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The developmental and genetic basis of ‘clubfoot’ in the peroneal muscular atrophy mutant mouse

J Martin Collinson†, Nils O. Lindström¹, Carlos Neves, Karen Wallace, Caroline Meharg², Rebecca Charles, Zoe Ross, Amy Fraser³, Ivan Mbogo⁴, Kadri Oras⁵, Masaru Nakamoto, Simon Barker$, Suzanne Duce^, Zosia Miedzybrodzka & Neil Vargesson

† Corresponding author. Email: m.collinson@abdn.ac.uk

School of Medicine, Medical Sciences and Nutrition
University of Aberdeen
Institute of Medical Sciences
Forsterhill
Aberdeen AB25 2ZD
UK

Royal Aberdeen Children’s Hospital
Forsterhill
Aberdeen AB25 2ZN
UK

^School of Life Sciences,
University of Dundee,
Dundee DD1 5EH

Current Addresses:

1. Eli and Edythe Broad CIRM Center for Regenerative Medicine and Stem Cell Research at USC, 1425 San Pablo Street, BCC 312, Los Angeles, CA 90033-9080, USA nils.lindstrom@med.usc.edu
2. Institute For Global Food Security, Queen’s University Belfast. caroline.meharg@qub.ac.uk
3. Division of Developmental Biology, Roslin Institute, University of Edinburgh, Easter Bush, Edinburgh, UK. amy.fraser@roslin.ed.ac.uk
4. Evolutionary Neurobiology Unit, Okinawa Institute of Science and Technology Graduate University, 1919-1 Tancha, Onna-son, Okinawa, 904-0495, Japan. ivan.mbogo@oist.jp
5. Department of Genetics, University of Cambridge, Downing Street, Cambridge CB2 3EH, United Kingdom. ko330@cam.ac.uk

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Summary Statement: The mutation in the ‘PMA’ mouse model of human clubfoot was mapped and a candidate gene, LIMK1, identified that was shown to cause sciatic nerve and limb abnormalities when overexpressed.
ABSTRACT

Genetic factors underlying the human limb abnormality congenital talipes equinovarus ('clubfoot') remain incompletely understood. The spontaneous autosomal recessive mouse 'peroneal muscular atrophy' mutant (pma) is a faithful morphological model of human clubfoot. In pma mice, the dorsal (peroneal) branches of the sciatic nerves are absent. In this study, the primary developmental defect was identified as a reduced growth of sciatic nerve lateral motor column (LMC) neurones leading to failure to project to dorsal (peroneal) lower limb muscle blocks. The pma mutation was mapped and a candidate gene encoding LIM-domain kinase 1 (Limk1) identified, that is upregulated in mutant lateral LMC motor neurons. Genetic and molecular analyses showed the mutation acts in the EphA4 – Limk1 – Cfl1/cofilin - actin pathway to modulate growth cone extension/collapse. In the chicken, both experimental upregulation of Limk1 by electroporation and pharmacological inhibition of actin turnover led to defects in hindlimb spinal motor neuron growth and pathfinding, and could mimic the clubfoot phenotype. The data support a neuromuscular aetiology for clubfoot and provide a mechanistic framework to understand clubfoot in humans.
INTRODUCTION

Congenital talipes equinovarus (CTEV, also known as 'clubfoot') is a human lower limb developmental defect with a worldwide prevalence of 1-3 cases for every 1000 live births, making it one of the most common paediatric orthopaedic problems (Cartlidge, 1984; Dobbs et al., 2000). Clubfoot is characterized by inward rotation and downward flexion of the foot which persists after birth and, unless corrected, causes permanent disability. However, understanding of the etiological and genetic factors underlying clubfoot is incomplete. The majority of clubfoot births, where the infant has unilateral or bilateral CTEV but no other clinical problems, are largely unexplained. Most patients respond well to serial casting (Ponseti manipulation) followed by Achilles tenotomy in infancy but a significant proportion (10-15%) experience relapse and most post-clubfoot individuals experience some degree of leg fatigue (Ippolito et al., 2009).

A number of hypotheses have been put forward to explain clubfoot aetiology. Although intuitively clubfoot may be supposed to arise from a skeletal patterning defect, in fact there is little evidence for this and the primary defect appears to be hindlimb developmental arrest from around day 44 of gestation (Miedzybrodzka, 2003). The hindlimb must rotate during late embryogenesis such that the sole of the foot, which lies initially in the plane of the body axis, faces down, with toes lying in the horizontal plane. Clubfoot is the consequence when this fails to complete, and it has been hypothesised that musculo-skeletal interrelationship is required for limb rotation (Isaacs et al., 1977; Stewart, 1951; Bechtol and Mossman, 1950; Dittrich, 1930; Ponseti and Campos, 1972). Several lines of clinical evidence show that weakness, inactivity of absence of calf muscles can be associated with clubfoot (Flynn et al., 2007; Ohno et al., 1986), and there is persistent loss of muscle density in the calves of clubfoot individuals (Duce et al., 2013).

Twin studies and population genetics have provided strong evidence for a genetic basis of clubfoot (Miedzybrodzka, 2003). Complex segregation analyses suggest that the most likely inheritance pattern is a single gene of major effect but low penetrance, with either dominant or recessive inheritance, operating against a polygenic background (Wang et al., 1988; de Andrade et al., 1988; Rebbeck et al., 1993; Chapman et al., 2000). However, the identity of the major gene(s) involved remains elusive. Mutations in the genes encoding the hindlimb transcription factors PITX1 and TBX4 mutations have been shown to lead to reduction of lower limb musculature and classic clubfoot phenotypes in humans and mice, though these may also be associated with syndromic long bone growth defects (Gurnett et al., 2008;
About 5% of human cases of familial isolated clubfoot are associated with microduplications of \( \text{TBX4} \) (Dobbs and Gurnett, 2013). There are other mouse models - for example one of the ephrin receptor \( \text{EphA4} \) knockout mice exhibits clubfoot as part of a syndrome of motor and cognitive abnormalities (Helmbacher et al., 2000) - but none of these have led to the discovery of other major human causative genes, and the overwhelming majority of human clubfoot cases are idiopathic i.e. with no known cause.

A spontaneous mouse mutant, the peroneal muscular atrophy (pma) mouse, shows an inherited, hindlimb-restricted, bilateral clubfoot-like phenotype at birth that is comparable to the human pathology (Nonaka et al., 1986; Duce et al., 2010) (Fig.1). The mutation is autosomal recessive with high penetrance, and has been previously mapped to mouse chromosome 5 (Katoh et al., 2003). However no candidate gene has been identified – at least 67 genes are contained within the 4.8 Mb mapped region.

Adult \( \text{pma/pma} \) mice have anterior-lateral regional defects of the hindlimbs only, including absence of the peroneal nerve and atrophy of the anterior-lateral calf muscle compartment (Nonaka et al., 1986; Ashby et al., 1993). It is hypothesised that because the outer (dorsal, lateral) calf muscles are atrophied but the inner (ventral, posterior) are normal, the foot remains in a CTEV-like position during development. It is not known why the peroneal branch of the sciatic nerve fails in pma mice. Normally, the motor neurones that contribute to the sciatic nerve project from the Lateral Motor Columns (LMCs) of the ventral neural tube. Those that will contribute to dorsal limb nerves project from the lateral LMC (lLMC) and those that will project ventrally originate in the medial region of the LMC (mLMC) (Landmesser, 1978). Both populations project through the ventral roots of the lumbar neural tube and coalesce at the lumbosacral plexus before entering the hindlimb (Wang and Scott, 2008; Jessell, 2000). Here they branch into the ventral limb, becoming the precursor of the tibial and sural nerves, or into the dorsal limb, becoming the peroneal nerve.

The pma mouse is potentially a clinically relevant model of clubfoot, but the genetic basis of the phenotype has not been described, and the primary defect, whether developmental or degenerative, is poorly understood. This work explores the aetiology and genetics of clubfoot using the pma animal model. By genetic mapping, sequencing and gene expression analysis, we identify \( \text{Limk1} \) as a candidate gene underlying the pma mutation and show, using mouse and chicken models, that overexpression of this gene delays neuronal growth and drives aberrant neuron-guidance, resulting in muscular defects, failure of foot rotation, and clubfoot.
RESULTS

Peroneal nerve development is aborted in pma/pma embryos.

It has been shown previously that the common peroneal nerve is absent in adult pma/pma mice (Nonaka et al., 1986). This is the dorsal branch of the sciatic nerve that innervates the anterior-dorsal ‘peroneal’ calf muscles that are atrophied in the pma mouse and in clubfoot patients (Duce et al., 2010; 2013). Immunohistochemistry on tissue sections and whole-mount preparations at E16.5 confirmed complete absence of motor innervation in the dorsal-anterior muscles of the lower leg of pma/pma homozygotes - the tibialis anterior, the extensor digitorum longus and the peroneus longus - with normal innervation of ventral calf muscles (Fig. 2). Immunohistochemical analysis showed that the anterior-dorsal muscles were normally vascularised in pma/pma homozygotes (Supplementary Fig. S1). The data confirm that clubfoot phenotype in pma mice is associated with neural and not vascular failure. In order to define the earliest developmental stage at which a defect is observed, homozygous pma/pma mice embryos were examined histologically at E10.5 – E16.5. It was found that at E11.5, the sciatic nerve had entered the hindlimb of wild-type mice homozygotes and the beginnings of branching were visible, but in stage-matched pma/pma homozygote embryos the nerve had neither entered the limb nor started to branch (Fig. 3A, B). At E12.5, wild-type sciatic nerves had forked into clearly demarcated, fasciculated dorsal peroneal and ventral tibial trunks (Fig. 3C, D). The E12.5 pma/pma littermates had a morphologically normal tibial trunk and no obvious peroneal nerve, suggesting failure of normal branching. However, whole-mount immunohistochemistry showed a dorsal projection of a small number of defasciculated axons projecting dorsally in E12.5 pma/pma embryos that may represent an abortive peroneal nerve (Fig. 3E, F). The fate of these axons is unknown, as no peroneal innervation was detectable at later stages, as described above (Fig. 3G, H). The peroneal nerve was confirmed by dissection to be absent postnatally in pma/pma mice consistent with Nonaka et al. (1986) (Supplementary Fig. S2).

We previously observed degeneration of the peroneal muscles in adult pma/pma hindlimbs (Duce et al., 2010; 2013). To determine whether this was secondary to the loss of innervating nerves we scrutinized early muscle development in pma/pma limbs. Abnormal myotube structures were observed specifically in myosin-positive developing dorsal muscles only at E16.5 with an approximate two-fold increase in apoptosis (Fig. 4). These results confirmed at a molecular level the previous observations of regional muscular defects in pma mice by our and other groups (Nonaka et al., 1986; Ashby et
al., 1993; Duce et al., 2010) and showed that muscle failure occurred subsequent to failure of innervation.

**Molecular specification is normal but axon growth is reduced and apoptosis increased in PMA motor neurones**

To delineate whether the eventual absence of the peroneal nerve in pma mice was due to death of putative dorsal-projecting LMC neurones or their misdirection, we measured the cross sectional area of the sciatic nerve in adult *pma/pma* and wild-type mice of equal weight. The mean area of the pma nerve (98.98 ±0.43 x10³ μm²; n = 5) was significantly reduced compared to controls (111.41 ±1.37 x10³ μm²; n = 9; *t*-test, *P* = 0.014), suggesting that motor neurone death occurred. Although TUNEL labelling at E11.5-E14.5 revealed no gross abnormal apoptotic events (Fig. 5A), by E16.5 the number of lumbar LMC nuclei was found to be reduced in *pma/pma* homozygotes compared to wild-type stage matched controls (Fig. 5B). Sacral LMC neurones were not affected. Patterns of proliferation were investigated by BrdU labelling and immunohistochemistry and were found to be normal in *pma/pma* embryos at E11.5 (Supplementary Fig. S3), suggesting dorsal motor neuron death at E14.5-E16.5, secondary to failure to target the dorsal muscle blocks.

Sciatic motor neurones of the hindlimb are derived from the lateral motor column (LMC) of the developing lumbar neural tube. The dorsal (peroneal) branch of the sciatic nerve is derived from neurones of the lateral component of the LMC (lLMC) that are characterised by expression of genes encoding marker LIM homeodomain proteins Lim1 and Islet 2 (Isl2) and the ventral (tibial/sural) branch is derived from the medial LMC neurones (mLMC) expressing Islet 1 and 2 (reviewed in Jessell, 2000). Lim1-deficient LMCs project illicitly along the ventral pathway (Kania et al., 2000). Dorso-ventral patterning of the neural tube and, specification of the LMC populations was found to be maintained in *pma/pma* embryos: Lim1-positive lLMC neurones that should form the peroneal nerve were present as normal (Fig. 6B). These data suggested that any loss of peroneal neurones in the *pma/pma* sciatic nerve is not a primary failure of patterning or cell proliferation at E11.5-E12.5, but is secondary to the cellular changes that prevent normal patterns of axonal projection.
No defect of genes required for normal dorso-ventral patterning of the hindlimb mesenchyme was detected in E11.5-E12.5 pma/pma embryos by western blot or qPCR (Supplementary Fig. S4). To investigate whether the motor neurones of the PMA mouse were inherently defective, E11.5 lumbar lateral motor columns (LMCs) were dissected from stage-matched pma/pma and wild-type embryos and cultured in identical media for 72 hours. Immunocytochemical analysis showed projecting axons with β-III-tubulin and cell body localisation of Lim1, confirming LMC identity (Fig. 6C, D). Time-lapse measurement of growth cone position showed that pma/pma axons projected about half the speed (2.09 μm/100 minutes; n = 50) than wild-type (4.03 μm/100 minutes; n = 97) (Fig. 6E and Supplementary Fig. S5). These data confirmed an autonomous defect of the pma/pma motor neurones.

The data suggest an autonomous qualitative axon growth defect of motor neurones in pma/pma embryos, such that limb innervation is delayed. The reasons why this could lead to failure of the peroneal branch but not of the tibial/sural are discussed below, but it is postulated that dorsal-specified axons miss their permissive time window for successful projection into the limb. It was concluded that the pma mutation has arisen in a gene required for normal extension and ultimate survival of lumbar motor neuron axons.

Genetic mapping of the pma mutation.

The pma mutation was previously mapped to a 4.8 Mb region of chromosome 5 (Katoh et al., 2003). This region contains 67 genes with no standout clubfoot candidate. Genomic PCR was performed for 20 genes spanning the candidate region in pma/pma animals and all genes were found to be present, suggesting no large deletion (Supplementary Fig. S6). We repeated the mapping using higher density of microsatellites across the candidate region (full details described in Supplementary Materials and Methods). The results are presented in Table 1. There were 12 potentially informative crossovers identified, which located the mutation to 2.5 Mb bounded by D5Mit166 (Chr5:133146598-133146706 bp) and D5Mit60 (Chr5:135715435-135715564 bp) (Fig. 7). This smaller candidate region contained 39 genes.

To further define the candidate region, targeted next-generation resequencing was performed on two recombinant mice (one with crossover distal to the mutation, and one proximal to the mutation), three parental pma/pma and three parental BALB/c mice.
3.04 Mb encompassing the entire candidate region between D5Mit166 and D5Mit60 was obtained from all mice. The full dataset with details of alleles, location, sequencing depth and reproducibility and inferred sites of crossovers is presented as Supplementary Table S1. This analysis defined crossovers at approximate positions Chr5: 134514245 and Chr5: 134483438, representing a 0.89 Mb candidate region containing 13 genes: Gatsl2; Wbscr16; Gtf2ird2; Ncf1; Gtf2i; Gtf2ird1; Cyln2 (Clip2); Lat2; Gm52; Rfc2; Eif4h; Limk1 and Eln (Fig. 7). Within this 0.89 Mb candidate region, nearly 4075 SNPs and small insertions or deletions (indels) were identified that were unambiguously homozygous for the pma allele in all clubfoot crossover animals and not found in BALB/c, any one of which could be the 'pma mutation'. Restricting the analysis only to predicted nonsynonymous SNPs and indels within annotated genes identified 23 SNPs that were predicted to cause either changes in amino acid sequence of the gene product (5 SNPs – Table 2) or changes in the untranslated region of the processed mRNA (18 SNPs, all located in 3' UTR) (Supplementary Table S2). Nearly all these changes were identifiable as known SNPs and could be identified as existing in the homozygous state in at least one of the C57BL/6, A/J, AKR/J, FVB/NJ and/or CAST/EiJ inbred strains without causing a clubfoot phenotype. The only novel nonsynonymous coding mutation was found in the Gm52 gene (Table 2). This gene corresponds to the envelope glycoprotein syncytin-A, a placenta-specific product required for normal architecture of the syncytiotrophoblast-containing labyrinth (Dupressoir et al., 2009). Gm52/syncytin-A knockout mice die in utero with placental failure, indicating that this gene is a weak functional candidate for the pma phenotype. These data suggest that the pma phenotype is not associated with an amino acid-coding mutation.

In addition, a microRNA, mmu-miR590-5p, is localised to this region (Accession number MIMAT0004895), nesting within Eif4h (Supplementary Fig. S7). No mutation was present within the mature 22 base miR sequence.

The possibility of gene amplification or deletion was directly tested. Genomic DNA was isolated from pma homozygotes and wild-type controls, and real-time PCR performed for using 5 primer pairs for Cyln2, Gtf2ird1, Limk1 (2 primer sets), and Pde6b, and gene 20 Mb outside the candidate region. No change in copy number in PMA mice was detected with any primer combination (Supplementary Fig. S8).

The data suggested the PMA mutation is not a null mutation (causing total loss of protein product or activity) in any of the genes in the candidate region. In addition to the lack of novel, potentially pathogenic coding mutations identified by sequencing,
published knockout mice exist for 10 of the genes, none of which produce a clubfoot phenotype or any other relevant defect of motor neurones (Table 2). Furthermore, the pma candidate region is syntenic to the region of human chromosome 7 that is heterozygously deleted in patients with Williams-Beuren syndrome (Francke, 1999), a morphological and neurodevelopmental disorder associated with developmental delay, mental retardation and behavioural abnormalities. Williams-Beuren syndrome is not associated with clubfoot (Morris, 1997). It was therefore hypothesised that the problem was one of misexpression of one or more genes due to a regulatory mutation – i.e. one of the several thousand SNPs and small indels affecting an important enhancer or promoter sequence. This was investigated.

Overexpression of Limk1 in pma homozygotes

Of the thirteen genes within the mapped region, Limk1, Cyln2 and Eif4h were the best candidates to underlie the motor neuron defects in the pma mouse on account of known or inferred function in CNS axon guidance. Limk1 encodes LIM-domain kinase 1, an enzyme which phosphorylates and inactivates the actin-depolymerising protein coflin (Yang et al., 1998). Limk1 activity modulates growth cone extension/retraction decisions and acts in a biochemical pathway that is downstream of EphA4, which was previously shown to be mutated in a clubfoot mouse model (Helmbacher et al., 2000). Cyln2 (Clip2) encodes the CAP-GLY domain containing linker protein 2 (also known as CLIP-115) which acts as a microtubule-associate linking protein in the CNS (Hoogenraad et al., 1998; Hoogenraad et al., 2000). Cyln2 was shown to be strongly expressed in the developing motor neurones of the sciatic nerve (Supplementary Fig. S9). Eif4h encodes a neurally expressed translation initiation factor responsible for modulating normal neuronal number and complexity in the CNS (Caposella et al., 2012).

Expression of Limk1, Eifh4 and Cyln2 was investigated in the pma mouse by qPCR on cDNA from limbs and neural tubes of E11.5 pma/pma embryos and stage-matched wild-types (Fig. 8A). No change in Cyln2 or Eif4h expression was detected, but the data suggested variable but significant 4 to 7-fold upregulation of Limk1 in both the neural tube ($P = 0.014$) and hindlimb ($P = 0.027$) of pma/pma homozygotes.

The putative upregulation of Limk1 was confirmed by immunohistochemistry and Western blot on protein extracts of E11.5 neural tube and hindlimbs. Normalised to β-actin, Limk1 and active (phosphorylated) p-Limk1 were localised at higher levels in
Furthermore, phosphorylation of Limk1’s major substrate, the actin-depolymerising protein, cofilin, was shown to be higher in pma/pma embryos. Total cofilin levels were unchanged. Cyln2 levels were unchanged in pma/pma homozygotes. This suggests that the observed increase in Limk1 leads to phosphorylation and inactivation of cofilin, which in turn could impact on the dynamics of actin cytoskeleton remodelling in the growth cones of the sciatic nerve.

We next wanted to understand the Limk1 profile throughout progression of clubfoot in the pma/pma mice. Repeating the western blots at E11.5-E16.5 showed that the upregulation of Limk1 expression was transient. Limk1 levels were increased in pma/pma mice at E11-12.5, at the time when the peroneal nerve fails to form and enter the hindlimb, but were reduced to wild-type levels by E16.5 (Supplementary Fig. S10).

To determine whether the upregulation of Limk1 detected by western blot indicated a genuine per-cell overexpression or misexpression of the gene in ectopic locations, immunohistochemistry was performed. In E11.5 wild-types, at the level of the lumbar neural tube, Limk1 protein was observed to be present at higher levels in neural tube, in particular the ILMC, of pma/pma homozygotes compared to controls (Fig. 8C). In pma/pma embryos processed in parallel with the wild-types, there was a general upregulation of the gene, but protein levels were very high in the ILMC, in the neurones that will form the peroneal branch of the sciatic nerve.

Limk1 protein was found to be differentially localised in the peroneal and tibial branches of the wild-type sciatic nerve at E12.5. Immunohistochemistry showed that in wild-type embryos, higher levels of p-LIMK1 are present in the dorsal (peroneal) branch of the sciatic nerve, compared to the ventral (tibial) branch (Fig. 8D and Supplementary Fig. S11).

Sciatic nerve plexus defects caused by jasplakinolide exposure mimic Limk1 overactivation

Regulatory mutations appear to play an important role in human pathology, particularly in hind-limb deformities (VanderMeer and Ahituv, 2011). Our data suggests that the pma mutation is in a cis-regulatory element of the Limk1 gene increasing its expression.
The predicted consequence of increased Limk1 activity is increased phosphorylation (hence inactivation) of cofilin leading to inhibition of actin treadmilling in the extending growth cone. This effect can be mimicked pharmacologically using jasplakinolide, a cell-permeable peptide that has been shown to inhibit actin depolymerisation \textit{in vivo} (Bubb et al., 2000; Rosso et al., 2004). For practical purposes, the pharmacological manipulation was performed in chicken embryos – we have recently shown that disruption of neuromuscular function in the hindlimb of chicken embryos causes a clubfoot-like phenotype (Vargesson et al., in prep), establishing the chicken as a useful model. 20 µg/ml jasplakinolide was therefore applied as a microsponge to the dorsal-lumbar region of HH stages 20-21 chicken embryos \textit{in ovo}, prior to nerve entry to the limb (approximately equivalent to E10.5 mouse). This was followed by whole-mount immunohistochemistry for \(\beta\)-III-tubulin to identify changes in sciatic nerve projection compared to the contralateral untreated limbs and vehicle-treated controls. Axon projection defects were induced after 24 hours in 11/29 jasplakinolide-treated limbs but in none of the 15 controls (Fisher Exact Test: \(P = 0.0079\)) (Fig. 9). In particular, the rostral-most roots exiting the neural tube, which are the main contributors to the peroneal nerve, were truncated. It was concluded that peroneal nerve disruption is effected by addition of jasplakinolide, consistent with a model that Limk1 over-activation can prevent normal peroneal nerve development \textit{in vivo}.

**Electroporation of Limk1 in chicken neural tube disrupts sciatic nerve innervation of the hindlimb**

Electroporation of a plasmid expressing \textit{Limk1} into the lumbar neural tube of stage 11 chicken embryos was performed to experimentally overexpress the gene in hindlimb motor neurones. This caused significant disruption and loss of plexus formation and nerve projection into the limb on the electroporated side of the embryo at stage 24, compared to the contralateral side (Fig. 10A). Electroporation of a control empty vector plasmid had no such effect. Cartilage preparations were made from 12 embryos that were incubated in ovo for a further 5 days. Although 9/12 were superficially normal, 3 showed unilateral clubfoot phenotype on the electroporated side (Fig. 10B). These data confirmed that overexpression of Limk1 alone disrupts sciatic nerve formation and that overexpression of Limk1 at the sensitive developmental period may lead to clubfoot.
Genetic interaction between EphA4 mutation and pma.

EphA4−/− mice show a very high incidence of clubfoot (Helmbacher et al., 2000). Analysis of EphA4−/−E16.5 hindlimbs, prior to the failure of limb rotation that characterises clubfoot, showed that only two muscle blocks, the tibialis anterior and the extensor digitorum longus, were aneural, suggesting that the peroneus longus is of lesser consequence for clubfoot (Supplementary Fig. S12).

We hypothesised that the EphA4 mutation may act on the sciatic nerve by modulating the Limk1-cofilin pathway. In support of this, increased labelling of p-cofilin was observed by immunohistochemistry in both E11.5 pma/pma and EphA4−/− mice compared to wild-type littermates, consistent with Limk1 regulating cofilin activity (Fig. 11A). To test a genetic interaction between EphA4 and pma mutations, EphA4−/− mice were bred with pma/pma homozygotes or wild-type C57BL/6 to generate littermates that were EphA4−/− pma/+, EphA4−/− pma/+ or EphA4−/−+/+ and EphA4−/−+/+. All progeny were scored as normal, unilateral or bilateral clubfoot at weaning on the basis of gross limb morphology, and dissections performed to score presence or absence of the peroneal nerve (Supplementary Fig. S13). No clubfoot or nerve loss was observed in either EphA4−/−+/+ mice (n = 37 from these litters) or EphA4−/− pma/+ (n = 44). Mice that were heterozygous only for EphA4 were usually normal (19/24 mice) but 20.8% (5/24) had unilateral loss of peroneal nerve (either complete loss in 4 cases or retention of an extremely thin remnant in 1 case). In contrast, only 32% (12/37) of double heterozygote EphA4−/− pma/+ littermates were normal; 45% (17/37) were scored as unilateral clubfoot and showed loss of peroneal nerve on the affected side. 21% (8/37) were bilateral clubfoot with loss or atrophy of nerves (Fig. 11B) (Full dataset: Supplementary Table S3). The occurrence of bilateral clubfoot in the double heterozygotes had never been observed in any of the pma/+ or EphA4−/− single heterozygotes bred over the course of the project. The increased incidence of peroneal nerve loss in the double heterozygotes was significant (5/24 EphA4−/−+/+ mice vs 25/37 EphA4−/− pma/+; Chi-squared = 12.43, P = 0.0004). Hence the phenotype of the double mutation is more severe than the sum of the phenotypes of the single mutations. This is formal genetic evidence that the pma mutation lies in the same molecular pathway as EphA4. It suggests that the phenotypic occurrence of clubfoot can be associated with the cumulative effect of heterozygosity for predisposing mutations in more than one gene.
DISCUSSION

Clubfoot is a complex heritable abnormality with a range of described anatomical defects and no simple association with any one genetic or environmental factor (Miedzybrodzka, 2003). In this work, the spontaneous autosomal recessive mouse model, peroneal muscular atrophy (pma), was used to understand the underlying developmental causes of clubfoot. The mutation was mapped, identifying a number of candidate genes, of which, Limk1, was upregulated in mutant mice. We showed in chickens that LIMK1 upregulation can cause sciatic nerve defects and a clubfoot phenotype. We also demonstrated proof of principle in mice that expression of the clubfoot phenotype can occur due to the cumulative effect of predisposing alleles at more than one locus.

Limk1 as the pma clubfoot gene

We have previously shown that Limk1 is dynamically expressed during embryogenesis (Lindström et al., 2011). Neural tube expression patterns of Limk1 and patterns of p-cofilin localisation described in this study are consistent with those previously reported in embryos (Phan et al., 2010). Limk1 is present in all neurones of the wild-type developing neural tube at E11.5, with higher levels in the lumbar LMC. P-cofilin is primarily localised to commissural neurones in wild-type mice, though with detectable staining in the LMC (this study). Immunohistochemical analysis confirmed upregulation of Limk1 throughout the E11.5 neural tube in pma/pma mutants, particularly obvious in the LMC. Limk1 is a serine/threonine kinase which phosphorylates cofilin, inhibiting its actin filament depolymerisation activity, so overexpression of Limk1 would be predicted to inhibit actin turnover (Sarmiere and Bamburg, 2004). Consistent with this, we showed that motor neurones from the LIMK1-overexpressing pma mouse have high levels of p-cofilin and extend axons poorly, in comparison with wild-type. In vivo, the most severe consequence of this would appear to be loss of the peroneal nerve. To determine empirically whether Limk1 over-activation can result in neuronal defect, Limk1 activity was mimicked in ovo pharmacologically using jasplakinolide to inhibit actin turnover. Delayed or repressed growth of the dorsal-projecting axons was observed in jasplakinolide-treated limbs, indicating that Limk1 pathway dysregulation may disrupt peroneal nerve development. These findings were further confirmed by additional electroporation of LMC in ovo to induce Limk1 overexpression during in vivo development of the chicken embryo.
Actin turnover, mediated by Limk1, ADF/cofilin, and slingshot proteins is essential to growth cone motility (Endo et al., 2003; Wen et al., 2007). The importance of Limk1 for CNS neuronal morphology and function has been established (Meng et al., 2002), but the effects of changing Limk1 dosage are context-dependent. Short-term overexpression of Limk1 in cultured hippocampal neurones promotes axon development through cofilin phosphorylation, but longer-term overexpression of Limk1 leads to growth cone collapse (Rosso et al., 2004). Similarly, in chicken dorsal root ganglia overexpressing Limk1, axon extension and growth cone mobility are inhibited (Endo et al., 2003). Phan et al. (2010) showed that overexpression of LIMK1 in chicken commissural neurones increases p-cofilin levels and stalls commissural growth cones, whereas lowering LIMK1 accelerates axon extension. The inhibition of commissural growth cone extension could be rescued by co-electroporation of a construct expressing a non-phosphorylatable cofilinS3A mutant. Hence excessive stabilisation of F-actin at the growth cone inhibits growth cone dynamics or facilitates myosin-driven growth cone retraction (Gallo et al., 2002).

The fact that Limk1 is widely expressed in CNS neurones makes it counterintuitive that the pma mouse has only a hindlimb phenotype. Similarly, Gdnf/Ret and Hoxc10/d10 double mutants all lead specifically to peroneal nerve loss, in spite of the expression of these genes in many other neurones (Tarchini et al., 2005; Kramer et al., 2006). Why the peroneal nerve should be overtly susceptible to failure is not known, but we show in this study that the peroneal nerve has higher basal levels of phosphorylated (active) Limk1 than the tibial nerve, which can suggest that the pma mutation raises this further to a level where nerve growth stalls. Limk1 activity should be assayed in the other models above, to determine whether this may be a general mechanism.

The timing of Limk1 overexpression overlaps the end of the previously described “waiting period” of the sciatic nerve plexus (Wang et al., 2008). This waiting period is characterized by arrest of axonal growth at the hindlimb sciatic nerve plexus, before limb innervations initiates, mediated through retinoic acid signalling. In Ret⁻/⁻ mice (Kramer et al., 2006), the ventral pathway choice becomes available to dorsal axons that are unable to project dorsally, indicating that delayed axons may also be able to select the ventral pathway. Timing of limb innervation is therefore crucial to correct patterning, and we hypothesise that reduced growth rate of pma motor neurones causes dorsal-fated axons to miss their time window for entry to the limb, resulting in atrophied peroneal muscles and clubfoot.
Limk1/EPHA4 interaction

*EphA4"*-/- animals show a similar phenotype to the pma mice and specifically lack peroneal nerves (Helmbacher et al., 2000). EPHA4/Ephrin-A receptor signalling is known to modulate Limk1 activity: it leads to phosphorylation of ephexin and Src kinases which signal through small GTPase RhoA and the Rho-associated protein kinase (ROCK) to modulate phosphorylation of Limk1 and Limk2 (Maekawa et al., 1999; Sahin et al., 2005; Sumi et al., 2001). In addition to the genetic and biochemical interaction between Limk1 and EPHA4 shown here in clubfoot mice, it has also been shown that over-activation of Src kinases redirects dorsal axons to the ventral mesenchyme in the limb (Kao et al., 2009), suggesting a potential ‘clubfoot pathway’ controlling innervation of the calf muscles.

The pma mouse as a model of human clubfoot.

Previous clinical candidate gene and genome-wide association studies have implicated several loci in the development of clubfoot (Basit and Khoshhal, 2017). These include hindlimb specifying genes *PITX1* and *TBX4* described above, members of the limb patterning *HOXA* and *HOXD* clusters (Ester et al., 2009), mutations in genes encoding pro-apoptosis caspases (Ester et al., 2009; Heck et al., 2005), and genes required for normal metabolism of environmental factors such as folic acid and tobacco smoke (Sharp et al., 2006; Hecht et al., 2007). Collagen (*COL9A1*) and a number of genes encoding muscle contractile proteins may also be mutated in clubfoot, supporting the model of hindlimb rotational arrest due to musculoskeletal dysfunction (Liu et al., 2007; Weymouth et al., 2011). The only other gene required for actin cytoskeletal function to have been associated with clubfoot is that encoding Filamin B (Yang et al., 2016). *LIMK1* has not directly been implicated in human clubfoot, however, a high incidence of clubfoot occurs in patients with microduplications in chromosome 7q11.23, syntenic with the pma candidate region on mouse chromosome 5. Although Williams-Beuren syndrome (deletion of 7q11.23) does not cause clubfoot, patients with duplications of the region have up to 25% incidence of clubfoot and all microduplication patients with clubfoot have duplicated *LIMK1* (Torniero et al., 2007; Van de Aa et al., 2009). This is consistent with the link between clubfoot and increased Limk1 activity shown in this study.
Circumstantial and direct anatomical evidence suggests that most human clubfoot patients do have a peroneal nerve but many cases, often associated with a drop-toe phenotype, have a neurological origin, including peroneal nerve palsy (Song et al., 2008; Edmonds and Frick, 2009; Yoshioka et al., 2010). Peroneal nerve dysfunction is rare in human clubfoot (8/837 patients in the study by Yoshioka et al., 2010) and the complete peroneal nerve loss described in pma/pma mice cannot be regarded as a common cause of clubfoot. Our work outlined in this study together with other work (Vargesson et al., in prep) is consistent with a hypothesis that muscle loss is the primary cause of clubfoot. Whether the muscle weakness is a direct failure of the muscle, or secondary to other defects such as failure of innervation or of nerve function, is perhaps of little consequence to the clubfoot phenotype presented, although the complex clubfoot cases with an underlying neurological basis are amongst those that do not respond well to normal treatment (Ponseti manipulation) (Song et al., 2009; Yoshioka et al., 2010). Identification of the genetic pathway that underlies neurological clubfoot is therefore central to screening and optimisation of patient care. Upregulation of LIMK1 in human tissue samples cannot be assayed by normal genomic sequencing, but the EPHA4 – ephexin- SRC-small GTPase-LIMK1-cofilin pathway has many components that can be screened by simple exome sequencing. Our data therefore support a general model that predisposing mutations in multiple genes in pathways affecting (directly or indirectly) muscle development in the lower limb are causal to clubfoot. The fact that so many such genes exist may well explain why the genetics of clubfoot is so complex in humans.
MATERIALS AND METHODS

Mice and genetic mapping

Experiments were performed under Licence after approval by Aberdeen University Animal Ethical Review Committee. The pma/pma homozygotes were obtained from Professor Cheryll Tickle, University of Bath, on an inbred CF1-derived background, and maintained by homozygous mating. The mice were poor breeders, so for generation of experimental animals and controls, the pma mutation was bred onto CD1 and C57BL/6 lines. Wild-type CD1 and C57BL/6 mice were used as controls. The PMA phenotype was qualitatively identical irrespective of genetic background, with full bilateral clubfoot. The data presented here are for CD1 background pma/pma mice unless stated otherwise e.g. in the genetic mapping and experiments investigating interaction with EphA4. Embryos were stage-matched according to date of gestation, crown-rump length and forelimb morphology. The pma mutation produced an idiopathic clubfoot phenotype on all pure and mixed genetic backgrounds.

Epha4+/− mice were obtained from Patrick Charnay, Ecole Normale Supérieure, Paris and maintained on a C57BL/6 background.

For timed matings, the date of vaginal plug was taken as embryonic day 0.5 (E0.5).

A preliminary pan-genomic screen for informative microsatellite alleles showed that the original pma inbred CF1-derived strain maintained by our group was genetically more distinct from BALB/c than from C57BL/6 (Supplementary Table S4). Balb/c crossing was therefore chosen for genetic mapping. Homozygous pma/pma male mice on the original inbred CF1-derived background were mated with wild-type BALB/c females, and the F1 pma/+ progeny crossed to the pma/pma males to derive a mixed pma/pma and pma/+ B1(F2) generation within which it was inferred would be some informative crossovers to map the pma mutation. All mice were scored at birth as ‘clubfoot’ (inferred pma/pma) or ‘normal’ (inferred pma/+). Mice were killed and dissected to confirm absence or hypotrophy of the peroneal nerve in clubfoot mice and presence of normal peroneal nerve in mice without clubfoot. Tail-tip DNA was isolated from all clubfoot F2 mice (also 3 non-clubfoot mice, 2 F1s and both parental strains) by proteinase K digestion (80 μg/ml at 56°C overnight) followed by ethanol precipitation (3:1 ethanol:sample, -20°C for several hours and centrifugation at 12,000G for 20 minutes). Microsatellite genotyping was performed by PCR, using 9 primer sets for microsatellites spanning and within the candidate region on chromosome 5 (see Supplementary Table S5). Fluorophore-conjugated forward primers (fluorophores
6FAM, VIC, PET or NED) were synthesised by Eurogentec (Southampton, UK). PCRs were multiplexed in groups of three using three different fluorophores conjugated to the forward primers (D5Mit218, D5Mit166 and D5Mit428 together; D5Mit282, D5Mit219 and D5Mit60; D5Mit33, D5Mit32 and D5Mit97) (Supplementary Table S5). Each PCR was run for 35 cycles with annealing temperature 61°C and the product sizes analysed using an Applied Biosystems3130 Series Genetic Analyzer. Expected band sizes were determined using DNA from founder pma/pma homozygotes and BALB/c mice, as well as their F1 progeny. All PCRs were run on the clubfoot backcross generation mice to identify clubfoot mice that were heterozygous at any of the microsatellite loci, indicative of an informative crossover event.

**EphA4 Genotyping and Crosses**

Genomic DNA was isolated from tissue samples by proteinase K digestion using the Qiagen DNA Mini Kit according to the manufacturer’s instructions, with elution in 100 μl of buffer AE. Genotyping PCR was performed on EphA4-mutant mice and littermates using primers SEK1.1 (5’-TTCTGCCACTGCTATTGGTCACGAG-3’), SEK1.2 (5’-AACTGGTTCTGAGCTCCAGAAGACC-3’) and SEK1.3 (5’-GATGGGCGCATCGTAACCGTGCATC-3’), with annealing temperature 65°C, 35 cycles. The EphA4 wild-type allele produced a band of ~200bp from primer combination SEK1.1/1.2, and the mutant allèd a ~300 bp band from primer combination SEK1.1/1.3 (Helmbacher et al., 2000).

For study of compound heterozygotes, EphA4+/− mice were crossed to pma/pma homozygotes to produce pma/+ heterozygotes that were either EphA4+/− or EphA4+/+. All mice were scored at or before weaning superficially as ‘normal’, unilateral clubfoot, or bilateral clubfoot. For statistical analysis, to avoid mis-diagnosis of gross morphology, all mice from these crosses were dissected and the peroneal nerve was scored as ‘normal’, ‘absent’, or ‘thin’ if a remnant of peroneal nerve was visible (Supplementary Fig. S13). Nerve scoring was performed blind by an observer unaware of the genotype of the mice.

**Gene detection and Copy number variation assay.**

Livers were dissected from 1 C57BL/6 control mouse and one pma/pma homozygote and genomic DNA isolated from 50 mg samples using the MagMAX™ DNA Multi-
Sample Kit (ThermoFisher Scientific) according to the manufacturer’s protocol, with elution into 300 μl volume. gDNA was diluted to 5 ng/μl.

Presence or absence of genes was assayed by genomic PCR using primers for 20 genes described in Supplementary Table S6.

Copy number variation was assayed by real-time PCR using the TaqMan(R) Copy Number Assay (Applied Biosystems) using manufacturer’s validated assays for murine Limk1 (Mm00561325_cn and Mm00165295_cn), Clip2/Cyln2 (Mm00149430_cn), Gtf2ird1 (Mm00158525_cn) and Pde6b – a linked gene that lies outside the pma candidate region (Mm00164399_cn). All genes were multiplexed and normalised to manufacturer’s internal reference assay (RNAse P).

**Histology and Immunohistochemistry**

Samples were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) overnight, washed several times in PBS and dehydrated through a series of ethanol and xylene washes for embedding in paraplast wax embedding medium (Sigma, Poole, UK). 7 μm wax sections were cut. For staining, the samples were de-waxed by immersing the slides in histoclear solution (National Diagnostics) and then re-hydrated through an ethanol series. Antigen retrieval was performed by boiling slides, 4 x 5 minutes, in 0.01 M sodium citrate, pH6. The samples were blocked in PBS, 4% normal serum, 0.3% BSA for 1 hour. Primary antibody was added, diluted in blocking buffer at 4ºC overnight. Primary antibodies used were: 1:1000 β-III-tubulin (T2200; Sigma); 1:400 PAX6 (PAX6c; Developmental Studies Hybridoma Bank, University of Iowa (henceforth DSHB)); 1:200 Cynl1/Clip2 (ab80976; abcam); 1:20 myosin heavy chain (MF20; DSHB); 1:200 myogenin (F5D-c; DSHB); 1:200 BrdU (ab8955; abcam); 1:200 Limk1 (611749; BD Transduction Laboratories. GWB-C4BF5E; Genway); phospho-LIMK1 (ab38508; abcam) 1:200 coflin (ab47401; abcam); 1:400 phospho-cofilin (sc-21867; Santa Cruz); 1:200 EPHA4 (AF641; R&D Systems); 1:100 EPHB4 (sc-5536; Santa Cruz); 1:100 Frizzled-9 (AF2440; R&D Systems); 1:400 Islet 1 (sc-23590; Santa Cruz.40.2D6, 39.4D%; DSHB); 1:400 Islet2 (sc-66454; Santa Cruz); 1:40 Pax3 (PAX3-sc-21231; Santa Cruz); 1:20 Lim1 (4F2; DSHB); 1:40 Neurofilament-associated antigen (3910-s; DSHB). No-primary-antibody controls were performed for each antibody. After washing with PBS, fluorescent secondary antibodies were used 1:250 in blocking buffer for 3 hours at room temperature as follows (all Molecular Probes, Invitrogen): A21442 Alexa594-conjugated chicken anti-rabbit; A21207 Alexa594-conjugated donkey anti-rabbit;
A21135 Alexa594-conjugated goat anti-mouse IgG2a; A21125 Alexa594-conjugated goat anti-mouse IgG1; A10037 Alexa568-conjugated donkey anti-mouse; A11078 Alexa488-conjugated rabbit anti-goat; A21141 Alexa488-conjugated goat anti-mouse IgG2b; A21121 Alexa488-conjugated goat anti-mouse IgG1; A21206 Alexa488-conjugated donkey anti-rabbit; A21093 Alexa350-conjugated goat anti-rat. After PBS washes, the samples were mounted in Vectashield with DAPI (H-1200; Vector Laboratories) to counterstain nuclei.

For immunohistochemistry with a diaminobenzidine (DAB) endpoint, the samples were treated as described above but before the antigen retrieval, the slides were immersed in 3% H₂O₂ (Sigma-Aldrich) in PBS for 15 minutes and then washed in PBS for 5 minutes. The secondary antibodies were biotin-conjugated rabbit anti-mouse (E0354; DakoCytomation) and biotin-conjugated goat anti-rabbit (B7389; Sigma). After PBS washes the samples were treated with avidin/biotin complex (ABC) reagent (‘R.T.U. Vectastain Kit’; Vector Laboratories) and then incubated in 830 µg/ml of 3, 3'-diaminobenzidine (DAB; Sigma-Aldrich), 20 mM Tris pH 7.4, 0.005% w/v H₂O₂. The DAB reaction was stopped by immersing the samples in running water. The samples were counterstained in haematoxylin.

Measurement of nerve diameters was performed after dissection and fixation of the sciatic nerve in adult mice approximately midway along the femur. Nerves were fixed in 4% paraformaldehyde, dehydrated and embedded vertically in wax. 7 µm-thick transverse sections were cut, rehydrated and stained with toluidine blue. Sections were imaged at x200 on a scale-calibrated Nikon Eclipse 400 light microscope. The diameter of tibial, sural and, if present peroneal branches of the nerve were measured in at least two places on each of 3 sections, and the mean total area calculated in µm².

**Whole Mount Antibody Staining (immunohistochemistry)**

Mouse or chicken embryos were fixed in Dent’s fixative (1 part DMSO: 4 parts methanol) for 24 hours at 4°C with rocking to permeabilise the tissue. Embryos were bleached with Dent’s bleach (1 part H₂O₂: 2 parts Dent’s fixative) for 24 hours at 4°C with rocking. Embryos were rinsed 5 times with methanol and post-fixed in Dent’s fixative for at least 24 hours. Embryos were washed 3 times with 1 x PBS for 1 hour, with rocking. Primary antibody, anti-β-III-tubulin (TuJ1, Sigma), was applied at a dilution of 1:1000 in blocking solution (75% 1 x PBS, 20% DMSO, 5% donkey serum). β-III-tubulin was used because it is highly expressed in differentiating neurons. Embryos were incubated overnight at room temperature. Embryos were rinsed 3 times with 1 x PBS then washed 5 times with 1 x PBS for 1 hour. Secondary antibody, Alexa 594-
donkey anti-rabbit (Molecular Probes), was applied at a dilution of 1:300 in blocking buffer. Embryos were incubated overnight at room temperature and in the dark. Embryos were rinsed 3 times with PBS, then washed, PBS, 5 x 1 hour, 1:1 PBS:methanol, 5 minutes, methanol, 3 x for 20 minutes. Following the third methanol wash, half of methanol was removed and replaced with BABB (1 part benzyl alcohol: 2 parts benzyl benzoate) for 5 minutes. This solution was replaced with 100% BABB and kept at 4°C in the dark. All analysis of the whole mount IHC results was carried out on a Nikon fluorescence dissecting microscope.

**In vivo chicken work**

Fertilized White Leghorn eggs (Henry Stewart & Co. Ltd, Louth, Lincolnshire) were stored at 14°C until ready to be used. To allow embryonic development, eggs were incubated in humidified incubator at 38°C for the required amount of time to reach the desired developmental stage.

**Jasplakinolide exposure:** A 1 mg/ml solution of jasplakinolide was prepared in DMSO and diluted to 20 μg/mL with PBS. The eggs were windowed and the stage of the embryo was visually confirmed. 1 mm x 1 mm microsponges soaked in 20 µg.mL⁻¹ of jasplakinolide were placed on the dorsal top of the lumbar and hind-limb regions of Hamilton-Hamburger stage 22 embryos (Supplementary Fig. S14). Sponges soaked in 2 % DMSO were used as vehicle controls. The eggs were closed with adhesive tape and incubated at 38 °C for 16 hours. Embryos were fixed using Dent’s fixative and processed for whole-mount β-III-tubulin immunohistochemistry as described above.

**Electroporation:** pWZL_Neo_Myr_Flag_LIMK1, expressing full length Flag-tagged human LIMK1, and control pWZL_Neo_Myr_Flag plasmids were obtained from addgene (Plasmids 20512 and 15300 respectively) and prepared to a concentration of 5 mg/ml using QIAGEN Endonuclease-free DNA Maxi Kit. In ovo electroporation was performed as described previously (Odani et al., 2008; Nakamura & Funahashi, 2001). LIMK1-Flag DNA or Flag DNA mixed with fast green (dye tracer) was injected into the lumbar region of the neural tube of HH11 (E1.5) chicken embryos in windowed eggs (Hamburger & Hamilton, 1951). Platinum-coated electrodes spaced at 4 mm were placed on both sides of the injection site, parallel to the long axis of the embryo. The anode was placed on the left of the neural tube and the cathode was placed on the right of the neural tube. Five square pulses (25 volts, 50 milliseconds/second) were
applied by a CUY21 electroporator (Nepagene, Japan) and the eggs resealed.

Surviving embryos were analysed 48-72 h later for whole-mount β-III-tubulin
immunohistochemistry, or after a further 5 days for cartilage preparation. Nerve
growth was blind-scored independently by two workers, for LIMK1 and control-
electroporated embryos, with ‘0’ representing abnormal plexus formation and/or
complete failure of axon to project towards the limb, ‘2’ representing normal projection
into the limb (Fig. 10) and ‘1’ representing complete or partial failure of projection from
at least one lumbar segment.

**Alcian blue cartilage staining**

Chicken embryos were fixed overnight in 5% trichloroacetic acid (TCA; Sigma) at 4 °C.
The next day the samples were incubated in 0.1% Alcian Blue (Sigma) in 70% ethanol
for 8 hours and destained overnight in 1% HCl in 70% ethanol at 4 °C. The embryos
were washed 3x1 hour in 100% ethanol and then cleared and stored in methyl
salicylate (Sigma).

**TUNEL**

Analysis of apoptosis was performed on tissue sections using the In situ cell death
detection kit (fluorescein) (11684795910; Roche Diagnostics) according to the
manufacturer’s instructions, with permeabilisation using Proteinase K according to
instructions. Apoptosis of muscle blocks was quantified manually on transverse
sections of the mid-calf of pma/pma homozygotes and wild-type controls. Each of 8
limbs was treated as an independent data point. Four sections were counted per limb,
and used to count a mean and standard error of % apoptotic cells for each muscle
block in wild-types and mutants.

**BrdU analysis**

Timed pregnant mice were given a single intraperitoneal injection of 10 mg/ml
bromodeoxyuridine (BrdU) to label all cells in S-phase, and killed 1 hour later. Embryos
were fixed in 4% PFA, wax-embedded and sectioned for anti-BrdU
immunohistochemistry using antibodies and conditions as described above.

**Motor neuron cultures**
E11.5 pma/pma and wild-type embryos were stage-matched on basis of crown-rump length. Lumbar neural tube was microdissected. The neural tube was further dissected by removing the dorsal region, allowing for purification of the ventral spinal cord, where the LMC neurons are located. A matrix made of bovine collagen (BD BioSciences), rat collagen (BD BioSciences) with DMEM (GIBCO) and 20 µL sodium bicarbonate was prepared on a 4 well plate. DEMEM/F12 with 5% foetal bovine serum and 1% pen/strep was used as culture medium. The dissected neural tube tissue was placed on the collagen matrix and incubated for 3 days at 37 °C, 5% CO₂. Fresh culture medium containing 25 mM of HEPES buffer was added to the explants before initiating the time lapse experiment. Time-lapse analysis of growing axons was performed by tracking movement of multiple growth cones in phase-contrast over a 16 hour period using a Leica Inverted microscope at 200x magnification with Volocity imaging software. Full culture conditions are provided in Supplementary Materials and Methods.

Next generation sequencing and analysis

Genomic DNA was isolated from livers of 3 Balb/c, 3 pma/pma and 2 crossover mice (identified by microsatellite analysis above) using DNAzol (ThermoFisher Scientific) according to the manufacturer’s instructions. Capture and sequencing of the 2.5 Mb Chr 5p23 region was performed on a contract basis by The GenePool (Next Generation Sequencing and Bioinformatics Platform at the University of Edinburgh, UK). In brief, Illumina sequencing libraries were prepared from each parental strain mouse and the two crossovers. The eArray web-based application (Agilent) was used to design custom DNA baits to capture candidate region sequences. The SureSelect system (Agilent) was used for sequence capture. Captured sequences from each sample were sequenced to at least 30x coverage using 50 bp-end reads on the Illumina platform (GAITx) in house at The GenePool. Raw sequences were returned to University of Aberdeen as .gz compressed text files for analysis.

BALB/c sequence reads were aligned to BALB/c reference genome mm9 using bwa (Li et al., 2009), discrepancies identified and a new BALB/c reference generated. The PMA mice and crossovers were then aligned against the new reference and all SNPs and indels identified. Reads were re-aligned around indels and the base quality score re-calibrated using GATK (McKenna et al., 2010).Duplicates were marked using Picard (http://picard.sourceforge.net). SNPs and indels were annotated as exon, intron, untranslated or intergenic sequence. Variant analysis was performed with samtools.
Annotation of variants was performed with seqgene v 2.3 (Li et al., 2009b). SNPs and Indels with Variant and Mapping quality >20 and present in all replicate samples were marked as potentially significant.

Quantitative PCR

For quantitative (qPCR) analysis, neural tubes and hind limbs were microdissected from E11.5 pma/pma and wild-type C57BL/6 mouse embryos. Total RNA was isolated using the peqGOLD total RNA kit (Peqlab, UK) according to the manufacturer’s instructions. cDNA was synthesized by reverse transcription using SuperScript® II Reverse Transcriptase kit (Life Technologies) using poly-T primer (Promega). Primers used to amplify genes in the pma candidate region were manufactured by Sigma-Aldrich (Poole, UK) as follows: Limk1 – Forward = 5’-GCTACTTTGTTGCACCTGGAG-3’. Reverse = 5’-CACACAGGCAACTCGCTTC-3’; Eif4h - Forward = 5’-TCAGGAAAGGTGGACCTGAT-3’. Reverse = 5’-TCCCATCCACCTCTAGATTCTC-3’; Cyln2 - Forward = 5’-CGGTTCTGCCAACGGTATT-3’. Reverse = 5’-GCGGTCAGTGTTTGTCCTC-3’. qPCR was performed using the Roche Universal Probe system. For each sample 1 μL of a probe, 0.2 μg of cDNA, 10 μL Sensimix (Bioline; London, UK) and 4 μL DEPC treated water were combined in each well of a 384-well plate. All reactions were run on a Roche Lightcycler 480 (Roche) and denatured at 95 °C for 15 min, followed by 50 cycles of 15 seconds at 95 °C and 30 seconds at 60 °C. All samples were in triplicate and normalized to Gapdh in the same run. Statistical analysis of changes in gene expression was performed using REST statistical package.

Western Blot

For western blotting, proteins were extracted from embryonic neural tube and hindlimbs in 5% SDS, 1:100 protease and phosphatase inhibitor cocktails (P2714 and P5726; Sigma Aldrich) and stored at −20 °C. SDS–PAGE was performed in 12% polyacrylamide gels with 10% SDS and proteins were blotted onto nitrocellulose. Membranes were blocked in Tris-buffered saline (TBS) with 0.3% Tween-20, 10% skimmed milk, and incubated in primary antibody as above diluted in 2% bovine serum albumin (BSA), 0.05% Tween-20, TBS overnight at 4 °C and washed. HRP conjugated species-specific secondary antibody was applied for 1 h before washing in TBS,
0.05% Tween-20, with detection using the enhanced chemiluminescence (ECL) kit (Amersham, Little Chalfont, UK). Peroxidase-conjugated anti-β-actin (A3854; Sigma) was used as an internal loading control. Secondary antibodies were peroxidase-conjugated anti-rabbit (#7074; Cell Signaling Technologies) and peroxidase-conjugated rabbit anti-mouse (A9044; Sigma).

Statistics

The randomization method of the Relative Expression Software Tool (REST) software was used for qPCR analysis. All error propagation calculations followed the equation:

\[
\frac{\Delta x}{z} = \sqrt{\frac{1}{\sum_{i=1}^{m} \left( \frac{\Delta x}{\Delta z} \right)^2}}
\]

where \( z \) is the mean calculated value and \( \Delta z \) the calculated standard error of the mean (EM), \( x \) the experimental value, \( \Delta x \) the experimental EM and \( m \) the categories considered.

Chi-squared test was performed online at [https://www.graphpad.com](https://www.graphpad.com).

Fisher Exact Test was performed online at [http://www.socscistatistics.com/tests/fisher/Default2.aspx](http://www.socscistatistics.com/tests/fisher/Default2.aspx)

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COMPETING INTERESTS

The authors declare no competing interests.

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Table 1: Microsatellite mapping of pma mutation in recombinant mice

<table>
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<tr>
<th>Phenotype</th>
<th>Genotype</th>
<th>D5Mit218</th>
<th>D5Mit166</th>
<th>D5Mit282</th>
<th>D5Mit219</th>
<th>D5Mit60</th>
<th>D5Mit33</th>
<th>D5Mit32</th>
<th>D5Mit97</th>
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<td>PMA founder</td>
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<td>[BALB/c x PMA] F1 x PMA</td>
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Legend: Results of microsatellite genotyping of BALB/c and pma/pma founders, their F1 progeny (top three rows) and the backcross F2 (bottom seven rows), for 8 informative microsatellites as described in Supplementary File 2 and main text, in chromosomal order. Microsatellite allele sizes are given. Red shading indicates homozygosity for the BALB/c alleles. No shading indicates homozygosity for the PMA founder alleles. Pink shading indicates heterozygosity for the BALB/c x PMA alleles. The number of animals tested that showed each phenotype/genotype combination is shown on right. Numbers in red indicate informative crossover events proximal or distal to the pma mutation that restricted the candidate region to between D5Mit166 and D5Mit60.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Encodes</th>
<th>Function</th>
<th>Amino acid change in PMA</th>
<th>dbSNP</th>
<th>Motor neuron expression</th>
<th>KO phenotype</th>
<th>References</th>
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<tr>
<td>GATS2</td>
<td>GATS Protein Like 2</td>
<td>Cellular arginine Sensor for MTORC1 protein</td>
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<td>Knockout</td>
<td>Chantratanong et al., 2016</td>
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<td>Wbscr16</td>
<td>Wbscr16-CCG-exchanging</td>
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<td>Jackson et al, 1995</td>
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<td>Hoogenraad et al., 2000; 2002</td>
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<td>Embryocidal</td>
<td>Dijkeman et al., 2016</td>
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<td>Baka et al., 2007</td>
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<td>Wang et al., 2010</td>
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Key: Gene = Gene symbol; Encodes = Name of protein; Function = brief description of major role; Amino acid change in PMA = predicted coding change based on identified SNP distinguishing pma/pma homozygotes from BALB/c (Supplementary Files 6, 7); dbSNP = database identification of SNP if previously catalogued; Motor neuron expression = indicated ‘Y’ if expression in developing motor neurones confirmed in literature; KO phenotype = brief description of published knockout phenotypes.
Figure Legends.

Figure 1: Clubfoot phenotype in pma/pma mice. Human newborn with congenital talipes equinovarus (CTEV – clubfoot) (top) compared to newborn pma/pma mouse (middle) and newborn wild-type mouse (bottom).

Figure 2: Absence of innervation of dorsal (peroneal) calf muscles in pma/pma homozygotes. Immunohistochemistry of cross-sections of stage-matched E16.5 wild-type (left) and pma/pma homozygotes (right) using antibodies against myosin heavy chain to label muscle blocks (green) and β-III-tubulin to label nerves (red). (A, B) Low magnification images with ventral muscles to left and dorsal muscles to right. (C, D) Higher magnification images of dorsal muscle blocks showing innervation of wild-type dorsal muscles (C) but not of pma/pma homozygotes (D). (E, F) Magnification of ventral muscles showing innervation of both wild-type (E) and pma/pma (F).

Abbreviations: tib, tibia; fib, fibula; TA, tibialis anterior; EDL, extensor digitorum longus; PL, peroneus longus. Scale bar represents 25 μm.

Figure 3: Retardation of nerve growth and abortive innervation of dorsal muscles in pma mice. (A, B) β-III-tubulin immunohistochemistry (green) in transverse sections of stage-matched E11.75 wild-type (A) and pma/pma mice (B). The wild-type sciatic nerve has projected further than pma/pma and, unlike the pma nerve, started to branch into discrete dorsal (peroneal, p) and ventral (tibial/sural, t) components. (C-D) Whole-mount β-III-tubulin immunohistochemistry (red) on wild-type (C, C’) and pma/pma (D, D’) embryos. (C’) and (D’) are detail of inset left-hand side nerves in (C) and (D) respectively. The peroneal (p) and tibial/sural (t) components are labelled. In pma/pma embryos, the tibial/sural branch is grossly normal, but only a few defasciculated axons are observable (*) in the place of the peroneal nerve, presenting a feather-like appearance. (E, F) Whole-mount β-III-tubulin immunohistochemistry (reverse colour - grey) on lower hindlimbs of E16.5 wild-type (left) and pma/pma embryos (right). Dorsal is to top – the dorsal muscles of the pma/pma foetuses are completely aneural, suggesting the putative peroneal axons noted at E12.5 have not survived. Scale bars represent 50 μm.

Figure 4: Increased apoptosis in dorsal muscle blocks of the pma hindlimb. (Top) TUNEL labelling (green) to visualise apoptotic cells in cross sections of E16.5 wild-type (left) and pma/pma foetuses (right), combined with immunohistochemistry for myosin.
Figure 5: Apoptosis and motor neuron survival in lumbar neural tube of pma homozygotes. (A) Immunohistochemistry on cross sections of lumbar neural of E14.5 wild-type (left) and pma/pma (right) foetuses. Islet 1 staining (yellow/magenta) is evident in the dorsal root ganglia and more weakly in the lateral motor columns of both genotypes. There are rare apoptotic events in both genotypes (green fluorescence - TUNEL labelling) but too few apoptotic cells were detected to allow quantification. (B) Quantification of motor neuron numbers in the neural tube of E16.5 wild-type and pma/pma foetuses. There are significantly fewer surviving motor neurones in pma/pma foetuses specifically at the level of the hindlimb (lumbar) but not more posteriorly (sacral). Scale bar represents 50 μm.

Figure 6: Normal neural tube patterning but reduced extension of motor neurons in the pma/pma hindlimb. (A) Immunohistochemistry on cross sections of the neural tube at E11.5 in pma/pma embryos and stage-matched wild-type controls. Pax6 most strongly labels medial-ventral progenitors at the level of the motor neurons. Pax3 is localised to more dorsal progenitors. Both genes are expressed normally in pma mice and there is no evidence of dorsalisation of the neural tube or loss of motor neuron identity. (B) Immunohistochemistry for ILMC marker Lim1 (green) and mLMC marker Islet1 (magenta) at E12.5 in wild-type and pma/pma stage matched embryos. Lim1-positive neurons are observable in the pma/pma ILMC indicating putative peroneal-fated neurones are correctly specified. DAPI nuclear counterstain is shown separately. There is perhaps some encroachment of Islet1-positive cells into the ILMC, but green Lim1-positive cells are discernible within the region of encroachment. (C-E) LMC axon growth in wild-type and pma/pma cultures. Double-immunohistochemistry for (C) β-III-tubulin (magenta) and (D) Lim1 (green) in a LMC culture from a wild-type embryo. Time-lapse analysis of axon extension in motor neuron cultures (E) showed
significantly reduced growth in pma/pma cultures. See also Supplementary Fig. S5).

Scale bar represents 50 μm.

Figure 7: Genetic mapping of the pma mutation.

The region of chromosome 5 linked to the pma mutation by Katoh et al. (2003) is represented by the green horizontal bar, with the location of all genes listed below. Positions of microsatellites used for genetic mapping are shown by black bars. The region eliminated from the candidate area by this study is shown by orange bars (see Table 1), with an additional orange bars showing the minimum area defined by Katoh et al. (2003). The 3.04 Mb region sequenced in crossover animals, PMA and BALB/c founders is indicated by the blue bar. The red bar and pink shaded area represents the region within the crossovers identified by sequencing, and delineates a 0.86 Mb region most likely to encompass the pma mutation, containing 13 genes.

Figure 8: Upregulation of Lim-kinase 1 (Limk1) in pma/pma mice.

(A) qPCR data of the three top candidate ‘clubfoot’ genes in the pma mouse – Cyln2, Eif4h, and Limk1. All data are means from three independent replicates, comparing E11.5 pma/pma homozygotes with C57BL/6 controls. cDNA was prepared separately from hindlimbs and neural tube of both genotypes. The error bars represent the 95% confidence interval from the statistical randomization method from the REST software. Limk1 transcripts were significantly overrepresented in both hindlimbs and neural tube of pma/pma homozygotes. (B) Western blot of E11.5 neural tube and hindlimbs of wild-type and pma/pma embryos. Limk1 and p-Limk1 were present at higher levels in pma/pma homozygotes. Total levels of cofilin (the main substrate of Limk1) were not affected but levels of phosphorylated cofilin increased, as would be expected with upregulation of Limk1. Cyln2 was not affected by pma genotype. β-actin was used as loading control. (C) Immunohistochemistry for Limk1 (brown) in the E11.5 neural tube of wild-type (left) and pma/pma embryos. Limk1 is present at higher levels in pma mice, especially in the medial and lateral LMC. (D) Immunohistochemistry for p-Limk1 in dorsal (peroneal) and ventral (tibial) branches of the sciatic nerve at E12.5. p-Limk1 is present at higher levels in the developing peroneal nerve. See also Supplementary Fig. S11.
Figure 9: Jasplakinolide addition to the developing chicken embryo can induce sciatic nerve guidance defects in vivo

Whole mount immunohistochemistry for β-III tubulin 16 hours after application of microsponge containing 20 μg/mL jasplakinolide (or 2% DMSO vehicle) to one side of the HH stage 22-23 chicken embryo at the level of the hindlimb. The spinal cord roots are numbered from I to VI, where root I (not shown) is associated with the anterior femoral nerve and roots III-V contribute to the posterior sciatic nerve. Root VI is only associated with the sciatic nerve later in development. Addition of jasplakinolide induced neuronal defects in the hindlimb. Top row shows jasplakinolide-treated (right) and contralateral untreated sides (left) highlighting loss of axons on treated side, especially from rostralmost root III, which contributes to the peroneal nerve (arrows). Shaded in grey is detail from root III. The vehicle, DMSO, was used as a control treatment and no defects were observed (bottom).

Figure 10. Electroporation of LIMK1 into chicken neural tube causes axon loss and clubfoot.

Electroporation of plasmids expressing LIMK1 or empty vector controls into HH stage 11 chickens followed by immunohistochemistry for β-III-tubulin 72 hours later or alcian blue cartilage staining after a further 5 days. (A) Nerve projection scored 0-2 as described in the methods for electroporated (Left-hand side – LHS) and non-electroporated contralateral sides (right-hand side – RHS) of each embryo, for LIMK1 and control vectors, shows significant inhibition of axon growth in LIMK1-treated nerves, but not in empty-vector electroporations. LIMK1 electroporation: RHS nerve score = 1.67 ± 0.25, n = 9; LHS score = 0.5 ± 0.22, n = 8 (one embryo damaged cf. RHS). Control FLAG electroporation: RHS nerve score = 1.75 ± 0.25, n = 4; LHS score = 2 ± 0.00, n = 3. ** Paired t-test: P = 0.001. (B) Whole mount β-III-tubulin immunohistochemistry (red) on chicken embryos showing normal sciatic plexus formation and axon projection (score 2) after an empty ‘FLAG’ vector transfection compared with representative failure of nerve plexus formation (score 0) after LIMK1 transfection. (C) Alcian blue cartilage preparation of one of the chickens (3/12) that exhibited a clubfoot phenotype after transfection with LIMK1.
Figure 11. Interaction between pma and Epha4 mutations. (A) p-cofilin immunohistochemistry (brown) on transverse sections of neural tube of E11.5 wild-type, pma/pma and Epha4−/− mouse embryos. Compared to wild-type, p-cofilin is at higher levels in both pma/pma and Epha4 mutant mice, suggesting a shared biochemical pathway. Similar levels of protein in the dorsal root ganglia provide an internal control for staining. (B) Representation of frequency occurrence of peroneal nerve loss in P21 mice that are compound heterozygotes for EphA4 and pma, compared to single heterozygous littermates for either gene, or littermates wild-type at both loci. See text for details. Scale bar represents 35 μm.
Apoptosis in dorsal muscle groups

Mean percentage of TUNEL positive cells

- **Peroneus longus**
- **Extensor digitorum longus**
- **Tibialis anterior**

WT vs PMA:
- **Peroneus longus**: PMA significantly higher than WT
- **Extensor digitorum longus**: PMA significantly higher than WT
- **Tibialis anterior**: PMA significantly higher than WT