LDL-receptor-related protein 4 is crucial for formation of the neuromuscular junction

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Low-density lipoprotein receptor-related protein 4 (Lrp4) is a member of a family of structurally related, single-pass transmembrane proteins that carry out a variety of functions in development and physiology, including signal transduction and receptor-mediated endocytosis. Lrp4 is expressed in multiple tissues in the mouse, and is important for the proper development and morphogenesis of limbs, ectodermal organs, lungs and kidneys. We show that Lrp4 is also expressed in the post-synaptic endplate region of muscles and is required to form neuromuscular synapses. Lrp4-mutant mice die at birth with defects in both presynaptic and postsynaptic differentiation, including aberrant motor axon growth and branching, a lack of acetylcholine receptor and postsynaptic protein clustering, and a failure to express postsynaptic genes selectively by myofiber synaptic nuclei. Our data show that Lrp4 is required during the earliest events in postsynaptic neuromuscular junction (NMJ) formation and suggest that it acts in the early, nerve-independent steps of NMJ assembly. The identification of Lrp4 as a crucial factor for NMJ formation may have implications for human neuromuscular diseases such as myasthenia syndromes.

KEY WORDS: Lrp4, Neuromuscular junction, Limb development, Lung hypoplasia, Oligosyndactyly, Mouse

INTRODUCTION
Members of the low-density lipoprotein (LDL) receptor-related protein (LRP) family are well known for their roles in lipid metabolism, cholesterol homeostasis and Wnt signaling (reviewed by He et al., 2004; Schneider and Nimtz, 2003). In addition, several members of the LRP family are also known to have specific roles in the development and function of the mammalian nervous system, as receptors for both known and unknown ligands (May and Herz, 2003). For example, mutant mice that lack the Wnt co-receptor Lrp6 have defects in the development of the thalamus and radial glia (Zhou et al., 2004) while Lrp2 (also known as Megalin) is required for normal forebrain development, perhaps through regulation of Bmp4 clearance (Spoelgen et al., 2005; Willnow et al., 1996). Vldlr and Lrp8 (also known as apoER2) are receptors for reelin and are required for normal layering of cortical neurons (D’Arcangelo et al., 1999; Hiesberger et al., 1999; Trommsdorff et al., 1999). Lrp1 is localized to postsynaptic specializations in the central nervous system, where it associates with NMDA-type glutamate receptors (Bu et al., 1994; Ishiguro et al., 1995; May et al., 2004; Moestrup et al., 1992). Taken together, the results of these studies indicate that Lrps have distinct roles in neural development and synaptic connectivity.

In an N-ethyl-N-nitrosourea (ENU) mutagenesis screen in the mouse, we identified two mutants that had striking and similar defects in limb development, as well as an intriguing set of other developmental defects. Both mutations proved to be alleles of Lrp4 [also known as Meg7 (Nakayama et al., 1998)]. Three other alleles of Lrp4 have been described that produce similar defects in limb development; however, those mutants are viable and no other developmental defects have been described (Johnson et al., 2005; Simon-Chazottes et al., 2006). Additionally, they appear to be hypomorphic alleles, whereas the ENU-induced alleles are null. The phenotypes of the null alleles reveal that Lrp4 is required for viability and for normal development of the lung, kidney and ectodermal organs. One of the striking phenotypes caused by a complete lack of Lrp4 is paralysis at birth, due to an early block in the development of a specialized synapse, the neuromuscular junction (NMJ).

During the formation of the NMJ, acetylcholine receptors (AChRs) are clustered in the central region of muscle before the nerve arrives at the site of the prospective synapse (Flanagan-Steet et al., 2005; Panzer et al., 2005; Panzer et al., 2006; Lin et al., 2001; Pun et al., 2002; Yang et al., 2001; Yang et al., 2000). Signals from the motor axon are required to refine and maintain this pre-pattern so that AChRs are stably restricted to synaptic sites (Lin et al., 2005; Misgeld et al., 2005). Agrin, a large glycoprotein that is expressed by motoneurons and deposited into the synaptic basal lamina, is a crucial nerve-derived signal (Denzer et al., 1997; Ruegg and Bixby, 1998; Rupp et al., 1991). Agrin regulates synaptic differentiation by activating MuSK (Musk – Mouse Genome Informatics) (Hopf and Hoch, 1998), a receptor tyrosine kinase that is expressed selectively by skeletal muscle and is concentrated at synaptic sites (Bowen et al., 1998; Glass et al., 1996; Jennings et al., 1993; Liyanage et al., 2002; Trinidad et al., 2000; Valenzuela et al., 1995). Mice lacking either Agrin or MuSK fail to form neuromuscular synapses, cannot move or breathe and die at birth (DeChiara et al., 1996; Gautam et al., 1999; Gautam et al., 1996; Gautam et al., 1995).

Previous studies have shown that MuSK plays a key role in all aspects of postsynaptic differentiation, including the early nerve-independent pre-patterning of AChRs and the subsequent stabilization of AChRs at synaptic sites (Lin et al., 2001; Yang et al., 2001; Yang et al., 2000). However, the mechanisms that lead to MuSK activation, both in the Agrin-independent step during pre-patterning and in the Agrin-dependent stage after contact with motor axons, are poorly understood.

Here we show that the phenotypes of the two ENU-induced alleles of Lrp4 define an essential role for Lrp4 in the initial establishment of the NMJ. Lrp4 is required for clustering of AChRs...
at the synapse, at both early and late stages of synapse formation. Expression of the *Lrp4* gene, like other genes that encode proteins enriched at the synapse, is upregulated in the postsynaptic endplate region, and *Lrp4* is required for this specialized transcription. *Lrp4* is required for proper localization of MuSK, and *Lrp4* and *Musk* mutants appear to have identical phenotypes at the neuromuscular junction. These studies indicate that *Lrp4* acts at the first known step of assembly of the neuromuscular junction.

**MATERIALS AND METHODS**

**Generation and mapping of mouse mutants**

Mutations were generated by using ENU as described (Garcia-Garcia et al., 2005; Kasarskis et al., 1998). The *mitaine* and *mitten* mutants (renamed *Lrp4mte* and *Lrp4mitt* after identification of the gene) were identified in two independent screens. Recombination mapping utilized simple-sequence length polymorphism markers to test for association between phenotypes and regions of the C57BL/6 genome (Kasarskis et al., 1998). Both mutations mapped on Chromosome 2 between markers D2Mit14 (49.6 cm) and D2Mit221 (50.3 cm).

**Myotube cultures**

Myoblasts and fibroblasts were dissociated from forelimb and hind limb muscles of embryonic day (E) 17.5 embryos and plated on collagen-coated tissue culture plates in Ham’s F10 Medium containing 20% fetal bovine serum, 2.5 ng/ml basic fibroblast growth factor (recombinant human, Promega) and antibiotics. Cells were selectively passaged twice over 8 days to enrich for myoblasts. Cultures were then switched to Matrigel (BD Biosciences)-coated tissue culture plates in Dulbecco’s modified Eagle Medium containing 5% horse serum and antibiotics for 6 to 7 days to promote fusion into myotubes. Cultures were then treated overnight with 20 ng/ml of C-terminal Agrin (recombinant rat, R&D systems). The next day (18 hours later), these cultures were incubated with α-bungarotoxin, rinsed in PBS, fixed in 2% paraformaldehyde (PFA) and mounted for immunofluorescence.

**Expression analysis**

In situ analysis was performed on whole-mount specimens, paraffin sections or cryosections of embryos fixed in 4% PFA at 4°C for 16-24 hours using published techniques (Holmes and Niswander, 2001). An antisense riboprobe (nucleotides 4481-5752) from mouse *Lrp4* cDNA was utilized for transcript detection. Immunofluorescence was performed using standard methods (Eggenschwiler and Anderson, 2000). Antibodies used were: anti-synaptophysin (polyclonal; Zymed), anti-α-syntrophin (monoclonal; Novacraft), anti-MuSK (polyclonal; Affinity BioReagents). The monoclonal antibodies SV2 (developed by Kathleen Buckley) and 2H3 (neurofilament, developed by Thomas Jessell and Jane Dodd) were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. Alexafluor594-conjugated α-bungarotoxin was obtained from Molecular Probes. For detection of β-galactosidase activity, embryos were stained with X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactoside) as described (Hogan and Lacy, 1994).

Antibodies used on lung sections (see Fig. S2 in the supplementary material) were: phospho-histone H3 (polyclonal, Upstate), ProSurfactant Protein C (polyclonal, Chemicon), PECAM-1 (polyclonal, Pharmingen), Clara Cell Protein 26 kDa (polyclonal, Chemicon) and acetylated α-tubulin (monoclonal, Zymed). The monoclonal antibody 8.1.1 (T1-tubulin antigens) was developed by Andrew Farr was obtained from the Developmental Studies Hybridoma Bank. TUNEL assay was performed as per manufacturer’s recommendations (Roche).

**Skeletal preparations**

Alican Blue/Alizarin Red staining was performed using standard methods (Hogan and Lacy, 1994). Briefly, embryos were eviscerated and skinned, followed by dehydration in ethanol. After processing for Alican Blue and Alizarin Red staining, embryos were cleared through a graded series of potassium hydroxide and glycerol.

Quantitative real-time RT-PCR

The quality of RNA was ensured before processing by analyzing 100 ng of each sample using the RNA 6000 NanoAssay and a Bioanalyzer 2100 (Agilent). One microgram of total RNA was reverse-transcribed using the iScript cDNA Synthesis Kit (Bio-Rad) at 52°C for 1 hour. Twenty nanograms of resultant cDNA was used in a quantitative real-time RT-PCR reaction using an iCycler (Bio-Rad) and pre-designed TaqMan Gene expression Assays for MuSK (Mm00448066_m1) and HPRT (Mm00449696_m1). Amplification was carried out for 40 cycles (95°C for 15 seconds, 60°C for 1 minute). Data from the linear phase of amplification were used to calculate MuSK and HPRT levels based on the standard curve method. MuSK levels were normalized to HPRT and triplicates values were averaged.

**RESULTS**

**The mte and mtt mutations are null alleles of *Lrp4***

The ENU-induced mutations *mitaine* (*mte*) and *mitten* (*mitt*) were first identified based on defects in limb development in the homozygotes, which included syndactyly and shortening of the autopod elements. Both mutations were linked to the same segment of chromosome 2 and failed to complement: *mte/mitt* transheterozygotes displayed the same spectrum of phenotypes as individual homozygotes, which demonstrated that the mutations are alleles of a single gene.

We used positional cloning to identify the locus disrupted by the *mte* and *mitt* mutations. The *mte/mitt* mutations were mapped by meiotic recombination to a 0.7 cM interval on Chromosome 2 (see Materials and methods). Sequencing of candidate genes in the interval showed that both mutant chromosomes had lesions in the *Lrp4* gene. The extracellular domain of *Lrp4* contains four YWTD β-propeller domains, five EGF-like domains and seven LDLR type A (LA; ligand binding) domains (Fig. 1A) (Springer, 1998). The *mitt* allele had two mutations: an early stop codon C-terminal to the LA domains, which should delete most of the protein, and a second splice site mutation that would introduce a second premature stop (Fig. 1A). The *mte* allele was associated with a missense mutation at a conserved position (Asp1436Gly) in the most C-terminal YWTD domain; mutations at the same position in human LDLR (Day et al., 1997; Ward et al., 1995; Webb et al., 1996) appear to disrupt protein folding and stability (Jeon et al., 2001). Based on the nature of these sequence changes and the identical phenotypes of *mte* and *mitt* homozygous embryos, it is likely that both of these mutations caused a complete loss of *Lrp4* function.

**Distal limb development depends upon *Lrp4***

The limb phenotype of *Lrp4mte* and *Lrp4mitt* homozygotes included a reduction in digit number, shortening of the autopod elements, ectopic phalanges or digits dorsal to the normal plane of the hand, and fusions of the central digits (Fig. 1B-E). The earliest defect in limb patterning of *Lrp4* mutants was an expansion of the apical ectodermal ridge (AER) along the dorsoventral axis of the limb bud. This was detected as an expanded domain of *Fgf8* (Fig. 1F,G) and *Msx2* (Fig. 1H,I) expression in the limb ectoderm. *Wnt7a*, a marker of dorsal limb ectoderm that normally extends to the dorsal boundary of the AER, was found only in proximal ectoderm (Fig. 1J,K), in a domain complementary to that of the dorsally expanded AER. A targeted allele and two insertion mutations in *Lrp4* produce skeletal defects similar to those seen in *Lrp4mte* and *Lrp4mitt* mutants (Johnson et al., 2005; Simon-Chazottes et al., 2006), and the gene expression changes we observed (Fig. 1; see Fig. S1 in the supplementary material) are consistent with the phenotype reported for the targeted *Lrp4* allele (Johnson et al., 2005).

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Expression and requirement for Lrp4 in a variety of tissues

Lrp4 was expressed in limb ectoderm at early stages (E9.5) (Fig. 2A) and later was restricted to the AER (E11.5) (Fig. 2B) (Johnson et al., 2005). We found that Lrp4 was also expressed in a variety of other tissues, and many of those tissues developed abnormally in the Lrp4mut and Lrp4mut mutants, although these defects have not been described for the targeted and insertional alleles. For example, Lrp4 was expressed throughout kidney development: it was expressed in the epithelial tips of the ureteric buds during formation of the ureteric bud (E11.5), (Fig. 2C); by E16.5, expression was restricted to the tips of the growing ureter in the nephrogenic zone (Fig. 2D). The majority of Lrp4mut and Lrp4mut mutants exhibited unilateral or bilateral kidney agenesis (66%, 59/91). The targeted allele of Lrp4 displayed variable craniofacial defects (Johnson et al., 2005), and we noted Lrp4 expression in tooth primordia (Fig. 2G), as well as in a variety of other ectodermal organs such as whisker vibrissae (Fig. 2E), hair follicles (Fig. 2F) and mammary buds (Fig. 2H); each of these tissues developed abnormally in mutant embryos (not shown).

By contrast to the previously described Lrp4 alleles, which are homozygous viable, homozygous Lrp4mut and Lrp4mut mutants died at birth. Although mutant pups were born at the expected frequency, they retained their in utero posture after birth, and did not move or breathe (Fig. 3A,B). Lrp4mut and Lrp4mut homozygous newborn mice showed respiratory defects, including a failure to expand the air passages in their lungs (Fig. 3C,D), which is most probably the cause of their early death. Lrp4 is expressed strongly in the proximal airways of the lungs as well as in the diaphragm (Fig. 2I). Primary and secondary branching of the lung appeared normal in Lrp4mut and Lrp4mut mutant animals (not shown). During later stages of embryonic development, overall organogenesis of the mutant lungs was preserved, but they were markedly reduced in size (50-75%) compared with wild-type siblings (see Fig. S2 in the supplementary material). Despite defects in lung size and sacculation, no abnormalities were detected in cell proliferation, cell death or the expression of molecular markers of lung differentiation (see Fig. S2 in the supplementary material). Thus, together these results indicate a broader role for Lrp4 than previously realized in the formation of multiple embryonic tissues.

Lrp4 is required for formation of the NMJ

The phenotypes of several mutants have pointed to the importance of muscle structure and activity in the proper morphogenesis of the lung (Inanlou et al., 2005). In the absence of skeletal and/or diaphragm muscles, embryos lack fetal breathing movements, which causes reduced lung size, reduced sacculation and lung patterning defects (Ackerman et al., 2005; Inanlou and Kablar, 2003; Inanlou and Kablar, 2005a; Inanlou and Kablar, 2005b). Similarly, muscles cannot contract in Musk mutants due to the absence of NMJs, and these embryos display pulmonary hypoplasia (DeChiara et al., 1996). The late lung hypoplasia phenotype observed in the Lrp4 mutants (Fig. 3D) did not appear to be the result of absence of normal muscles, as the intercostal and diaphragm muscles appeared macroscopically normal (data not shown). However, the failure of Lrp4 mutant mice to move at birth suggested that the mutants might have a general defect in neuromuscular function. Although Lrp4 was not expressed in motoneurons (Fig. 2I), Lrp4 transcripts were detected in the diaphragm and other muscles at E12.5, before neuromuscular synapse formation (Fig. 2I and data not shown), which would be consistent with a postsynaptic requirement for Lrp4.

To assess whether normal synaptogenesis took place at the NMJ of Lrp4 mutants, we examined the organization of AChRs in mutant mice. In normal muscles, each muscle fiber bears a single AChR cluster asayed by a nerve terminal (Fig. 3I-K). By contrast, no AChR clusters could be detected by staining with labeled α-bungarotoxin in either Lrp4mut or Lrp4mut mutant diaphragms (Fig. 3L-N) or intercostal muscles (Fig. 3E,F) at E18.5. The absence of AChR clusters in these muscles is sufficient to account for the failure of the mutants to breathe, and therefore can explain the abnormal morphology of the mutant lungs. No AChR clustering was detected in limb muscles of Lrp4 mutants (Fig. 3G,H), and the lack of embryonic and perinatal movement suggests that Lrp4 is required globally for NMJ organization.

To confirm that Lrp4 was acting in the postsynaptic cell, we derived myotubes from E18.5 wild-type and Lrp4mut or Lrp4mut mutant forelimb and hind limb muscles. In wild-type cultured myotubes, spontaneous clustering of AChRs occurs in the absence...
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Fig. 2. Lrp4 is expressed in multiple tissues and organs during mouse development. (A) Brightfield image of a radioactive in situ hybridization for Lrp4. A cross-section of an E9.5 forelimb bud shows expression of Lrp4 (dark grains) throughout the ectoderm. (B) Dorsal view of an E11.5 hind limb bud shows strong Lrp4 expression (blue) in the AER. (C,D) Whole-mount in situ hybridizations show expression of Lrp4 at the tips of the ureteric buds at E11.5 (C, black arrows) and E16.5 (D). (E) A lateral view of the snout of an E12.5 embryo shows Lrp4 is expressed in whisker primordia. (F) Transverse section through the dorsolateral back region of an E14.5 embryo, counter-stained with Fast Red (pink). Lrp4 is expressed in developing hair follicles (blue). Inset, high magnification view of a hair follicle. (G) Upper palate of an E12.5 embryo. Lrp4 is strongly expressed in the developing incisors and molars. (H) Whole-mount in situ hybridization shows expression of Lrp4 in mammary buds (white arrowheads) at E13.5. (I) Radioactive in situ hybridization (darkfield) for Lrp4 on a sagittal section of a wild-type E12.5 embryo. Lrp4 is expressed strongly in the proximal airways of the lungs (red arrows) and in the diaphragm (white arrow). (J) Transverse section through an E10.5 embryo counter-stained with Fast Red (pink) shows expression of Lrp4 in the dorsal neural tube and floorplate. di, diaphragm; he, heart; in, incisor; lb, limb bud; li, liver; lu, lung; mb, mammary bud; mn, motoneuron; mo, molar; wh, whisker vibrissae.

Lrp4 is required for the initiation of the NMJ

The absence of AChR clusters in E18.5 Lrp4 mutants suggested that Lrp4 was required for either the formation or stabilization and maintenance of AChR clusters. By E18.5, Agrin is required to stabilize synaptic AChR clusters, but the initial formation of AChR clusters occurs in the absence of Agrin and can occur in the absence of nerve terminals (Lin et al., 2001). We therefore examined AChR clusters in wild-type and Lrp4 mutant mice at E13.5 and 14.5. In wild-type animals, AChR preclustering in the endplate region was visible at E13.5 diaphragms, as motor axons were exiting the main body of the phrenic nerve (Fig. 4B). However, Lrp4 mutants lack this early AChR clustering before innervation (Fig. 4E). At E14.5, as at E18.5, AChR clusters were prominent in wild type (Fig. 4H), but could not be detected in Lrp4 mutant muscle (Fig. 4K). This early NMJ defect is similar to that observed in Musk mutants (Lin et al., 2001). Also similar to Musk mutants, the motor axons in Lrp4 mutants did not stop progressively at the main intramuscular nerve, but instead grew excessively across the muscle (Fig. 4L, Fig. 3N). These phenotypes suggest that Lrp4 is essential for AChR clustering and acts at the early, Agrin-independent, MuSK-dependent step in this process.

Lrp4 is required for synaptic protein localization at the NMJ

AChR clustering depends on the cytoplasmic, peripheral membrane protein Rapsn (also known as rapsyn), which localizes to NMJs in response to MuSK (DeChiara et al., 1996) and binds directly to AChRs (Bartoli et al., 2001; Burden et al., 1983). To examine whether Lrp4 might act with Rapsn, we tested whether Rapsn was localized correctly in Lrp4 mutant muscles. We marked synaptic sites with antibodies to synaptophysin or SV2, which are components of presynaptic vesicles (Gautam et al., 1995). Rapsn was concentrated at postsynaptic regions in wild-type muscles, but was not localized in Lrp4 mutants (Fig. 5A,‘D’). Utrophin, a component of the muscle cytoskeleton, is specifically localized with AChRs at the crests of postjunctional folds (Blake et al., 1994; Blake et al., 1996) and depends upon a scaffold of proteins, including MuSK and Rapsn, for its localization. In Lrp4 mutant muscle, utrophin was not detectable at the synapse (Fig. 5B,‘E’). Thus Lrp4 is required for proper localization of Rapsn at the synapse and because Rapsn fails to localize correctly, neither AChRs nor utrophin (Gautam et al., 1995) localize to the NMJ of Lrp4 mutants.

Failure of postsynaptic specialization in Lrp4 mutants

The subset of muscle cell nuclei located near synaptic sites is specialized to express high levels of proteins enriched at the postsynaptic membrane (Sanes et al., 1991). Synapse-specific transcription is disrupted in Musk, but not in Rapsn, mutants (Gautam et al., 1999); thus MuSK has a second activity in addition to its role in localization of Rapsn and Rapsn-dependent proteins. Transcripts encoding the AChR α and δ subunits and MuSK were concentrated in the central region of diaphragm muscles in E14.5 controls (Fig. 6A-C); however, these transcripts were more broadly expressed in Lrp4mut and Lrp4mut mutants (Fig. 6E-G, data not shown), indicating that their expression had not been restricted to synaptic nuclei. By E18.5, the Musk, AChRa and AChRDδ genes were expressed only at very low levels in Lrp4 mutant muscles and were not localized to the endplate region (Fig. 6M-O).

Moreover, Lrp4 mRNA itself was highly enriched in the synaptic region of diaphragm and intercostal muscles from wild-type animals (Fig. 6D, and data not shown), as seen for proteins that are enriched at the synapse. Lrp4 RNA was not properly localized in Lrp4mut and Lrp4mut mutant muscles (Fig. 6H, data not shown), although it was detectable via quantitative RT-PCR (data not shown). Thus Lrp4 is required for both aspects of MuSK activity, assembly of the AChR scaffold and specialization of postsynaptic nuclei.

The similarity of the Musk and Lrp4 phenotypes suggested that Lrp4 and MuSK regulate a common step in formation of the NMJ. We found that MuSK was not concentrated at synapses in Lrp4 mutant muscles (Fig. 5C,‘F’), even though MuSK transcripts were...
present at similar levels in wild type and mutants before postsynaptic specialization based on quantitative real-time RT-PCR (see Fig. S3 in the supplementary material). Thus, Lrp4 is required for the first known step of postsynaptic assembly, the accumulation of MuSK at the synapse.

**DISCUSSION**

We have shown that at the NMJ, Lrp4 is required for the first step in the development of postsynaptic specialization. All signs of postsynaptic specialization are lost in the Lrp4 mutants, just as in *Musk* mutants. As in *Musk* mutants, the Rapsn and utrophin-dependent scaffold fails to assemble and AChRs do not localize to the synapse in the Lrp4 mutants. In Lrp4 and *Musk* mutants, but not *Agrin* mutants, AChR clusters are absent when clusters first assemble at E13.5. In both Lrp4 and *Musk* mutants, motor axons grow past the normal endplate and branch extensively across the surface of the muscle. Lrp4, like MuSK, is also required for synapse-specific transcription in muscle nuclei. In addition to the similar phenotypes of Lrp4 and *Musk* mutants, MuSK protein is not localized to the synapse in the Lrp4 mutants. Thus, Lrp4 is required...
for all aspects of postsynaptic specialization and therefore acts either upstream of MuSK or together with MuSK to establish the NMJ.

The phenotypes of the spontaneous and targeted alleles of Lrp4 differ markedly from those of the ENU-induced alleles described here. The most obvious difference is the completely penetrant perinatal lethality of homozygous Lrp4mte and Lrp4mut alleles, by contrast to the spontaneous, retrovirally derived (Lrp4mdig and Lrp4dan, respectively) (Simon-Chazottes et al., 2006) and targeted (Johnson et al., 2005) alleles of the gene, which are homozygous viable and fertile. One of the ENU-induced alleles has an early stop codon that would remove over 80% of the protein, and the second ENU-induced allele carries a missense mutation at the same position as an allele of the LDL receptor that probably destabilizes the protein (Jeon et al., 2001). These two ENU-induced mutations are therefore likely to be null alleles. The Lrp4mdig and Lrp4dan alleles appear to be hypomorphic, as some wild-type transcript is present in each mutant (Simon-Chazottes et al., 2006). The targeted allele of Lrp4 removes the transmembrane and cytoplasmic domains of Lrp4, but could express an intact extracellular domain. Approximately 35% of homozygous targeted mutants die between P0 and P5, although the cause of lethality was not determined (Johnson et al., 2005). Based on the phenotypes of the Lrp4mte and Lrp4mut mutant alleles, the incompletely penetrant lethality of the targeted mutants could be due to renal agenesis or defects in postsynaptic specialization. In principle, differences in genetic background could account for the phenotypic differences between the ENU-induced and targeted alleles, as the Lrp4mte and Lrp4mut alleles were analyzed on mixed C3H-C57Bl/6 and congenic C3H backgrounds, while the targeted allele was studied on a mixed 129SvEv-C57Bl/6 background. In either case (different allele strength or different genetic background), we conclude that reduced levels of Lrp4 cause a common limb phenotype, and complete loss of Lrp4 function causes additional phenotypes that include defects in the formation of multiple embryonic tissues, perinatal lethality and failure to organize the NMJ.

The diverse phenotypes caused by loss of Lrp4 have no obvious common molecular basis. Musk mutants do not show limb defects, and it has been suggested that Lrp4 limb phenotypes are the result of excessive canonical Wnt signaling (Johnson et al., 2005), which is supported by our in vivo analysis of a Wnt reporter in Lrp4mte and Lrp4mut mutants (see Fig. S1A,G in the supplementary material), although the mechanism by which Lrp4 could antagonize Wnt signaling has not been defined. By contrast, the kidney hypoplasia and agenesis phenotype is more akin to Wnt loss of function (Carroll et al., 2005; Majumdar et al., 2003; Pinson et al., 2000; Stark et al., 1994) than to excess Wnt signaling. Thus it appears that Lrp4 affects different signaling pathways in specific tissues.

Defects in NMJ formation, maintenance or function can lead to human disorders such as muscular dystrophy and myasthenia syndromes (Engel et al., 2003; Ervasti and Campbell, 1993; Hughes et al., 2005). Myasthenia syndromes are characterized by muscle weakness; severe cases can affect breathing and thus are life-threatening. Several congenital myasthenia syndromes have been mapped to mutations in proteins localized in the presynaptic and postsynaptic regions of the NMJ. Myasthenia gravis is a group of acquired autoimmune disorders wherein some patients produce...
antibodies against AchR subunits, while others test positive for antibodies that recognize MuSK or Rapsn. Targeting of NMJ components by the host immune system results in a reduction in the number of AchR clusters (up to an 80% decrease) in voluntary muscles. In some cases of Myasthenia gravis, the autoantigen has not been identified, and the genetic basis of a subset of congenital myasthenia syndromes has not been elucidated. It will be important to test whether any of these individuals have mutations in the Lrp4 gene or generate antibodies against the Lrp4 protein.

The requirement of Lrp4 for NMJ formation is reminiscent of the role of Lrp1 at central nervous system synapses. Lrp1 associates with postsynaptic proteins, including neurotransmitter receptors, and is required for normal synaptic function (reviewed by May et al., 2005). The biochemical activities of other members of the Lrp family suggest two possible mechanisms for the function of Lrp4 at the synapse. Like Lrp5 and Lrp6, Lrp4 might act as a co-receptor that, together with MuSK, binds a ligand that activates MuSK activity. No ligand has been identified that activates MuSK kinase activity. No ligand has been identified that activates MuSK kinase activity. In fact, we have not found any biochemical evidence that MuSK is activated by any ligand. In addition, there is no evidence that MuSK is activated by any ligand. Instead, MuSK is activated by the tyrosine kinase MuSK. MuSK is required for neuromuscular junction formation in vivo. Cell 85, 501-512.


