

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

Scott D. Weatherbee<sup>1,2,\*</sup>, Kathryn V. Anderson<sup>1</sup> and Lee A. Niswander<sup>2,\*</sup>

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 Nimpf, 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 *Megf7* (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

<sup>1</sup>Developmental Biology Program, Sloan-Kettering Institute, New York, NY 10021, USA. <sup>2</sup>Howard Hughes Medical Institute, University of Colorado Health Sciences Center, Department of Pediatrics, Section of Developmental Biology, Aurora, CO 80045, USA.

\* Authors for correspondence (e-mail: s-weatherbee@ski.mskcc.org; Lee.Niswander@uchsc.edu)

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 *Lrp4<sup>mte</sup>* and *Lrp4<sup>mitt</sup>* 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  $\alpha$ -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-utrophin (monoclonal; Novacastra), 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  $\alpha$ -bungarotoxin was obtained from Molecular Probes. For detection of  $\beta$ -galactosidase activity, embryos were stained with X-Gal (5-bromo-4-chloro-3-indolyl  $\beta$ -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  $\alpha$ -tubulin (monoclonal, Zymed). The monoclonal antibody 8.1.1 (T1- $\alpha$ ) developed by Andrew Farr was obtained from the Developmental Studies Hybridoma Bank. TUNEL assay was performed as per manufacturer's recommendations (Roche).

### Skeletal preparations

Alcian 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 Alcian 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 (Mm00448006\_m1) and HPRT (Mm00446966\_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

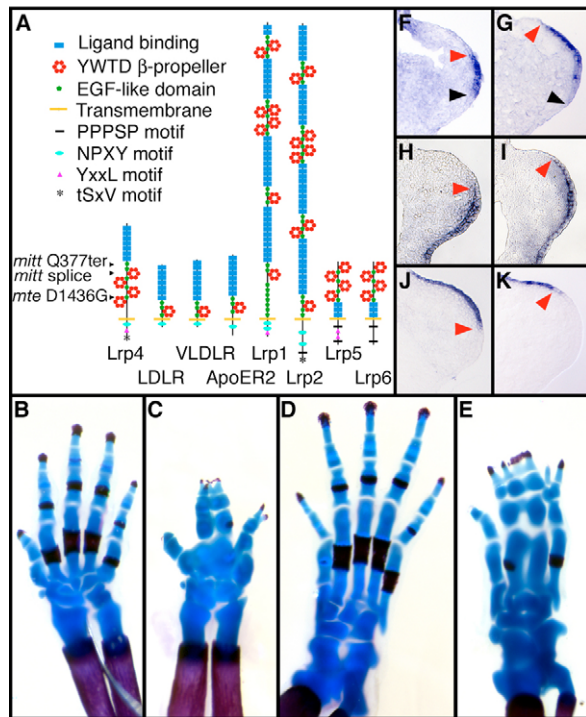
### The *mte* and *mitt* mutations are null alleles of *Lrp4*

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  $\beta$ -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 *Lrp4<sup>mte</sup>* and *Lrp4<sup>mitt</sup>* 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 insertional mutations in *Lrp4* produce skeletal defects similar to those seen in *Lrp4<sup>mte</sup>* and *Lrp4<sup>mitt</sup>* 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).



**Fig. 1. *mte* and *mitt* mouse embryos have limb defects due to mutations in the *Lrp4* gene.** (A) Schematic diagram of mouse Lrp proteins. Positions of mutations in the *mitt* and *mte* alleles of Lrp4 are indicated next to the proposed Lrp4 structure. The intracellular region of Lrp4 contains motifs predicted to mediate clustering to clathrin-coated pits and a terminal SXV motif predicted to mediate interactions with PDZ domain-containing proteins. (B-E) E18.5 Limb skeletal preparations highlight the abnormalities in *Lrp4*<sup>mitt</sup> autopods (C,E) compared with wild type (B,D). (B,C) Forelimbs; (D,E) hind limbs. Digit numbers are reduced, phalanges and metacarpals/metatarsals are fused, and ossification is decreased in *Lrp4*<sup>mitt</sup> mutant autopods (C,E). At earlier stages of limb development, *Lrp4* mutants display AER expansion and a failure of AER refinement and maturation. (F-K) Longitudinal sections through E9.5 limb buds. Dorsal is to the top, distal to the right. *Fgf8*, a marker of AER cells, is normally restricted to the distal region of E9.5 forelimbs (F), but is expanded both dorsally (red arrowhead) and ventrally (black arrowhead) in *Lrp4* mutants (G) and no morphologically distinct AER forms (see Fig. S1 in the supplementary material). In parallel, dorsal expression of *Msx2* (H) is expanded in *Lrp4* mutants (I, red arrowhead) and there is a loss of *Wnt7a* in the most distal region of mutant forelimbs (compare K to J).

### 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 *Lrp4*<sup>mte</sup> and *Lrp4*<sup>mitt</sup> 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 *Lrp4*<sup>mte</sup> and *Lrp4*<sup>mitt</sup> mutants exhibited unilateral or bilateral kidney agenesis (66%, 59/89). 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 *Lrp4*<sup>mitt</sup> and *Lrp4*<sup>mte</sup> 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). *Lrp4*<sup>mitt</sup> and *Lrp4*<sup>mte</sup> 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 *Lrp4*<sup>mitt</sup> and *Lrp4*<sup>mte</sup> 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 sacculcation, 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 sacculcation 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. 2J), *Lrp4* transcripts were not 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 muscles. In normal muscles, each muscle fiber bears a single AChR cluster apposed by a nerve terminal (Fig. 3I-K). By contrast, no AChR clusters could be detected by staining with labeled  $\alpha$ -bungarotoxin in either *Lrp4*<sup>mitt</sup> or *Lrp4*<sup>mte</sup> 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.

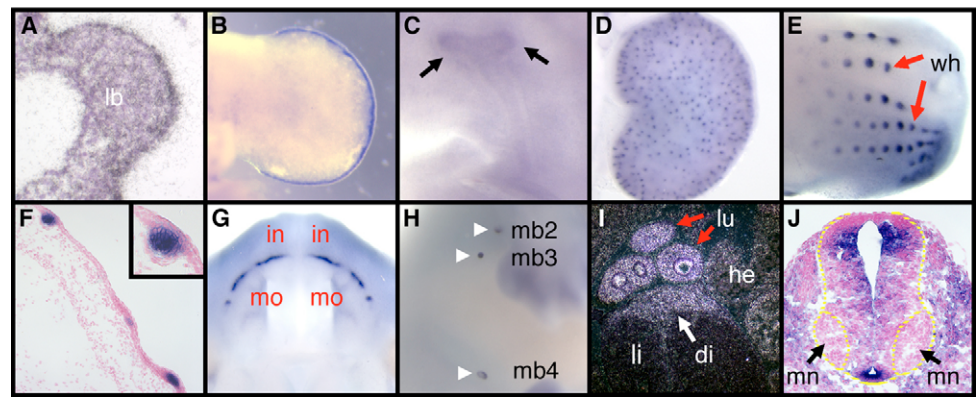
To confirm that *Lrp4* was acting in the postsynaptic cell, we derived myotubes from E18.5 wild-type and *Lrp4*<sup>mitt</sup> or *Lrp4*<sup>mte</sup> mutant forelimb and hind limb muscles. In wild-type cultured myotubes, spontaneous clustering of AChRs occurs in the absence

## 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, motorneuron; mo, molar; wh, whisker vibrissae.



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, motorneuron; mo, molar; wh, whisker vibrissae.

of motor axons (Fig. 4M) and treatment with Agrin increases the number and frequency of AChR clusters (Fig. 4O) (Campanelli et al., 1991). However, in *Lrp4* mutant myotubes, clusters of AChRs were not observed forming spontaneously or following stimulation with Agrin (Fig. 4N). These results confirm that *Lrp4* is required in the postsynaptic cell for the clustering of AChRs.

### *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 in 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 adjacent to 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

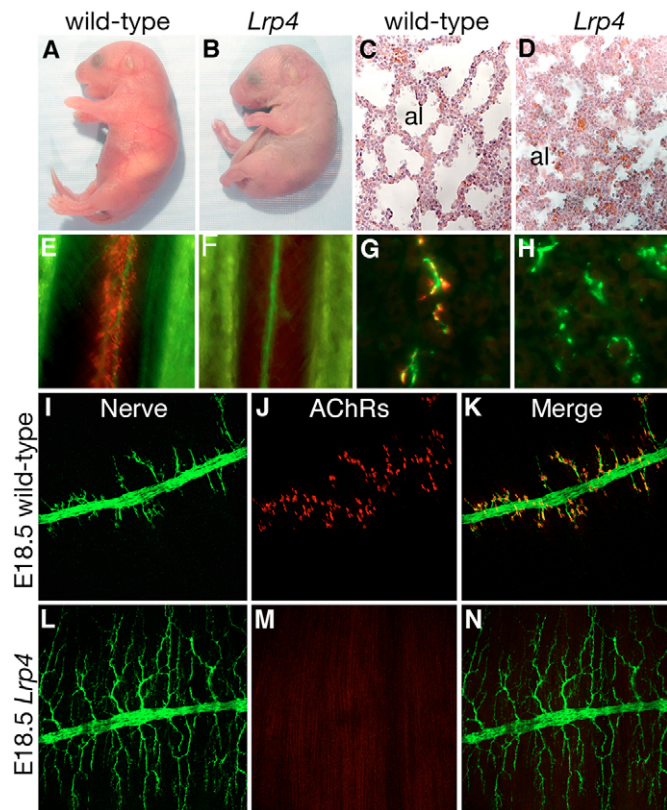
was concentrated at postsynaptic regions in wild-type muscles, but was not localized in *Lrp4* mutants (Fig. 5A',D'). Utrophin, a component of the muscle cytoskeleton, is specifically localized with AChRs at the crests of postjunctional folds (Blake et al., 1994; Blake et al., 1996) and depends upon 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  $\alpha$  and  $\delta$  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 *Lrp4<sup>mie</sup>* and *Lrp4<sup>mitt</sup>* mutants (Fig. 6E-G and data not shown), indicating that their expression had not been restricted to synaptic nuclei. By E18.5, the *Musk*, *AChR $\alpha$*  and *AChR $\delta$*  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,L and data not shown), as seen for proteins that are enriched at the synapse. *Lrp4* RNA was not properly localized in *Lrp4<sup>mie</sup>* and *Lrp4<sup>mitt</sup>* mutant muscles (Fig. 6H,P and 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



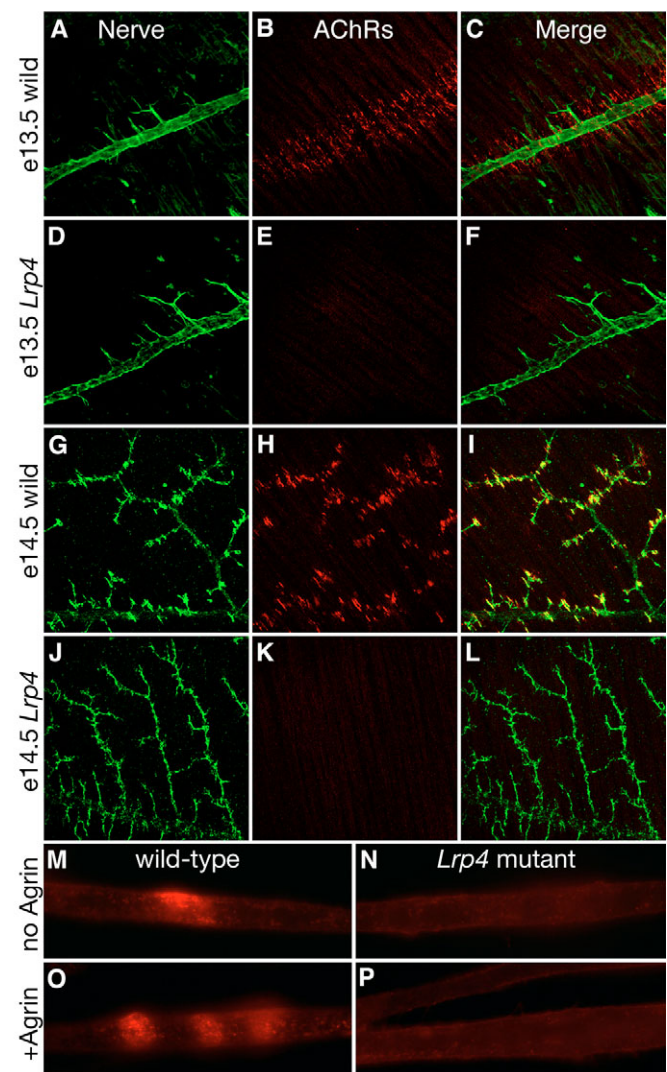
**Fig. 3. *Lrp4* is required for neuromuscular synaptogenesis.**

(A) Newborn wild-type pups display motile activity and have begun feeding shortly after birth (indicated by the presence of a milkspot). By contrast, *Lrp4* mutant pups (B) are cyanotic, do not move, feed or breathe and retain their in utero posture. (C,D) Longitudinal histological sections through the left lung lobe of E18.5 wild-type (C) and mutant embryos (D). The future alveolar spaces (al) are smaller and the interstitial mesenchyme is thicker in *Lrp4* mutant (D) embryos compared with wild type (C). E18.5 intercostal (E,F) and hind limb (G,H) muscles stained with  $\alpha$ -bungarotoxin (red) to mark acetylcholine receptors and co-visualized with HB9-GFP (E-F, green), which is expressed specifically in motoneurons, or synaptophysin (G-H, green) which marks synaptic vesicles. E18.5 wild-type (I-K) and mutant (L-N) diaphragm muscles stained with  $\alpha$ -bungarotoxin (red) and co-visualized with HB9-GFP (green). *Lrp4* mutants show extensive branching of the phrenic nerve in diaphragms and lack AChR clustering in the synaptic endplate (N). In all types of muscle examined, *Lrp4* mutants lack normal AChR clustering in the synaptic endplate.

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

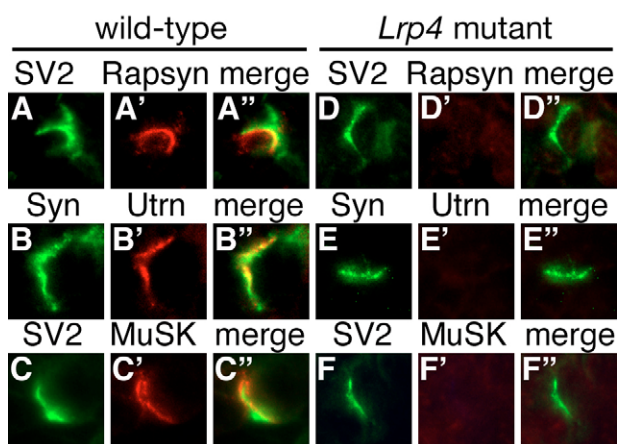


**Fig. 4. *Lrp4* is required for the initiation of AChR clustering.** E13.5 (A-F) and E14.5 (G-L) wild-type (A-C,G-I) and mutant (D-F,J-L) diaphragms, with neuronal projections visualized with neurofilament (A-F, green) or HB9-GFP (G-L, green) and AChRs marked by  $\alpha$ -bungarotoxin (red). Before innervation, pre-clustering of AChRs does not occur in *Lrp4* mutants (compare B to E). At E14.5, mutant phrenic nerves show longer primary branches that do not stop in the endplate region. AChR clusters are still undetectable in *Lrp4* mutants at this stage of NMJ initiation (K). (M-P) Wild type (M,O) and *Lrp4* mutant (N,P) cultured myotubes, either untreated (M,N) or following overnight treatment with recombinant Agrin (O,P). AChR clusters (visualized with  $\alpha$ -bungarotoxin in red) form in myotubes derived from wild type but not *Lrp4* mutants.

*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.

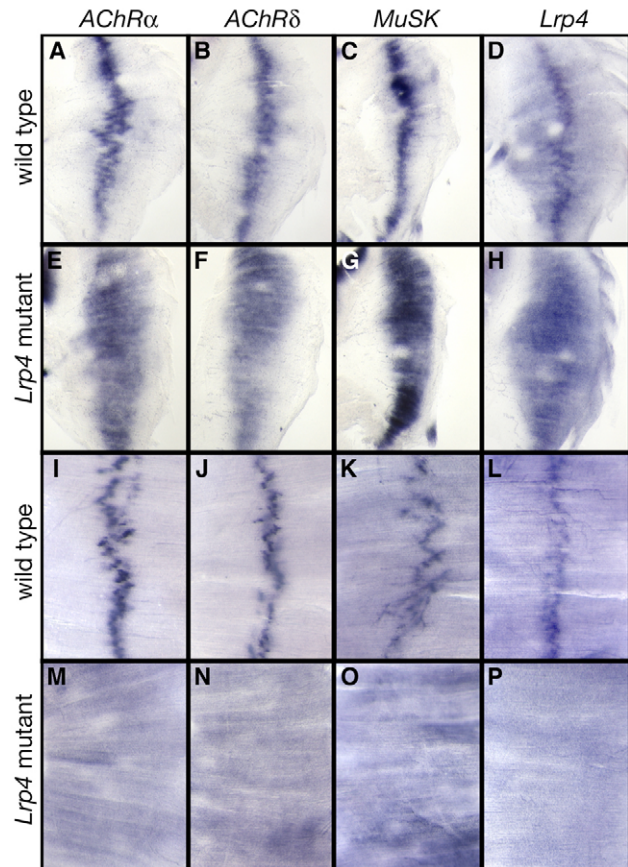
The phenotypes of the spontaneous and targeted alleles of *Lrp4* differ markedly from those of the ENU-induced alleles described here. The most obvious difference is the completely penetrant perinatal lethality of homozygous *Lrp4<sup>mte</sup>* and *Lrp4<sup>mitt</sup>* mutants, by contrast to the spontaneous, retrovirally derived (*Lrp4<sup>mdig</sup>* and *Lrp4<sup>dan</sup>*, 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 *Lrp4<sup>mdig</sup>* and *Lrp4<sup>dan</sup>* 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 *Lrp4<sup>mte</sup>* and *Lrp4<sup>mitt</sup>* 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 *Lrp4<sup>mte</sup>* and *Lrp4<sup>mitt</sup>* 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.



**Fig. 5. Rapsyn, utrophin and MuSK localization is disrupted in *Lrp4* mutants.** Immunofluorescence analysis of NMJs in control (A-C) and *Lrp4* mutant (D-F) embryos. Sections of E18.5 hind limb muscles were stained with antibodies against Rapsyn (A,D) and utrophin (Utrn; B,E). MuSK localization was visualized on sections of E18.5 intercostal muscles (C,F). Synaptic sites were identified by co-staining with antibodies against synaptic vesicle proteins, SV2 (A,D,C,F) or synaptophysin (B,E).

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 *Lrp4<sup>mte</sup>* and *Lrp4<sup>mitt</sup>* 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



**Fig. 6. Synaptic nuclei are not transcriptionally specialized in *Lrp4* mutant muscles.** E14.5 (A-H) or E18.5 (I-P) diaphragms showing *in situ* hybridizations using probes specific to *AChRα* (A,E,I,M), *AChRδ* (B,F,J,N), *Musk* (C,G,K,O) and *Lrp4* (D,H,L,P). At E14.5, transcripts of these genes become restricted to the endplate band region in wild-type muscles (A-D) but are more broadly expressed in *Lrp4* mutants (E-H). At E18.5, transcripts of these genes continue to be upregulated in the endplate band region in wild-type muscles (I-L) but are expressed at low, uniform levels in mutants (M-P).

antibodies against AChR subunits, while others test positive for antibodies that recognize MuSK or Rapsyn. 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 activity early in the formation of the synapse, and it is possible that Lrp4 helps bind that ligand. Alternatively, because the cytoplasmic domain of Lrp4, unlike Lrp5 and Lrp6, contains clustering motifs (NPxY, di-leucine YxxL), similar to those in Ldlr, Lrp1 and Lrp2 (He et al., 2004), Lrp4 might facilitate its own clustering or clustering of another protein such as MuSK. Before our work, the only proteins previously known to be required to initiate postsynaptic differentiation was MuSK and a recently described binding partner of MuSK, Dok7 (Okada et al., 2006). The discovery that Lrp4 is a crucial component for the localization of MuSK provides a new entry point to define the early events that lead to synaptic specialization. The findings that Lrp4 has been isolated from synaptic fractions of rat brain extracts and that Lrp4 interacts with postsynaptic scaffold proteins in neurons (Tian et al., 2006) suggests that Lrp4 may play a broad role in synapse formation.

We thank Natalie Kim and Steve Burden for Rapsyn antibodies, AChR subunit constructs, protocols, helpful suggestions and comments on the manuscript. We thank Thomas Jessell for the HB9-GFP transgenic mouse line. We thank Katia Manova and the MSK Core Cytology Facility for the radioactive in situ of Lrp4 and confocal image capture and analysis. We thank Agnes Viale and the MSK Core Genomics Facility for quantitative RT-PCR analysis. This work was supported by an NRSA fellowship (S.D.W.), NIH RO1 HD32427(L.A.N.) and NIH U01 HD43478 (K.V.A.). L.A.N. is an Investigator of the Howard Hughes Medical Institute.

#### Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/133/24/4993/DC1>

#### References

- Ackerman, K. G., Herron, B. J., Vargas, S. O., Huang, H., Tevosian, S. G., Kochilas, L., Rao, C., Poerber, B. R., Babiuk, R. P., Epstein, J. A. et al. (2005). Fog2 is required for normal diaphragm and lung development in mice and humans. *PLoS Genet.* **1**, 58-65.
- Bartoli, M., Ramarao, M. K. and Cohen, J. B. (2001). Interactions of the rapsyn RING-H2 domain with dystroglycan. *J. Biol. Chem.* **276**, 24911-24917.
- Blake, D. J., Tinsley, J. M. and Davies, K. E. (1994). The emerging family of dystrophin-related proteins. *Trends Cell Biol.* **4**, 19-23.
- Blake, D. J., Tinsley, J. M. and Davies, K. E. (1996). Utrophin: a structural and functional comparison to dystrophin. *Brain Pathol.* **6**, 37-47.
- Bowen, D. C., Park, J. S., Bodine, S., Stark, J. L., Valenzuela, D. M., Stitt, T. N., Yancopoulos, G. D., Lindsay, R. M., Glass, D. J. and DiStefano, P. S. (1998). Localization and regulation of MuSK at the neuromuscular junction. *Dev. Biol.* **199**, 309-319.
- Bu, G., Maksymovitch, E. A., Nerbonne, J. M. and Schwartz, A. L. (1994). Expression and function of the low density lipoprotein receptor-related protein (LRP) in mammalian central neurons. *J. Biol. Chem.* **269**, 18521-18528.
- Burden, S. J., DePalma, R. L. and Gottesman, G. S. (1983). Crosslinking of proteins in acetylcholine receptor-rich membranes: association between the beta-subunit and the 43 kd subsynaptic protein. *Cell* **35**, 687-692.
- Campanelli, J. T., Hoch, W., Rupp, F., Kreiner, T. and Scheller, R. H. (1991). Agrin mediates cell contact-induced acetylcholine receptor clustering. *Cell* **67**, 909-916.
- Carroll, T. J., Park, J. S., Hayashi, S., Majumdar, A. and McMahon, A. P. (2005). Wnt9b plays a central role in the regulation of mesenchymal to epithelial transitions underlying organogenesis of the mammalian urogenital system. *Dev. Cell* **9**, 283-292.
- D'Arcangelo, G., Homayouni, R., Keshvara, L., Rice, D. S., Sheldon, M. and Curran, T. (1999). Reelin is a ligand for lipoprotein receptors. *Neuron* **24**, 471-479.
- Day, I. N., Whittall, R. A., O'Dell, S. D., Haddad, L., Bolla, M. K., Gudnason, V. and Humphries, S. E. (1997). Spectrum of LDL receptor gene mutations in heterozygous familial hypercholesterolemia. *Hum. Mutat.* **10**, 116-127.
- DeChiara, T. M., Bowen, D. C., Valenzuela, D. M., Simmons, M. V., Poueymirou, W. T., Thomas, S., Kinetz, E., Compton, D. L., Rojas, E., Park, J. S. et al. (1996). The receptor tyrosine kinase MuSK is required for neuromuscular junction formation in vivo. *Cell* **85**, 501-512.
- Denzer, A. J., Brandenberger, R., Gesemann, M., Chiquet, M. and Ruegg, M. A. (1997). Agrin binds to the nerve-muscle basal lamina via laminin. *J. Cell Biol.* **137**, 671-683.
- Eggenschwiler, J. T. and Anderson, K. V. (2000). Dorsal and lateral fates in the mouse neural tube require the cell-autonomous activity of the open brain gene. *Dev. Biol.* **227**, 648-660.
- Engel, A. G., Ohno, K. and Sine, S. M. (2003). Sleuthing molecular targets for neurological diseases at the neuromuscular junction. *Nat. Rev. Neurosci.* **4**, 339-352.
- Ervasti, J. M. and Campbell, K. P. (1993). Dystrophin-associated glycoproteins: their possible roles in the pathogenesis of Duchenne muscular dystrophy. *Mol. Cell. Biol. Hum. Dis. Ser.* **3**, 139-166.
- Flanagan-Steet, H., Fox, M. A., Meyer, D. and Sanes, J. R. (2005). Neuromuscular synapses can form in vivo by incorporation of initially aneural postsynaptic specializations. *Development* **132**, 4471-4481.
- Garcia-Garcia, M. J., Eggenschwiler, J. T., Caspar, T., Alcorn, H. L., Wyler, M. R., Huangfu, D., Rakeman, A. S., Lee, J. D., Feinberg, E. H., Timmer, J. R. et al. (2005). Analysis of mouse embryonic patterning and morphogenesis by forward genetics. *Proc. Natl. Acad. Sci. USA* **102**, 5913-5919.
- Gautam, M., Noakes, P. G., Mudd, J., Nichol, M., Chu, G. C., Sanes, J. R. and Merlie, J. P. (1995). Failure of postsynaptic specialization to develop at neuromuscular junctions of rapsyn-deficient mice. *Nature* **377**, 232-236.
- Gautam, M., Noakes, P. G., Moscoso, L., Rupp, F., Scheller, R. H., Merlie, J. P. and Sanes, J. R. (1996). Defective neuromuscular synaptogenesis in agrin-deficient mutant mice. *Cell* **85**, 525-535.
- Gautam, M., DeChiara, T. M., Glass, D. J., Yancopoulos, G. D. and Sanes, J. R. (1999). Distinct phenotypes of mutant mice lacking agrin, MuSK, or rapsyn. *Brain Res. Dev. Brain Res.* **114**, 171-178.
- Glass, D. J., Bowen, D. C., Stitt, T. N., Radziejewski, C., Bruno, J., Ryan, T. E., Gies, D. R., Shah, S., Mattsson, K., Burden, S. J. et al. (1996). Agrin acts via a MuSK receptor complex. *Cell* **85**, 513-523.
- He, X., Semenov, M., Tamai, K. and Zeng, X. (2004). LDL receptor-related proteins 5 and 6 in Wnt/beta-catenin signaling: arrows point the way. *Development* **131**, 1663-1677.
- Hiesberger, T., Trommsdorff, M., Howell, B. W., Goffinet, A., Mumby, M. C., Cooper, J. A. and Herz, J. (1999). Direct binding of Reelin to VLDL receptor and ApoE receptor 2 induces tyrosine phosphorylation of disabled-1 and modulates tau phosphorylation. *Neuron* **24**, 481-489.
- Hogan, B. and Lacy, E. (1994). *Manipulating the Mouse Embryo: a Laboratory Manual*. Plainview, NY: Cold Spring Harbor Laboratory Press.
- Holmes, G. and Niswander, L. (2001). Expression of slit-2 and slit-3 during chick development. *Dev. Dyn.* **222**, 301-307.
- Hopf, C. and Hoch, W. (1998). Tyrosine phosphorylation of the muscle-specific kinase is exclusively induced by acetylcholine receptor-aggregating agrin fragments. *Eur. J. Biochem.* **253**, 382-389.
- Hughes, B. W., Kusner, L. L. and Kaminski, H. J. (2005). Molecular architecture of the neuromuscular junction. *Muscle Nerve* **33**, 445-461.
- Inanlou, M. R. and Kablar, B. (2003). Abnormal development of the diaphragm in mdx:MyoD<sup>-/-</sup>(9th) embryos leads to pulmonary hypoplasia. *Int. J. Dev. Biol.* **47**, 363-371.
- Inanlou, M. R. and Kablar, B. (2005a). Abnormal development of the intercostal muscles and the rib cage in Myf5<sup>-/-</sup> embryos leads to pulmonary hypoplasia. *Dev. Dyn.* **232**, 43-54.
- Inanlou, M. R. and Kablar, B. (2005b). Contractile activity of skeletal musculature involved in breathing is essential for normal lung cell differentiation, as revealed in Myf5<sup>-/-</sup>:MyoD<sup>-/-</sup> embryos. *Dev. Dyn.* **233**, 772-782.
- Inanlou, M. R., Baguma-Nibasheka, M. and Kablar, B. (2005). The role of fetal breathing-like movements in lung organogenesis. *Histol. Histopathol.* **20**, 1261-1266.
- Ishiguro, M., Imai, Y. and Kohsaka, S. (1995). Expression and distribution of low density lipoprotein receptor-related protein mRNA in the rat central nervous system. *Brain Res. Mol. Brain Res.* **33**, 37-46.
- Jennings, C. G., Dyer, S. M. and Burden, S. J. (1993). Muscle-specific trk-related

- receptor with a kringle domain defines a distinct class of receptor tyrosine kinases. *Proc. Natl. Acad. Sci. USA* **90**, 2895-2899.
- Jeon, H., Meng, W., Takagi, J., Eck, M. J., Springer, T. A. and Blacklow, S. C.** (2001). Implications for familial hypercholesterolemia from the structure of the LDL receptor YWTD-EGF domain pair. *Nat. Struct. Biol.* **8**, 499-504.
- Johnson, E. B., Hammer, R. E. and Herz, J.** (2005). Abnormal development of the apical ectodermal ridge and polysyndactyly in Megf7-deficient mice. *Hum. Mol. Genet.* **14**, 3523-3538.
- Kasarskis, A., Manova, K. and Anderson, K. V.** (1998). A phenotype-based screen for embryonic lethal mutations in the mouse. *Proc. Natl. Acad. Sci. USA* **95**, 7485-7490.
- Lin, W., Burgess, R. W., Dominguez, B., Pfaff, S. L., Sanes, J. R. and Lee, K. F.** (2001). Distinct roles of nerve and muscle in postsynaptic differentiation of the neuromuscular synapse. *Nature* **410**, 1057-1064.
- Lin, W., Dominguez, B., Yang, J., Aryal, P., Brandon, E. P., Gage, F. H. and Lee, K. F.** (2005). Neurotransmitter acetylcholine negatively regulates neuromuscular synapse formation by a Cdk5-dependent mechanism. *Neuron* **46**, 569-579.
- Liyanage, Y., Hoch, W., Beeson, D. and Vincent, A.** (2002). The agrin/muscle-specific kinase pathway: new targets for autoimmune and genetic disorders at the neuromuscular junction. *Muscle Nerve* **25**, 4-16.
- Majumdar, A., Vainio, S., Kispert, A., McMahon, J. and McMahon, A. P.** (2003). Wnt11 and Ret/Gdnf pathways cooperate in regulating ureteric branching during metanephric kidney development. *Development* **130**, 3175-3185.
- May, P. and Herz, J.** (2003). LDL receptor-related proteins in neurodevelopment. *Traffic* **4**, 291-301.
- May, P., Rohlmann, A., Bock, H. H., Zurhove, K., Marth, J. D., Schomburg, E. D., Noebels, J. L., Beffert, U., Sweatt, J. D., Weeber, E. J. et al.** (2004). Neuronal LRP1 functionally associates with postsynaptic proteins and is required for normal motor function in mice. *Mol. Cell. Biol.* **24**, 8872-8883.
- May, P., Herz, J. and Bock, H. H.** (2005). Molecular mechanisms of lipoprotein receptor signalling. *Cell Mol. Life Sci.* **62**, 2325-2338.
- Misgeld, T., Kummer, T. T., Lichtman, J. W. and Sanes, J. R.** (2005). Agrin promotes synaptic differentiation by counteracting an inhibitory effect of neurotransmitter. *Proc. Natl. Acad. Sci. USA* **102**, 11088-11093.
- Moestrup, S. K., Gliemann, J. and Pallesen, G.** (1992). Distribution of the alpha 2-macroglobulin receptor/low density lipoprotein receptor-related protein in human tissues. *Cell Tissue Res.* **269**, 375-382.
- Nakayama, M., Nakajima, D., Nagase, T., Nomura, N., Seki, N. and Ohara, O.** (1998). Identification of high-molecular-weight proteins with multiple EGF-like motifs by motif-trap screening. *Genomics* **51**, 27-34.
- Okada, K., Inoue, A., Okada, M., Murata, Y., Kakuta, S., Jigami, T., Kubo, S., Shiraishi, H., Eguchi, K., Motomura, M. et al.** (2006). The muscle protein Dok-7 is essential for neuromuscular synaptogenesis. *Science* **312**, 1802-1805.
- Panzer, J. A., Gibbs, S. M., Dosch, R., Wagner, D., Mullins, M. C., Granato, M. and Balice-Gordon, R. J.** (2005). Neuromuscular synaptogenesis in wild-type and mutant zebrafish. *Dev. Biol.* **285**, 340-357.
- Panzer, J. A., Song, Y. and Balice-Gordon, R. J.** (2006). In vivo imaging of preferential motor axon outgrowth to and synaptogenesis at prepatterned acetylcholine receptor clusters in embryonic zebrafish skeletal muscle. *J. Neurosci.* **26**, 934-947.
- Pinson, K. I., Brennan, J., Monkley, S., Avery, B. J. and Skarnes, W. C.** (2000). An LDL-receptor-related protein mediates Wnt signalling in mice. *Nature* **407**, 535-538.
- Pun, S., Sigrist, M., Santos, A. F., Ruegg, M. A., Sanes, J. R., Jessell, T. M., Arber, S. and Caroni, P.** (2002). An intrinsic distinction in neuromuscular junction assembly and maintenance in different skeletal muscles. *Neuron* **34**, 357-370.
- Ruegg, M. A. and Bixby, J. L.** (1998). Agrin orchestrates synaptic differentiation at the vertebrate neuromuscular junction. *Trends Neurosci.* **21**, 22-27.
- Rupp, F., Payan, D. G., Magill-Solc, C., Cowan, D. M. and Scheller, R. H.** (1991). Structure and expression of a rat agrin. *Neuron* **6**, 811-823.
- Sanes, J. R., Johnson, Y. R., Kotzbauer, P. T., Mudd, J., Hanley, T., Martinou, J. C. and Merlie, J. P.** (1991). Selective expression of an acetylcholine receptor-*lacZ* transgene in synaptic nuclei of adult muscle fibers. *Development* **113**, 1181-1191.
- Schneider, W. J. and Nimpf, J.** (2003). LDL receptor relatives at the crossroad of endocytosis and signaling. *Cell Mol. Life Sci.* **60**, 892-903.
- Simon-Chazottes, D., Tutois, S., Kuehn, M., Evans, M., Bourgade, F., Cook, S., Davison, M. T. and Guenet, J. L.** (2006). Mutations in the gene encoding the low-density lipoprotein receptor LRP4 cause abnormal limb development in the mouse. *Genomics* **87**, 673-677.
- Spoelgen, R., Hammes, A., Anzenberger, U., Zechner, D., Andersen, O. M., Jerchow, B. and Willnow, T. E.** (2005). LRP2/megalin is required for patterning of the ventral telencephalon. *Development* **132**, 405-414.
- Springer, T. A.** (1998). An extracellular beta-propeller module predicted in lipoprotein and scavenger receptors, tyrosine kinases, epidermal growth factor precursor, and extracellular matrix components. *J. Mol. Biol.* **283**, 837-862.
- Stark, K., Vainio, S., Vassileva, G. and McMahon, A. P.** (1994). Epithelial transformation of metanephric mesenchyme in the developing kidney regulated by Wnt-4. *Nature* **372**, 679-683.
- Tian, Q. B., Suzuki, T., Yamauchi, T., Sakagami, H., Yoshimura, Y., Miyazawa, S., Nakayama, K., Saitoh, F., Zhang, J. P., Lu, Y. et al.** (2006). Interaction of LDL receptor-related protein 4 (LRP4) with postsynaptic scaffold proteins via its C-terminal PDZ domain-binding motif, and its regulation by Ca/calmodulin-dependent protein kinase II. *Eur. J. Neurosci.* **23**, 2864-2876.
- Trinidad, J. C., Fischbach, G. D. and Cohen, J. B.** (2000). The Agrin/MuSK signalling pathway is spatially segregated from the neuregulin/ErbB receptor signalling pathway at the neuromuscular junction. *J. Neurosci.* **20**, 8762-8770.
- Trommsdorff, M., Gotthardt, M., Hiesberger, T., Shelton, J., Stockinger, W., Nimpf, J., Hammer, R. E., Richardson, J. A. and Herz, J.** (1999). Reeler/Disabled-like disruption of neuronal migration in knockout mice lacking the VLDL receptor and ApoE receptor 2. *Cell* **97**, 689-701.
- Valenzuela, D. M., Stitt, T. N., DiStefano, P. S., Rojas, E., Mattsson, K., Compton, D. L., Nunez, L., Park, J. S., Stark, J. L., Gies, D. R. et al.** (1995). Receptor tyrosine kinase specific for the skeletal muscle lineage: expression in embryonic muscle, at the neuromuscular junction, and after injury. *Neuron* **15**, 573-584.
- Ward, A. J., O'Kane, M., Young, I., Nicholls, D. P., Nevin, N. C. and Graham, C. A.** (1995). Three novel mutations in the EGF precursor homology domain of the low-density lipoprotein receptor gene in Northern Irish patients with familial hypercholesterolemia. *Hum. Mutat.* **6**, 254-256.
- Webb, J. C., Sun, X. M., McCarthy, S. N., Neuwirth, C., Thompson, G. R., Knight, B. L. and Soutar, A. K.** (1996). Characterization of mutations in the low density lipoprotein (LDL)-receptor gene in patients with homozygous familial hypercholesterolemia, and frequency of these mutations in FH patients in the United Kingdom. *J. Lipid Res.* **37**, 368-381.
- Willnow, T. E., Hilpert, J., Armstrong, S. A., Rohlmann, A., Hammer, R. E., Burns, D. K. and Herz, J.** (1996). Defective forebrain development in mice lacking gp330/megalin. *Proc. Natl. Acad. Sci. USA* **93**, 8460-8464.
- Yang, X., Li, W., Prescott, E. D., Burden, S. J. and Wang, J. C.** (2000). DNA topoisomerase IIbeta and neural development. *Science* **287**, 131-134.
- Yang, X., Arber, S., William, C., Li, L., Tanabe, Y., Jessell, T. M., Birchmeier, C. and Burden, S. J.** (2001). Patterning of muscle acetylcholine receptor gene expression in the absence of motor innervation. *Neuron* **30**, 399-410.
- Zhou, C. J., Pinson, K. I. and Pleasure, S. J.** (2004). Severe defects in dorsal thalamic development in low-density lipoprotein receptor-related protein-6 mutants. *J. Neurosci.* **24**, 7632-7639.