proteins are upregulated, and ostes/ostes mice show signs of abnormal lipid metabolism. This work shows the first role for a TRPP channel in neuromuscular integrity and disease.

INTRODUCTION

Discovering the molecular mechanisms underlying neuromuscular degenerative diseases is pivotal to the development of therapies for such heterogenous disorders. Model organisms have assisted in the identification of novel neuromuscular disease genes and genetic pathways (reviewed in 1–3). We have exploited mouse mutagenesis to identify new neuromuscular mutants. In phenotype-driven screens of mutagenized mice, simple measures of muscle strength such as wheel running or grip strength have failed to consistently identify mutants that show muscular defects at the histological level. In addition, measuring progressive motor neuron loss in mutagenized animals is not a suitable protocol for large-scale screening programmes. In contrast, overt phenotypes such as muscle atrophy, tremors, gait defects and growth retardation have been used to signpost abnormal neuromuscular function in mice (4,5). Using this simple paradigm, we identified ostes in an N-ethyl N-nitrosourea (ENU) screen (6). The aim of this work was to characterize this novel neuromuscular phenotype in the mouse and to provide insights into the underlying molecular mechanism. We present evidence that the product of the genetically linked Pkd1l2 (polycystic kidney disease gene 1-like 2) gene is grossly overexpressed in ostes/ostes mice, and that ectopic expression of PKD1L2 in transgenic mice leads to similar neuromuscular features. To date, no analysis of PKD1L2 protein expression, localization

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or in vivo function has been done. Here, we show that PKD1L2 is the first transient receptor potential polycystic (TRPP) channel to be associated with neuromuscular function and disease. Additionally, we show that abnormal lipid metabolism in PKD1L2-dysregulated mice is a key feature of this disease.

RESULTS

Ostes mice show a complex neuromuscular phenotype

We assessed the neuromuscular system in ostes/ostes mice at three levels of analysis. Grossly, ostes/ostes mice become recognizable by their small size and reduced weight between 3 and 5 weeks of age (Fig. 1A) and remain small and light throughout life (see weights of 12-week-old mice, Fig. 1B). Ostes/ostes mice also show a moderate tremor of the hind limbs upon suspension by the tail and have a poor reaching response. Conversely, ostes/+ mice do not display any overt abnormal phenotype. Sections of 24 ostes/ostes tissues including heart did not reveal any pathology except for muscle atrophy (Supplementary Material, Fig. S2). Additionally, insets in Fig. 1C). Despite the lack of gross phenotype in ostes/ostes mice, similar pathological hallmarks are evident in ostes/+ hind limb muscle, but the pathology is milder and there is no evidence of muscle atrophy (Fig. 1I and Supplementary Material, Fig. S3), indicating a semi-dominant action of the ostes mutation in muscle.

Pyknotic nuclear clumps have been associated with denervation of muscle fibres in amyotrophic lateral sclerosis and bulbospinal muscular atrophy. Therefore, endplates, axons and presynaptic axon terminals in lumbrical muscle whole-mounts from 45-day-old ostes/ostes, ostes/+ and wild-type mice were immunostained and examined for signs of denervation. Confocal microscopy revealed many denervated endplates in ostes/ostes muscles, characteristically showing a plaque-like morphology, with or without contact to a visible degenerating nerve terminal, or only partially occupied by a small number of nerve terminal boutons (Fig. 1F). Additionally, ~40% of ostes/ostes motor endplates were polynervously innervated by collateral branches of two or three axons. All endplates in wild-type littersmates had only one axon terminal per endplate. We assessed the function of polynervinated junctions in ostes mice by intracellular recordings of nerve-evoked endplate potentials (EPPs) in extensor digitorum longus (EDL) and soleus muscle from ostes/ostes (n = 3) mice. Recordings from ostes/ostes muscle in response to graded stimulation of the nerve supply resolved two components to the EPP with distinct thresholds in 20–30% of muscle fibres (n = 15–18 fibres per muscle), indicating functional polynervous innervation (Fig. 1G). These recordings corroborated the morphological evidence of enduring polyneuronal innervation in ostes/ostes mice. Fibre type distribution in the soleus muscle region did not show any differences between ostes/ostes and wild-type staining. Fibre size asymmetry did not correlate with specific fibre type, and there was no evidence of fibre type grouping (Fig. 1I and Supplementary Material, Fig. S4).

Figure 1. Neuromuscular phenotypes of ostes and Tg(Pkd1l2) mice. (A) Photograph of representative 5-week-old wild-type (+/+ ) and ostes/ostes mice. Ostes/ ostes mice are small and weigh less than wild-type mice from 3–5 weeks of age. (B) Weight of 12-week-old wild-type (+/+ ) and ostes/ostes mice. Both male and female ostes/ostes mice weigh significantly less than wild-type mice at 12 weeks of age (females: P = 5.873E−16, n = 9/ostes, 22 wild-type; males: P = 1.14E−12, n = 11/ostes, 22 wild-type). Data are represented as mean ± SEM. (C) Representative sections of gastrocnemius skeletal muscle from 45-day-old wild-type (+/+ ), ostes/ostes and Tg(Pkd1l2)/Tg(Pkd1l2) mice, stained with H&E. Evidence of degenerative changes are present in ostes/ostes muscle, including muscle atrophy, small fibres, striking fibre size asymmetry, pyknotic nuclei and centrally nucleated fibres (arrows). Muscles from 45-day-old Tg(Pkd1l2)/Tg(Pkd1l2) mice show similar pathological hallmarks. Scale bar: × 20 magnification, 100 μm, × 40, 50 μm. Inset: H&E-stained levelled cross-sections of whole hind limbs from the same mice show the reduced size and muscle atrophy in ostes/ostes and Tg(Pkd1l2)/Tg(Pkd1l2) hind limbs. (D) Mean fibre size according to fibre type in soleus muscle from wild-type (+/+ ), ostes/ostes and Tg(Pkd1l2)/Tg(Pkd1l2) mice. Mean fibre size in both ostes/ostes and Tg(Pkd1l2)/Tg(Pkd1l2) skeletal muscle is reduced compared with wild-type. Type-2 fibres show a small but significant reduction in size in ostes/ostes and Tg(Pkd1l2)/Tg(Pkd1l2) muscle. Fibre size was analysed in MYH7-stained sections (slow twitch) of soleus muscle from three 45-day-old mice containing at least 80 fibres each. Data are represented as mean ± SEM. (E) Fibre size distribution in 45-day-old wild-type (+/+ ), ostes/ostes and Tg(Pkd1l2)/Tg(Pkd1l2) skeletal muscle. Fibre size was analysed in five H&E-stained sections of gastrocnemius from 45-day-old mice containing at least 80 fibres each. (F) Immunofluorescence photographs of NMJs in wholemount lumbrical muscle from wild-type (+/+ ) and ostes/ostes mice. Acetylcholine receptors are labelled in red, and presynaptic boutons and axons in green. Wild-type (+/+ ) endplates are mononeuronally innervated, with one axon leading to each endplate. Ostes endplates show both normal mononeuronal innervation (μ), alongside abnormal polynervous innervation (ν) where two or more axon terminals innervate one endplate, in both 45-day-old and 9-month-old mice. In addition, some ostes/ostes endplates are denervated (δ), characterized by a plaque-like morphology of acetylcholine receptor staining and only partial or absent occupation by axon terminals. Scale bar, 20 μm. (G) Example of an intracellular recording of nerve-evoked EPPs from an ostes/ostes EDL muscle, showing functional polynervous innervation. (H) Immunofluorescence photographs of NMJs in wholemount hind limb muscle from 26-day-old Tg(Pkd1l2)/Tg(Pkd1l2) mice. Examples of an abnormal polynervous innervation (ν) and a plaque-like partially denervated endplate (δ) are shown. (I) Fibre type analysis of levelled cross-sections of 45-day-old +/+ , ostes/ostes and Tg(Pkd1l2)/Tg(Pkd1l2) hind limb skeletal muscle. There is no evidence of fibre type grouping, and fibre size asymmetry does not correlate with specific fibre type in either ostes/ostes or Tg(Pkd1l2)/Tg(Pkd1l2) mice. (J) Representative sections of gastrocnemius skeletal muscle from aged (>6 months) +/+ , Tg(Pkd1l2)/0 and +/+ , Tg(Pkd1l2)/0 mice, stained with H&E. Ostes/+ skeletal muscle shows similar but much milder pathological hallmarks than ostes/ostes muscle, and no evidence of overall muscle atrophy. Skeletal muscle from hemizygous Tg(Pkd1l2)/0 mice does not show any obvious myopathic changes or atrophy. However, skeletal muscle from aged ostes/+ , Tg(Pkd1l2)/0 mice shows marked fibre size asymmetry, very small fibres and centrally nucleated regenerating fibres. Scale bar: × 20, 100 μm, × 40, 50μm. Inset: H&E-stained cross-sections of whole hind limbs from the same mice show no significant differences in size.
To assess whether this polyinnervation was due to a delay in developmental synapse elimination at the neuromuscular junction (NMJ), we examined motor endplates in 9-month-old ostes/ostes mice (Fig. 1F). Polyinnervated endplates were also observed in these mice, suggesting that this phenotype was persistent and not due to prolongation of the period over which synapse elimination would normally occur (1–2 weeks postnatal). Ostes/+ mice also showed evidence of polyinnervation, but at lesser incidence than ostes/ostes mice, and polyinnervation was not unequivocally found to be functional (n = 2 mice, data not shown). In both young and old ostes/ostes and ostes/+ mice, normal mononeuronal innervation of endplates was also observed (Fig. 1F). Therefore, three distinctive types of endplates were characteristically observed in lumbrical muscles from ostes/ostes mice: monoinnervated (~50%, n = 3), denervated (~10%, n = 3) and polyinnervated (~40%, n = 3).

The specific effects of the morphological changes in ostes/ostes muscle on muscle function were also established. We found that the hind limb muscles of ostes/ostes mice were weaker than the corresponding muscles in age-matched wild-type controls. Whereas tibialis anterior (TA) muscles in wild-type mice produced a maximum tetanic force of 137 g (± 5.3 SEM, n = 6), this was reduced to only 32 g (± 2.1 SEM, n = 10) in ostes/ostes mice (Fig. 2A). Similarly, the maximum force in EDL muscles of wild-type mice was 25 g (± 3.1 SEM, n = 8) compared with only 13 g (± 0.8 SEM, n = 7) in ostes/ostes mice (Fig. 2A). The contractile characteristics of these muscles in ostes/ostes mice were also assessed. Although the time to peak force and the time taken to reach half relaxation force were the same in TA and EDL muscles of ostes/ostes and wild-type mice (Fig. 2B), there was a significant change in the fatigue characteristics of EDL muscles in ostes/ostes mice (Fig. 2C). EDL is normally a fast muscle thatfatigues rapidly when repeatedly stimulated (Fig. 2C). However, EDL in ostes/ostes mice has a higher fatigue index than that of wild-type (Fig. 2C), indicating that it has become more fatigue resistant than normal.

Despite the presence of some denervated NMJs, the loss of muscle force and change in fatigue characteristics observed in hind limb muscles of ostes/ostes mice were not due to a loss of motor units. Physiological assessment of motor unit survival showed that EDL muscles in wild-type and ostes/ostes mice were innervated by an average of 38.8 (± 1.17 SEM, n = 6)
and 36.7 (± 0.88 SEM, n = 6) motor neurons, respectively (Fig. 3A). This finding, indicating that there was no loss of motor axons in osteos/mice, was supported by morphological assessment of motor neuron survival (Fig. 3B). We found no difference in the number of motor neurons present in the lumbar spinal cord of osteos/mice and wild-type littermates (Fig. 3B). This was supported by the lack of muscle paralysis in either osteos/+ or osteos/mice at any age. Additionally, cross-sections of sciatic nerve showed no evidence of demyelination in osteos/mice (n = 3) (Fig. 3C).

The osteos mutation maps to a 0.78 Mb region on distal chromosome 8

The osteos mutant was identified in a complex recessive mouse mutagenesis screen that involved ENU treatment of a hybrid (C3H/HeH × 101/H) strain (6). To facilitate genetic and functional analysis, the osteos line was crossed to wild-type BALB/c mice for 20 generations to generate a congenic osteos line. Genetic mapping on osteos progeny from these crosses established linkage to distal chromosome 8 between D8Mit213 and D8Mit13. Two further outcrosses, to the C57BL/6J and FVB inbred strains, also independently confirmed significant linkage to the same region on chromosome 8 (data not shown). Since the FVB strain showed the highest rate of polymorphism with the initial mutagenized background, this strain was selected for high-resolution mapping. We generated and genotyped approximately 500 intercross progeny including both affected (osteos/) and non-affected (+/+) and osteos/+ offspring to utilize all recombination events. This analysis refined the non-recombinant region to 772 kb, between D8Mit89 and the in-house marker FM3 (see Materials and Methods). Pkd1l2, Gcsh, Bcmo1, Dynlrb2 and five additional predicted genes lie within this region, comprising 94 coding exons in total (Ensembl v49, May 2008; Supplementary Material, Table S1). All exons and splice acceptor/donor sites were amplified by PCR from genomic osteos DNA and screened for mutation by sequencing, but intriguingly, no mutation was revealed. To confirm this finding, we used a commercial next-generation sequencing approach (NGS) to sequence all exons in the interval. As no mutation in coding sequence was identified, this analysis confirmed our previous results (see Materials and Methods). To screen for cDNA variants, cDNA of all candidate genes except Pkd1l2 was successfully amplified and sequenced from primary cDNA libraries from wild-type and osteos tissues. This analysis revealed no osteos-specific splicing variants of these genes. Therefore, this raised the possibility that the osteos mutation could lie in a regulatory element and that misregulation of one of these genes, rather
than a coding mutation, could underlie the *ostes* phenotype. Intriguingly, 30 sets of primers in a large number of combinations failed to amplify overlapping segments of the large *Pkd1l2* cDNA from several tissues including the liver, kidney and muscle; therefore, a complete sequence scan for this particular cDNA was not achieved.

To evaluate expression, we performed microarray analysis on adult *ostes*/*ostes* and wild-type hind limb skeletal muscle using the RNG-MRC Mouse 25k array (MRC Harwell) which contained all of the nine genes from the non-recombinant region. No significant differences in the expression of these genes between *ostes*/*ostes* and wild-type samples were found (Supplementary Material, Fig. S5A; data deposited at ArrayExpress, accession number E-MEXP-1873). *Pkd1l2* was the only gene in this region for which analysis of splicing variants or differences in expression could not be established despite its reported expression in skeletal muscle (7). This was not due to technical problems, because detection of *Pkd1l2* to levels amenable for analysis failed in direct cDNA amplifications, Q-PCR (see what follows) and microarray experiments, whereas cDNAs from other genes in the non-recombinant region were efficiently detected using the same techniques and templates. However, we considered *Pkd1l2* to be a good candidate on the basis of

Figure 3. Motor neuron survival in *ostes*. (A) Assessment of motor unit number in *ostes*/*ostes* EDL. Examples of stepwise increments in twitch force from wild-type (+/+) and *ostes*/*ostes* EDL in response to gradual intensity increases of stimulation of the motor nerve to the EDL. Motor unit number in *ostes*/*ostes* EDL is not significantly different to wild-type EDL (*n = 6* per genotype). Data shown as mean motor unit number ± SEM. (B) Motor neuron survival in *ostes*/*ostes* mice. Shown are representative 20 μm-thick transverse sections of spinal cord from wild-type and *ostes*/*ostes* mice. Motor neurons are stained with galloacyanin (blue). Data shown as mean motor neuron counts ± SEM. The number of galloacyanin-stained motor neurons present in the sciatic motor pool of the ventral horn of each spinal cord was determined by counting every third serial section (*n = 60* sections per mouse; *n = 6* per genotype). (C) Representative cross-section of proximal regions of sciatic nerve from 6-week-old wild-type (+/+) (*n = 3*) and *ostes*/*ostes* mice (*n = 3*) fixed with osmium tetroxide and stained with methylene blue. No demyelination of *ostes*/*ostes* motor neurons as evidenced by reduced myelin-to-lumen staining or by ‘onion-bulb’ morphology is seen. Note that *ostes*/*ostes* motor neurons are markedly smaller than wild-type, reflecting the overall small size of *ostes*/*ostes* mice. Scale bar: 25 μm.
the association of the homologous PKD1 gene with disease and the fact that all genes in the non-recombinant region except Pkd1l2 had been effectively ruled out. Thus, for all these other genes, no coding or splicing mutations and no significant expression differences between the mutant and control were found. Therefore, we decided to assess expression of Pkd1l2 in ostes/mice at the protein level.

**PKD1L2 protein is upregulated in ostes mice**

We assessed Pkd1l2 as a candidate gene for ostes by generating PKD1L2-specific antibodies and examining PKD1L2 protein in ostes/mice. Two rabbit polyclonal antibodies, APKD1L2_1 and APKD1L2_2, were generated and thoroughly characterized (Fig. 4). Western blot analysis of soluble skeletal muscle extracts from wild-type mice showed that both antibodies recognized a band migrating at ~268 kDa (Fig. 4B), consistent with the PKD1L2 expected size (272 kDa). Western blot analysis of multiple tissues showed that this band was the largest protein recognized in several tissues (Supplementary Material, Fig. S6A), suggesting that this protein is likely to be full-length PKD1L2. We used immunoprecipitation to confirm the specificity of these antibodies. Antibody APKD1L2_1 specifically and efficiently immunoprecipitated a protein migrating at ~268 kDa band from wild-type skeletal muscle lysates (Fig. 4C). Analysis of the corresponding band by peptide mass fingerprinting identified PKD1L2 among other proteins (see below and Fig. 4C). In addition, the immunoprecipitated protein was also recognized by the second antibody, APKD1L2_2 in membrane fractions (bottom panels). IP, immunoprecipitation; WB, western blot; Lys, lysate; IP+, immunoprecipitated sample; IP−, negative control.

The association of the homologous PKD1 gene with disease and the fact that all genes in the non-recombinant region except Pkd1l2 had been effectively ruled out. Thus, for all these other genes, no coding or splicing mutations and no significant expression differences between the mutant and control were found. Therefore, we decided to assess expression of Pkd1l2 in ostes/mice at the protein level.
soluble and membrane fractions (Fig. 5). These results suggested that the upregulation of PKD1L2 underlies the ostes phenotype.

In search of a putative regulatory mutation within the vicinity of the Pkd1l2 gene that would explain the upregulation of this gene in mutant mice, we used NGS to sequence unique non-coding sequences in a 250 kb genomic segment encompassing Pkd1l2 and flanking genes Bcmol and Gcsh, that is, all genomic DNA except repetitive elements in that interval. This analysis produced 149 kb of sequence from ostes/ostes DNA, but no change was revealed, suggesting that a regulatory mutation could exist in a non-conserved regulatory element somewhere else within the non-recombinant interval.

Transgenic overexpression of PKD1L2 causes ostes-like myopathic and innervation changes

We tested the hypothesis that the overexpression of PKD1L2 found in ostes/ostes mice causes neuromuscular dysfunction in these mice by generating a transgenic mouse overexpressing PKD1L2. Since a full-length Pkd1l2 cDNA could not be amplified (discussed earlier), we opted to use a bacterial artificial chromosome (BAC) to generate a transgenic line. A BAC (RP23-269J16) was selected which contained the full-length genomic sequence of Pkd1l2 and the small flanking genes Bcmol and Gcsh. These flanking sequences were required to include the promoter and other possible regulatory elements in the vicinity of the Pkd1l2 gene. Following microinjection of the BAC into fertilized oocytes, a transgenic founder was identified (Tg(Pkd1l2)_#1). Subsequent crosses showed that the BAC was transmitted to 57.5% (57 of 99) of offspring from this mouse, thus indicating a single integration site. We examined hemizygous Tg(Pkd1l2)/0 mice for evidence of neuromuscular dysfunction. Tg(Pkd1l2)/0 mice were indistinguishable from wild-type littersmates and did not show any overt phenotype, and histological analysis did not reveal any anomalies on H&E-stained hind limb skeletal muscle sections (Fig. 1J). However, on intercrossing Tg(Pkd1l2)/0 mice, we found that homozygous Tg(Pkd1l2)/Tg(Pkd1l2) mice showed extreme growth retardation and developed progressive hind limb dragging and paralysis between 4 and 6 weeks of age. Nineteen per cent of intercross offspring displayed this phenotype (13/64), and 3% were found dead around this age (2/64), indicating that some perinatal loss may be occurring. H&E-stained skeletal muscle sections from these mice showed extreme fibre size asymmetry, pyknotic nuclei, small centrally nucleated fibres and no evidence of macrophage infiltration, strongly recapitulating the pathological hallmarks seen in ostes/ostes mice (Fig. 1C, D and E). Similar pathology was seen in pharyngeal muscle and diaphragm, but not in any other 23 tissues analysed including the heart (not shown). Moreover, fibre type analysis revealed a similar distribution of fast and slow fibres to ostes/ostes muscle, again showing a lack of both fibre-specific hypertrophy and clustering (Fig. 1I and Supplementary Material, Fig. S4). Therefore, both ostes and Tg(Pkd1l2) homozygotes showed a small but statistically significant reduction of type-2 fibre size in soleus muscle (Fig. 1D). Additionally, polynerviated and denervated endplates were also evident in homozygous Tg(Pkd1l2)/Tg(Pkd1l2) mice (n = 4, representative examples shown in Fig. 1H).

Given the similarities between the ostes and Tg(Pkd1l2) myopathies, these results theoretically reduced the genetic cause of the ostes disease to the upregulation of any of the three genes in the BAC: Bcmol, Pkd1l2 or Gcsh. We had already established that PKD1L2 protein was elevated in ostes mice. To unequivocally establish how expression levels of these three candidate genes correlated with the ostes neuromuscular disease, further expression analysis was carried out. Real-time quantitative PCR was performed using skeletal muscle from 10 ostes/ostes mice and 10 age- and sex-matched wild-type controls. The results were consistent with the microarray results described earlier: expression of Bcmol or Gcsh was not significantly different in ostes/ostes mice (Supplementary Material, Fig. S5B), whereas Pkd1l2 was not detected at sufficient levels for a reliable analysis. Therefore, Bcmol and Gcsh have no coding or regulatory mutations in ostes/ostes mice. Western blot analysis of skeletal muscle from Tg(Pkd1l2)/Tg(Pkd1l2) mice showed that full-length PKD1L2 was indeed overexpressed in this line (Fig. 5). Further proof that the myopathies described in ostes and Tg(Pkd1l2) homozygous mice share a common pathogenic mechanism could be obtained from making double heterozygotes. Thus, if ectopic expression of PKD1L2 as a result of an ENU mutation or expression from a BAC clone was respectively underlyng myopathic changes in ostes/ostes and Tg(Pkd1l2)/Tg(Pkd1l2) mice, we could expect double-heterozygous mice (ostes/+, Tg(Pkd1l2)/0) to show a more severe pathology than the mild changes observed in ostes/+ mice. In young mice, approximately 45 days old, the presence of the BAC clearly exacerbated the mild myopathic changes of ostes heterozygotes without adding any new distinctive feature.

Figure 5. Western blot analysis of skeletal muscle extracts from wild-type, ostes/ostes and Tg(Pkd1l2)/Tg(Pkd1l2) mice. (A) PKD1L2 and FASN show increased levels in the membrane fraction of ostes/ostes and Tg(Pkd1l2)/Tg(Pkd1l2) skeletal muscle. Samples shown are from individual mice (n = 2 per genotype). (B) PKD1L2 and FASN show increased levels in the soluble fraction of ostes/ostes skeletal muscle. Samples shown are from individual mice (n = 2 per genotype). PKD1L2 and FASN were detected, respectively, with antibodies APKD1L2_1 and mAFASN. +/+ wild-type; ost, ostes/ostes; Tg, Tg(Pkd1l2)/Tg(Pkd1l2).
PKD1L2 interacts with fatty acid synthase in vivo

Our data showed that overexpression of PKD1L2 was associated with neuromuscular dysfunction in two mouse mutants. However, neither PKD1L2 nor other TRPP channels have previously been shown to have roles in muscle. To explore a molecular role for PKD1L2 in muscle, we examined PKD1L2 protein complexes by immunoprecipitation from adult mouse skeletal muscle lysates. Immunocomplexes precipitated with antibody APKD1L2_1 were analysed by SDS–PAGE and mass spectrometry to identify PKD1L2-interacting proteins. Upon gel staining, a very prominent band was observed migrating at ~268 kDa, which was absent in the negative control lane (Fig. 4C). A peptide mass fingerprint was generated from this band by mass spectrometry. Database search using ProFound identified PKD1L2, as expected, but additionally the enzyme fatty acid synthase (FASN) was identified as the top scorer. FASN was also identified from bands of smaller molecular weight, probably reflecting partial degradation (Fig. 4C). The identity of this protein was confirmed by western blot with a monoclonal antibody against FASN (gAFASN). PKD1L2 and FASN were found localized to the membrane on cross-sections (Fig. 6A). On longitudinal sections, both antibodies produced overlapping localizations at striations overlying the Z-discs on the surface of the fibre (Fig. 6A, middle panel) but not at the centre (Fig. 6A, bottom panel). A similar result was obtained with mAFASN antibody (Supplementary Material, Fig. S7), suggesting a costameric localization of both proteins. Costameric distribution was supported by colocalization of PKD1L2 with the costameric transmembrane protein β-dystroglycan (8) on serial views of longitudinal sections (Fig. 6C). An identical pattern was observed with antibody APKD1L2_2, and for both PKD1L2 antibodies, the fluorescent signal was effectively blocked upon pre-incubation with their respective antigenic peptides (data not shown). On the other hand, expression of PKD1L2 was also detected at slightly higher levels at the postsynaptic membrane of the NMJs (Fig. 7A and B).

FASN is overexpressed in ostes mice

We speculated that the overexpression of PKD1L2 in ostes/ostes skeletal muscle could affect the expression or activity of FASN in this tissue. Surprisingly, we found that FASN was overexpressed in ostes/ostes and Tg(Pkd1l2)/Tg(Pkd1l2) skeletal muscle by western blot analysis, mirroring the overexpression of PKD1L2 (n = 12, representative samples shown in Fig. 5). Overexpression of FASN was observed in both soluble and membrane fractions (Fig. 5) and was also confirmed to occur at the transcriptional level (Supplementary Material, Fig. S8A). The growth defect exhibited by the ostes mouse is counterintuitive to an overexpression of FASN, as the latter is generally found in conditions of metabolic overload such as obesity (reviewed in 9). We therefore hypothesized that PKD1L2 acts as a negative regulator of FASN and that the upregulation of PKD1L2 in the ENU and transgenic lines results in decreased FASN activity. In this hypothesis, the increased expression of FASN in skeletal muscle of ostes mice would result from a positive feedback regulatory mechanism. Relative activity of FASN to total amount of (Supplementary Material, Fig. S3). This result suggests that increasing the copy number of the Pkd1l2 gene results in worsening of the pathology caused by the ENU mutation. Moreover, ostes/+ Tg(Pkd1l2)/0 mice (n = 3) showed a late-onset muscular atrophy and weakness of the hind limbs between 6 and 10 months old, with foot-curling, unstable gait and similar myopathic changes to those observed in ostes/ostes and Tg(Pkd1l2)/Tg(Pkd1l2) muscles (Fig. 1J). These features were not found in aged ostes/+ (n = 6) or Tg(Pkd1l2)/0 mice (n = 3) (Fig. 1J). These results indicated that ostes and Tg(Pkd1l2) interact genetically and strongly supported the hypothesis that the upregulation of PKD1L2 causes neuromuscular disease. These results did not strictly exclude the possibility that a new mutation caused by the insertion of the BAC and not the BAC itself was responsible for the phenotype observed in the transgenic line. However, the specificity of the pathological changes in the ENU-induced and BAC-induced mutant lines and the genetic interaction between the underlying genetic loci made that possibility unlikely.

that the APKD1L2_1 antibody does not recognize FASN (Supplementary Material, Fig. S6A). Analysis of the same samples using non-denaturing conditions produced a different pattern for both antibodies on western blots (data not shown), suggesting that PKD1L2_1 antibodies did not cross-react against the FASN native form either. Moreover, affinity-purified antibody APKD1L2_2, raised against a different PKD1L2 epitope, also immunoprecipitated FASN (Supplementary Material, Fig. S6B), and the reciprocal immunoprecipitation using mAFASN antibody also produced a ~268 kDa protein recognized by antibody APKD1L2_2, confirming the co-immunoprecipitation of FASN and PKD1L2 (Supplementary Material, Fig. S6B).

Despite PKD1L2 being a predicted transmembrane protein and FASN a soluble cytoplasmic enzyme, their association as revealed by immunoprecipitation of endogenous proteins suggested that these proteins must have overlapping subcellular localizations. To assess this, immunostainings on cross- and longitudinal sections of fresh-frozen skeletal muscle samples were performed with APKD1L2_1 and a goat polyclonal antibody against FASN (gAFASN). PKD1L2 and FASN were localized to the membrane on cross-sections (Fig. 6A). On longitudinal sections, both antibodies produced overlapping localizations at striations overlying the Z-discs on the surface of the fibre (Fig. 6A, middle panel) but not at the centre (Fig. 6A, bottom panel). A similar result was obtained with mAFASN antibody (Supplementary Material, Fig. S7), suggesting a costameric localization of both proteins. Costameric distribution was supported by co-localization of PKD1L2 with the costameric transmembrane protein β-dystroglycan (8) on serial views of longitudinal sections (Fig. 6C). An identical pattern was observed with antibody APKD1L2_2, and for both PKD1L2 antibodies, the fluorescent signal was effectively blocked upon pre-incubation with their respective antigenic peptides (data not shown). On the other hand, expression of PKD1L2 was also detected at slightly higher levels at the postsynaptic membrane of the NMJs (Fig. 7A and B).
FASN protein was measured in soluble fractions of *ostes*/*ostes* and wild-type muscle by spectrophotometric analysis of NADPH > NADP+ oxidation. Although there is a clear trend showing reduced relative activity in *ostes*/*ostes* mice, FASN activity varied widely in individual samples of the same genotype, making the difference between *ostes*/*ostes* and control statistically not significant (*P* = 0.236) (Supplementary Material, Fig. S8B). Therefore, we looked for other biochemical markers of lipid metabolism to determine whether fatty acid biosynthesis was abnormal in *ostes*/*ostes* mice. We assessed the blood plasma lipid profile of *ostes*/*ostes* mice (Fig. 8A). Both triglyceride (TG) and free fatty acid (FFA) levels were significantly and markedly decreased in plasma from both male and female *ostes*/*ostes* mice, compared with
wild-types (Fig. 8A). Furthermore, total body fat content of ostes/ostes mice was analysed by dual-energy X-ray absorptiometry (DEXA) analysis (Fig. 8B). The total fat percentage of body mass was significantly decreased in ostes/ostes mice from a mean wild-type percentage of ~20 to ~17% in female ostes/ostes mice and from ~17 to ~14% in male ostes/ostes mice (Fig. 8B). These results indicated a reduction of lipogenesis in ostes mice.

**DISCUSSION**

In this work, we have characterized the neuromuscular phenotype of ostes mice and explored the underlying molecular mechanism. Indeed, the genetic mechanism leading to the upregulation of the linked Pkd1l2 gene in ostes mice remains elusive despite meticulous sequencing efforts. However, as indicated by the total protein levels, Pkd1l2 is
the only gene in the non-recombinant region that is upregulated in *ostes/*ostes mice, and there are identical pathological findings in *ostes/*ostes and Tg(Pkd1l2)/Tg(Pkd1l2) mice. Common histological hallmarks include muscle fibre asymmetry, scattered regenerating fibres, a lack of fibre type grouping, whole-muscle atrophy and an irregular innervation pattern. Conversely, typical dystrophic changes such as excessive connective tissue or macrophage infiltration are not present in either mutant. Disease manifestation is more severe in Tg(Pkd1l2)/Tg(Pkd1l2) probably because the BAC transgene does not exactly replicate the changes in Pkd1l2 gene expression caused by the *ostes* ENU mutation. Intriguingly, neither *ostes/+* nor Tg(Pkd1l2)/0 mice showed any overt phenotype at young or old age (>9 months), but the double heterozygote suffered from a more severe myopathy that resulted in late-onset gait defects. Hind limb muscle histology of the latter showed changes qualitatively similar to those found in young *ostes/*ostes mice and much more profound than those of age-matched single heterozygotes. Altogether, these results suggested that overexpression of *Pkd1l2*, which was demonstrated at the protein level in both mutants, provokes neuromuscular disease in mice. Pathological changes were not found in any other tissue, including sciatic nerve and spinal cord, suggesting that skeletal muscle is the primary target tissue of this disease. The polyinnervation defects found in *ostes/*ostes mice could thus be secondary to the myopathic changes, but since PKD1L2 accumulates at NMJs on the postsynaptic membrane, a role for this protein in regulating the stability of NMJs is possible and merits further investigation.

PKD1L2 belongs to the TRPP family of proteins. The transient receptor potential (TRP) superfamily comprises a large group of cation channel proteins usually containing six transmembrane domains, which, despite having diverse functions and expression, appear to function as critical sensors of environmental stimuli, at both organism and cellular levels (reviewed in (10 and 11). The TRPP family includes two genes that are associated with human disease: mutation of TRPP1/PKD1 or TRPP2/PKD2 cause polycystic kidney disease in humans (12,13) and mice (14,15). Other TRPP1-like genes have been shown to have roles in taste reception (*Pkd1l3* (16,17)) and reproductive success (*Pkdrej* (18)). Thus far, no TRPP genes have been associated with neuromuscular function or disease. PKD1L2 is a large, complex protein of unknown function. It is predicted to act as an ion channel (7) and has also been reported to bind *in vitro* to two G-proteins (19). The presence of FASN as the major component of the PKD1L2-immunoprecipitated complex in skeletal muscle suggests a functional relationship between these proteins in this tissue. In mammals, FASN synthesizes *de novo* long-chain, saturated fatty acids from malonyl-CoA and acetyl-CoA, and is therefore a critical enzyme in lipogenesis (20). In muscle, both imported and *de novo*-synthesized fatty acids are used as an energy source via beta oxidation. The increased levels of FASN protein in the skeletal muscle of *ostes* mice are due to the upregulation at the transcriptional level. This is not a non-specific response of skeletal muscle to a disease condition because the actively regenerating muscles of the muscular dystrophy *ky* mutant (21) do not show FASN upregulation (Supplementary Material, Fig. S9). However, the finding that FASN is overexpressed in *ostes/*ostes mice is intriguing because *ostes/*ostes mice are small and lean. Intriguingly, the dramatic accumulation of FASN in *ostes/*ostes skeletal muscle did not result in an increase of FASN activity. In fact, blood measurements of TGs and FFAs as well as total fat deposition would suggest that lipid metabolism is indeed compromised in the *ostes* homozygote. Given that muscle has a particularly high metabolic need for fatty acids driven by its high-energy demand, a reduction in FASN activity would predictably compromise muscle function. Mice homozygous for a null allele of FASN show embryonic lethality (22), but skeletal muscle was not examined in surviving *Fasn* heterozygous mice. A direct involvement of FASN activity in muscle disease or indeed the pathogenic mechanism of *ostes* cannot be tested at the

Figure 8. Lipid metabolism in *ostes* mice. (A) Lipid profiling in *ostes/*ostes mice. Blood lipids were analysed from fasted *ostes/*ostes (n = 9 female, 11 male) and wild-type (n = 12 female, 12 male) mice for triglyceride (TG) and free fatty acid (FFA). Both male and female *ostes/*ostes mice showed significantly decreased levels of both TG and FFA (TG: female: P = 3.107E – 05; male: P = 0.0001; FFA: female: P = 0.0005; male: P = 2.22E – 06). Data represented are mean blood plasma levels ± SEM. (B) Total percentage body fat in *ostes/*ostes mice. Total body fat was analysed by DEXA analysis in *ostes/*ostes (n = 9 female, 11 male) and wild-type (n = 22 female, 22 male) mice. Total percentage body fat was significantly decreased in both female and male *ostes/*ostes mice, compared with wild-type (female: P = 1.922E – 03; male: P = 5.023E – 06). Data represented are mean percentage body fat ± SEM.
present time, as a Fasn skeletal-muscle condition
knotton mouse has not been reported. However, a rare case of de
novo fatty acid synthesis deficiency resulting in persistent
myopathy and poor growth has been reported in humans.
This defect was due to the deficiency of the acetyl-CoA car-
boxylase enzyme, which catalyses the formation of the
FASN substrate malonyl-CoA (23), indicating that insufficient
fatty acid synthesis can cause pathological changes in skeletal
muscle. Moreover, the microarray data generated in this work
identified lipid metabolism as the major biological pathway
altered in ostes/ostes skeletal muscle: eight out of the top
nine genes upregulated in ostes/ostes muscle have functions
in lipid metabolism (Supplementary Material, Table S2; see
also deposited data ArrayExpress, accession number E-MEXP-1873).
Therefore, lipogenesis is altered at skeletal
muscle and systemic levels in ostes/ostes mice. The precise
reasons for the tissue specificity of this disease remain to be
established. Concomitant upregulation of FASN and
PKD1L2 was also detected in the kidney, but here the increase
in FASN is smaller than that observed in muscle; overexpres-
sion of both PKD1L2 and FASN was not detected in other
tissues from ostes/ostes mice, including the brain, spinal
cord and liver (Supplementary Material, Fig. S10A). In con-
clusion, our data suggest that PKD1L2-induced dysregu-
lation of lipid metabolism is a cause of complex neuromuscular
cord and liver (Supplementary Material, Fig. S10A). In con-
clusion, our data suggest that PKD1L2-induced dysregu-
lation of lipid metabolism is a cause of complex neuromuscular
defects in mice. On that basis, we propose that PKD1L2 and
defective fatty acid synthesis should be assessed, respectively,
as a genetic risk factor and as a precipitating cause of neu-
romuscular disorders in humans.

MATERIALS AND METHODS
Additional methods are detailed in Supplementary Material.

Antibodies
Polyclonal antibodies to mouse PKD1L2 were generated in
rabbit using two PKD1L2 peptides (APKD1L2_1: LVASTKVPEDORRQEATRA; APKD1L2_2: GNSKLV
GSAHIRQVRVRE) and independently affinity-purified
(NeoMPS Inc., San Diego, CA, USA).

Genetic mapping
The in-house-generated marker used was a microsatellite
marker FM3 (forward primer: 5′-TCTGGGTTGGAGATGGA-
CATTT-3′; reverse primer: 5′-CCCAGATGATGGAAGGAGA
GAA-3′). The ostes locus was genotyped on an FVB genetic
background using D8Mit89 and D8Mit120 (MGI), and
rs37591388 (NCBI).

BAC transgenesis
A BAC (identifier RP23-269J16) containing full-length
genomic sequence of Pkd1l2 was obtained from BACPAC
Resource Center, Children’s Hospital Oakland Research Insti-
tute (CHORI), Oakland, CA 94609, USA. DNA was prepared
from culture using the QIAGEN Large-Construct Kit, diluted
to 1 ng/μl and microinjected into fertilized FVB oocytes.

Resulting mice were screened for BAC integration by PCR
with primers designed against the vector: forward 5′-ataaat
ggatccctgctgta-3′; reverse 5′-ttcaaccgtagctgctt-3′. One
positive founder for the BAC was identified and bred to the
FVB strain to generate a colony. The line was submitted to
the Mouse Genomic Nomenclature Committee (The Jackson
Laboratory) and designated the identifier Tg(Pkd1l2)1Blac
(accession number MGI:3811813). For brevity, we refer to
this line in this publication by the shortened identifier
Tg(Pkd1l2).

FASN activity assay and HeLa cell culture
FASN-specific activity in soluble fraction of skeletal muscle
from 3-month-old female wild-type (n = 4) and ostes/ostes
(n = 4) mice was measured as described in Nepokroeff et al.
(24), except that mice were fed ad libitum before sacrificing.
Untreated, and serum-deprived and insulin-treated, HeLa
cells were used as positive control. HeLa cell culture, treat-
ment and protein isolation were performed as described in
Albers et al. (25). FASN protein amounts were quantified
by densitometric analysis using QuantityOne software
(Bio-Rad) from western blot of equally loaded cytosolic frac-
tions and were normalized to GAPDH. FASN relative activity
was calculated using the following formula: FASN activity =
FASN-specific activity (units/mg/min)/FASN total protein
(arbitrary units). Statistical testing was performed using a two-
tailed Student’s t-test.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

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