Diverging roles for Lrp4 and Wnt signaling in neuromuscular synapse development during evolution

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Motor axons approach muscles that are prepatterned in the prospective synaptic region. In mice, prepatterning of acetylcholine receptors requires Lrp4, a LDLR family member, and MuSK, a receptor tyrosine kinase. Lrp4 can bind and stimulate MuSK, strongly suggesting that association between Lrp4 and MuSK, independent of additional ligands, initiates prepatterning in mice. In zebrafish, Wnts, which bind the Frizzled (Fz)-like domain in MuSK, are required for prepatterning, suggesting that Wnts may contribute to prepatterning and neuromuscular development in mammals. We show that prepatterning in mice requires Lrp4 but not the MuSK Fz-like domain. In contrast, prepatterning in zebrafish requires the MuSK Fz-like domain but not Lrp4. Despite these differences, neuromuscular synapse formation in zebrafish and mice share similar mechanisms, requiring Lrp4, MuSK, and neuronal Agrin but not the MuSK Fz-like domain or Wnt production from muscle. Our findings demonstrate that evolutionary divergent mechanisms establish muscle prepatterning in zebrafish and mice.

[Keywords: MuSK; Wnt; Frizzled; Lrp4; neuromuscular; synapse]

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Consistent with these findings, forced, uniform expression of MuSK in mammalian muscle disrupts prepatterning and leads to exuberant motor axon growth and synapse formation throughout the muscle [Kim and Burden 2008]. Together, these findings indicate that muscle prepatterning biases motor axon growth and synapse formation toward the central, prepatterned region of muscle.

Once motor axons make contact with muscle, motor axons provide opposing signals that refine and sharpen the prepatterned arrangement of AChRs. Agrin binds Lrp4, which stimulates further association between Lrp4 and MuSK and increases MuSK phosphorylation [Kim and Burden 2008; Zhang et al. 2008, 2011], essential to induce and maintain AChR clustering at nascent synaptic sites (for review, see Kummer et al. 2006; Burden et al. 2013). In contrast, ACh, acting in an antagonist manner, depolarizes muscle and extinguishes AChR clusters that are not directly apposed to nerve terminals that supply Agrin locally [Kummer et al. 2006; Burden et al. 2013].

The extracellular region of MuSK contains three Ig-like domains, a Frizzled (Fz)-like domain, and a short, unstructured juxtamembrane region [Burden et al. 2013]. The first Ig-like domain is crucial for MuSK to associate with Lrp4 [Zhang et al. 2011]. Autoantibodies to this first Ig-like domain disrupt binding between Lrp4 and MuSK and cause autoimmune MuSK myasthenia gravis [Huijbers et al. 2013; Koneczny et al. 2013]. Because Fz receptors bind Wnts, the presence of a Fz-like domain in MuSK raised the possibility that Wnts may bind to the MuSK Fz-like domain and function as alternative ligands for MuSK [Koles and Budnik 2012]. Several findings are consistent with the idea that Wnts promote one or more steps in synapse formation. First, Wnt proteins can bind MuSK [Jing et al. 2009; Strochlic et al. 2012; Zhang et al. 2012] in a manner that depends on the Fz-like domain [Jing et al. 2009, Strochlic et al. 2012]. Second, Wnts can stimulate clustering of AChRs in cultured muscle cells in a MuSK-dependent manner [Henriquez et al. 2008; Zhang et al. 2012]. Third, prepatterning of AChRs in zebrafish is dependent on the MuSK Fz-like domain as well as two Wnts, Wnt11r and Wnt4a, which are expressed by muscle [Jing et al. 2009; Gordon et al. 2012]. Fourth, the development of neuromuscular synapses in Drosophila requires Wnt/Fz signaling and Neto [neuropilin and tolloid-like], an auxiliary subunit of glutamate receptors [Koles and Budnik 2012; Kim and Serpe 2013].

Although Wnts are required for AChR prepatterning in zebrafish [Jing et al. 2009], a role for Wnt signaling in prepatterning in mice has not been investigated. Because Lrp4 can bind and activate MuSK [Kim and Burden 2008], direct activation of MuSK by Lrp4 may be sufficient to stimulate MuSK phosphorylation and establish muscle prepatterning in mammals [Burden et al. 2013]. An alternative model, supported by the findings in zebrafish, suggests that additional ligands, such as Wnts, assist or cooperate with Lrp4 and MuSK to activate MuSK and stimulate prepatterning in mammals. Moreover, although Wnt signaling is not required for synapse formation in zebrafish, a potential role for Wnt signaling in neuromuscular synapse formation in mammals has not been studied.

Here, we show that muscle prepatterning in zebrafish and mammals is established by different mechanisms. First, Lrp4 is required for prepatterning in mice but is dispensable in zebrafish. Second, Wnt signaling through the MuSK Fz-like domain is essential for prepatterning AChRs in zebrafish but is expendable in mice. The mechanisms for synapse formation, however, appear similar, as synapse formation in zebrafish and mice requires Lrp4 and MuSK, whereas the MuSK Fz-like domain and Wnt production from muscle are dispensable.

**Results**

**Generation of a mouse MuSK mutant lacking the MuSK Fz-like domain**

The MuSK Fz-like domain extends from Ser307 to Asp454 and is encoded by exons 9–12 [Fig. 1A,B]. Exons 9–12 encode for two of the five amino acids between the third Ig-like domain and the Fz-like domain, the entire Fz-like domain, and seven of the 38 amino acids from the unstructured extracellular, juxtamembrane region [Fig. 1A,B]. We used CRISPR/Cas9 to introduce double-strand breaks in introns 8 and 12, deleting 16.2 kb of DNA encoding the Fz-like domain and allowing splicing of RNA encoded by exons 8 and 13, which restores the reading frame [Fig. 1B].

To generate double-strand breaks in introns 8 and 12, we selected sgRNAs that were predicted to have a low probability to hybridize to off-target sites and tested and confirmed this expectation experimentally [see Supplemental Fig. S1A]. We used PCR and DNA sequencing to analyze 25 mice born from zygotes injected with the sgRNAs and Cas9 mRNA [Fig. 1C]. We found that 15 mice were heterozygous and that five mice were homozygous for the desired mutation in MuSK [Supplemental Fig. S1B]. All mutations deleted exons 9–12 and led to nonhomologous end-joining of breaks in introns 8 and 12 and in-frame splicing of mRNA encoded by exons 8 and 13 [Supplemental Fig. S1C]. Founder mice were mosaic for the MuSK mutation and harbored deletions of varying size at the double-strand break sites. As such, we backcrossed founder mice to wild-type mice to obtain lines that contained the expected sequence and only 2-, 11-, or 18-base-pair [bp] deletions at the double-strand break sites in introns 8 and 12 [Supplemental Fig. S1C].

Prepatterning of AChRs is very sensitive to MuSK expression levels and is nearly abolished in mice that are heterozygous for MuSK [Yang et al. 2001]. Moreover, MuSK overexpression can lead to severe defects in motor axon growth, synapse formation, and motor performance [Kim and Burden 2008]. As such, it was important to determine whether the MuSK ΔFz protein was expressed at normal levels. We generated primary muscle cell cultures from control and MuSKΔFz/ΔFz mice, immunoprecipitated MuSK and MuSK AΔFz proteins with antibodies to the first Ig-like domain in MusK, and detected MuSK and MuSK ΔFz proteins by probing Western blots with antibodies to the N terminus of MuSK. MuSK ΔFz protein expressed in either MuSKΔFz/ΔFz or MuSKΔFz/ΔFz myotubes migrated...
in SDS-PAGE at the predicted, truncated size (Fig. 1D; Supplemental Fig. S1D). We quantified expression of the MuSK ΔFz protein by comparing expression levels of the wild-type and MuSK ΔFz protein in MuSK<sup>ΔFz/+</sup> primary myotubes. This assay provided a reliable and accurate comparison of protein expression, as we measured wild-type and MuSK ΔFz protein expression within individual myotube cultures and avoided the inevitable variability caused by immunoprecipitating and comparing MuSK expression in separate cultures of wild-type and MuSK<sup>ΔFz/ΔFz</sup> myotubes. Importantly, we found that MuSK ΔFz and wild-type MuSK proteins are expressed at the same level (Fig. 1D), ensuring that potential defects in patterning or synapse formation in MuSK<sup>ΔFz/ΔFz</sup> mice would be caused by a loss of the Fz-like domain rather than an alteration in MuSK expression.

We measured the responsiveness of MuSK ΔFz to neural Agrin by measuring the number and size of AChR clusters that formed in primary myotubes following Agrin treatment. We found that the number and size of Agrin-induced AChR clusters were similar in myotubes expressing MuSK ΔFz or wild-type MuSK (Fig. 1E). These findings indicate that deletion of the Fz-like domain did not impair the ability of MuSK to associate with Lrp4 and initiate downstream signaling pathways that organize and anchor AChRs in response to Agrin.

The MuSK Fz-like domain is not essential for muscle prepatternning

In mammals, motor axons arrive and make contact with muscle shortly after muscle prepatternning is established. Motor axons then provide multiple signals that refine and sharpen the muscle prepattern [Flanagan-Steet et al. 2005; Kummer et al. 2006]. For these reasons, it is difficult to define and unravel requirements for prepatternning AChRs separately from mechanisms for clustering AChRs during synapse formation by studying wild-type mice with intact innervation. Therefore, to study the role of the MuSK Fz-like domain in muscle prepatternning, we generated mice that were homozygous mutant for MuSK ΔFz and also lacked motor neurons. As in the past, we generated “motor neuron-less” mice by expressing diphtheria toxin A (DTA) selectively in motor neurons, which ablates motor neurons before they extend motor axons into the periphery [Yang et al. 2001]. We dissected muscle from embryonic day 17.5–18.5 (E17.5–E18.5) mice, 1–2 d before birth, and stained diaphragm muscles with probes that labeled axons and AChRs. Figure 2 shows that AChRs are clustered and enriched in the central region of muscle in wild-type and MuSK<sup>ΔFz/ΔFz</sup> mice that lack motor axons, demonstrating that the MuSK ΔFz-like domain is not essential for muscle prepatternning. The distribution of prepatternned AChR clusters, however, is modestly broader in mice lacking the MuSK Fz-like domain: In wild-type mice, 50% of AChR clusters are contained in a 567-µm-wide zone in the center of the muscle, whereas the width of this zone is modestly expanded to 750 µm in MuSK<sup>ΔFz/ΔFz</sup> mice (Fig. 2B). These findings indicate that the MuSK Fz-like domain is not required for muscle prepatternning but contributes to shaping the prepatternned zone within the central region of the muscle [see the Discussion].

Figure 1. Generation of mice lacking the MuSK Fz-like domain. (A) The extracellular region of mammalian MuSK contains three Ig-like domains, a Fz-like domain encoded by exons 9–12, and a juxtamembrane (JM) region. Following the transmembrane (TM) domain, the intracellular region of MuSK contains a juxtamembrane region and a kinase (K) domain. (B) The schematic shows the strategy for deleting exons (E) 9–12, which encode the Fz-like domain (S307 to D454). The positions of the sgRNAs used to define the double-strand break sites (X) are indicated. The arrows show the positions of the primers (1–3) used for genotyping. Splicing of RNA encoded by exons 8 and 13 produces a protein that joins A304 to F463 and lacks the Fz-like domain. An A residue between A304 and F463 replaces two residues (EW) between the third Ig-like domain and the Fz-like domain as well as eight residues [YKKENITIT] from the juxtamembrane region, which are encoded by exons 9 and 12. (C) Wild-type MuSK and MuSK<sup>ΔFz</sup> mutant alleles were detected by PCR. (D) Western blots from primary cultures of MuSK<sup>ΔFz/+</sup> myotubes show that MuSK ΔFz migrates at the predicted, truncated size and is expressed at the same level as wild-type MuSK (n = 2; each measurement is shown) [see also Supplemental Fig. S1]. (E) Agrin stimulates AChR clustering in a similar manner in wild-type and MuSK<sup>ΔFz/ΔFz</sup> primary myotubes (wild-type, n = 4; MuSK<sup>ΔFz/ΔFz</sup>, n = 5).
Synaptic function is normal in mice lacking the MuSK Fz-like domain

To determine whether synaptic transmission was normal in mice lacking the MuSK Fz-like domain, we recorded from individual muscle fibers with intracellular micro-electrodes to measure the amplitude and frequency of spontaneous miniature end-plate potentials (mepps). In addition, we used extracellular electrodes to measure nerve-elicted compound muscle action potentials (CMAPs) in muscle. We found that the muscle resting potential and the amplitude and frequency of mepps were indistinguishable between wild-type and MuSKΔFz/ΔFz mice [Fig. 4A]. Likewise, CMAPs, following stimulation at 10–50 Hz, were normal in MuSKΔFz/ΔFz mice [Fig. 4B; Supplemental Fig. S2]. Thus, the MuSK Fz-like domain is not required for the cardinal features of synaptic transmission.

To determine whether motor function was perturbed in mice lacking the MuSK Fz-like domain, we measured motor performance using multiple motor function tests. We found that the performance of MuSKΔFz/ΔFz mice on RotaRod, wire hang, all limb, and forelimb grip strength assays were normal, consistent with the idea that synaptic transmission and muscle function do not require signaling through the MuSK Fz-like domain [Fig. 4C].

Wnt secretion from muscle is dispensable for synaptic formation in mice

Binding of Wnts to MuSK depends on the MuSK Fz-like domain [Jing et al. 2009; Strochlic et al. 2012]. Because the MuSK Fz-like domain is expendable for synaptic differentiation [Fig. 3], our findings demonstrate that Wnt signaling through MuSK is not required for synaptic formation. We wondered, however, whether Wnts were also dispensable or might act through bona fide Fz receptors to regulate synaptic formation in mice. Because 19 Wnt genes are expressed in mammals, we studied mice that that were deficient in Wnt secretion by generating mice that were conditionally mutant for Wntless (Wls), which is required for secretion of lipid-modified Wnt proteins [Port and Basler 2010]. We studied mice that were deficient in Wnt secretion from muscle tissue by generating Pax3cre; WlsloxP/loxP mice to inhibit Wnt secretion from skeletal muscle cells and Scx:cre; WlsloxP/loxP mice to inhibit Wnt secretion from tendon precursors and muscle interstitial cells. Pax3cre; WlsloxP/loxP mice were runted and displayed several overt structural abnormalities, including malformations of the head, the absence of a tail, and incomplete closure of the neural tube [Fig. 5A], consistent with described roles for Wnt-3a, Wnt-5a, and Dvl1/2 during mouse development [Takada et al. 1994; Gofflot et al. 1998; Yamaguchi et al. 1999; Hamblet et al. 2002]. However, the pattern of AChR expression, axon branching, and nerve terminals was normal [Fig. 5B]. Moreover, each AChR cluster was apposed by a nerve terminal, indicating that ACh-mediated disassembly of noncontacted AChR clusters occurred normally [Fig. 5C]. Similarly, synaptic formation was normal in Scx:cre; WlsloxP/loxP mice.

Synapse formation is normal in mice lacking the MuSK Fz-like domain

Neuromuscular function becomes essential for survival at birth, when mice first need to breathe on their own. To determine whether neuromuscular synapses failed to form or function, we intercrossed MuSKΔFz/+ mice and measured lethality. We found that homozygous MuSKΔFz/ΔFz mice were born at the expected Mendelian frequency [Supplemental Fig. S1E], suggesting that neuromuscular function was not severely perturbed. To study the structure of neuromuscular synapses, we intercrossed MuSKΔFz/+ mice and stained muscle from postnatal day 0 (P0), P21, and P60 mice with probes that label axons, nerve terminals, and AChRs. Plaque-shaped synapses, indistinguishable from those in wild-type mice, were present in MuSKΔFz/ΔFz mice at birth and matured to form complex, pretzel-shaped synapses over the next month. The structure of the synapse in MuSKΔFz/ΔFz mice continued to evolve over the following month in a manner indistinguishable from wild-type mice and was maintained in adults. We measured the width of the endplate zone at birth and the synaptic size and the density of synaptic AChRs at each age. We found that MuSKΔFz/ΔFz mice were normal in each aspect [Fig. 3]. Thus, the formation and maturation of neuromuscular synapses do not depend on signaling through the MuSK Fz-like domain.
and tendon cells, namely, skeletal muscle cells, muscle interstitial cells, muscles from P0 wild-type, and nerve terminals, AChRs, and synapses are similar in diaphragm muscles from P0 wild-type, Lrp4 mutant mice (Fig. 5C). Although we cannot exclude the possibility that a low level of Wnt secretion from muscle occurs in the absence of Wls, these findings indicate that Wnt secretion from the major cell types in skeletal muscle tissue is not required for neuromuscular synapse formation in mice.

Zebrafish Lrp4 is required for synapse formation but not prepatternning

Previous studies reported that Lrp4 is necessary for prepatternning in mice [Weatherbee et al. 2006]. In zebrafish, Wnt signaling through the MuSK Fz-like domain is required for prepatternning AChRs. We therefore wondered whether muscle prepatternning in zebrafish is controlled exclusively by a Wnt-dependent mechanism or whether Lrp4 also has a role in prepatternning in zebrafish. We used TALENS to generate two zebrafish lrp4 mutant alleles with premature stop codons following the LDLa repeats, thus deleting most of the MuSK- and Agrin-binding domains (Fig. 6A,B). Importantly, these zebrafish lrp4 alleles closely resemble the mouse Lrp4 mutant allele lacking prepatternning and synapse formation [Weatherbee et al. 2006].

In zebrafish embryos, prepatternning AChR clusters form in the central region of adaxial muscle cells prior to the arrival of motor axons, ∼16 h post-fertilization [hpf] [Flanagan-Steeet et al. 2005; Panzer et al. 2006]. As motor neuron growth cones traverse the muscle territory, between 17 and 29 hpf, some of the aneural AChR clusters become incorporated into stable en passant neuromuscular synapses [Flanagan-Steeet et al. 2005].

We stained lrp4 mutant zebrafish embryos at 19.5 hpf with α-bungarotoxin (α-BGT) and an antibody (znp-1) to Synaptotagmin2 and found that AChR clusters were present in lrp4 mutant and wild-type sibling embryos (Fig. 6C,C′). We measured the width of the band of prepatternning AChR clusters relative to the width of each hemisegment and found no difference between wild-type and lrp4 mutant embryos (wild-type, n = 17 hemisegments, 20.17% ± 1.24%; lrp4 mutant, n = 30 hemisegments, 21.73% ± 1.16%; mean ± SEM, P = 0.4, unpaired Student’s t-test). These findings indicate that zebrafish use a Wnt–MuSK signaling mechanism, exclusive of Lrp4, for muscle prepatternning. However, Lrp4 is required for neuromuscular synapse formation in zebrafish, as en passant synapses between motor axons and axial muscles fail to form in the absence of Lrp4 (Fig. 6D,D′), demonstrating that Lrp4 has a conserved and essential role in synapse formation in lower vertebrates.
and higher vertebrates. A few abnormally shaped AChR clusters remain on the surface of muscle pioneer cells in lrp4 mutants (Fig. 6D′, white arrowheads), although the significance of these clusters is unknown.

In mammals, Lrp4 is expressed as myoblasts begin to fuse to form multinucleated fibers and prior to innervation (Kim et al. 2008). We therefore wondered whether lrp4 is expressed in early muscle development and during the prepatterning stage in zebrafish embryos. Using RNA-scope, which is optimized to detect low-level transcripts, we found that lrp4 mRNA is not detectable at the prepatterning stage (Fig. 6E) but is readily detectable when neuromuscular synapses are forming (Fig. 6E′). These findings are consistent with our data showing that Lrp4 is not required for AChR prepatterning in zebrafish and indicate that the timing of lrp4 expression in muscle differs in zebrafish and mice.

**Lrp4 is required for AChR prepatterning in mice**

The differing roles for Lrp4 in prepatterning in zebrafish and mice led us to revisit the reported role for Lrp4 in muscle prepatterning in mice. Although AChR clustering is absent in Lrp4 mutant mice at E14.5 (Weatherbee et al. 2006), motor axons are already present within the muscle at this stage. Because neuronal ACh disassembles AChR clusters that are not sustained by Agrin/MuSK signaling (Flanagan-Steet et al. 2005; Kummer et al. 2006), we wondered whether an acceleration of ACh-mediated disassembly rather than a failure to initially prepattern AChRs might be responsible for an absence of AChR clusters in Lrp4 mutant mice at this stage. To study the role of Lrp4 in nerve-independent prepatterning, we generated mice that lack Lrp4 as well as...
motor neurons. Figure 7 shows that AChRs fail to cluster in muscle from mice that lack Lrp4 and motor innervation. These findings confirm and extend the earlier findings and demonstrate that Lrp4 has a critical role in prepatterning AChRs in mice but not in zebrafish (Weatherbee et al. 2006).

Discussion

The extracellular region of mammalian MuSK possesses three Ig-like domains and a Fz-like domain. One face of the first Ig-like domain is hydrophobic and has a critical role in MuSK dimerization, whereas the opposing, solvent-exposed surface is critical for association of MuSK with Lrp4 (Stiegler et al. 2006). As such, the first Ig-like domain plays an essential role in stimulation of MuSK by Agrin, which is crucial for synapse formation. The presence of a Fz-like domain in MuSK led to speculation that Wnts may bind to MuSK and regulate MuSK activity at one or more stages during synapse formation (Masaiakowski and Yancopoulos 1998; Xu and Nusse 1998; Koles and Budnik 2012; Barik et al. 2014). Consistent with this idea, the MuSK Fz-like domain as well as Wnt4 and Wnt11r play important roles in muscle prepatterning in zebrafish (Jing et al. 2009). Here, however, we show that the MuSK Fz-like domain is dispensable for muscle prepatterning and synapse formation in mice. Moreover, we show that Lrp4 is required for prepatterning in mice but not in zebrafish. These findings indicate that prepatterning is established by different mechanisms in mice and zebrafish. In mice, Lrp4, which binds to MuSK and stimulates MuSK phosphorylation (Kim et al. 2008), is essential for muscle prepatterning. In contrast, prepatterning in zebrafish depends on Wnt signaling through the MuSK Fz-like domain and does not require Lrp4. Thus, different cis-acting ligands activate MuSK during prepatterning in zebrafish and mice: Muscle-derived Wnts function as MuSK ligands in zebrafish, while Lrp4 serves as the cis-acting ligand for MuSK in mice. The requirements for synapse formation,
however, are similar in zebrafish and mice, as Lrp4 and MuSK, but not the MuSK Fz-like domain, play essential roles in synapse formation.

We found that synapse formation and motor performance are normal in mice lacking the MuSK Fz-like domain, similar to findings in zebrafish [Jing et al. 2009]. A recent study reported that prepatterned AChRs were almost undetectable and that synapse formation and motor performance were severely impaired in mice that are mutant for the MuSK Fz-like domain [Messéant et al. 2015]. However, Messéant et al. [2015] did not examine the patterning of AChR expression prior to or independent of innervation. Even at the earliest stage examined by Messéant et al. [2015], motor axons had innervated the diaphragm muscle, precluding investigation of the role of the MuSK Fz-like domain in muscle prepatternning. It is unclear what accounts for the strikingly differing findings on synapse formation and motor performance reported here and in the study from Messéant et al. [2015]. We note that the MuSK ΔFz mutant described in Messéant et al. [2015] lacked amino acids 305–453 and was substantially overexpressed, as the 85-kDa mutant protein was shown to be much more abundant than the ~110-kDa wild-type protein. In contrast, the MuSK Δ305–461 protein described here is expressed at normal levels. We speculate that overexpression of the MuSK Δ305–453 protein in Messéant et al. [2015] may be due to an abnormal stability of the mutant protein or mRNA; alternatively, splicing of RNA encoded by exons 8 and 12 may be unusually efficient, resulting in higher levels of mRNA expression. Increases in MuSK expression cause severe defects in AChR clustering, motor axon growth, synapse formation, and motor performance [Kim and Burden 2008], similar to the defects reported by Messéant et al. [2015]. Thus, MuSK overexpression rather than the loss of the Fz-like domain may be responsible for the defects in synapse formation and motor function described in the previous study. Future studies—in particular to determine whether MuSK Δ305–453 heterozygotes display a phenotype indicating a gain-of-function mutation rather than a loss-of-function mutation—will be important for resolving these issues.

We found that AChR clusters are prepatternned in a modestly broader zone in mice lacking the MuSK Fz-like domain. These results indicate that signaling through the MuSK Fz-like domain is not necessary but participates to restrict AChR expression to the central region of mammalian muscle. These findings raise the possibility that a remnant of the older, Wnt-dependent mechanism, critical for prepatternning AChRs in fish, may be retained in mammals but has become subservient to Lrp4.

Injection of cells that express Sfrp, which binds and sequesters Wnts and inhibits Wnt-dependent signaling, in chick embryos leads to a twofold reduction in the number of AChR clusters in muscle [Henriquez et al. 2008]. Because Sfrp-expressing cells were injected at Hamburger-Hamilton stage 24/25, before synapses form in chick embryos, and analyzed at Hamburger-Hamilton 27/28, when synapses first begin to form [Landmesser and Morris 1975], Wnts may have a role in regulating AChR expression and prepatternning during muscle development in chicks as well as zebrafish.

Lrp4 functions bidirectionally at mammalian neuromuscular synapses. Lrp4 binds neuronal Agrin to stimulate postsynaptic differentiation and signals in turn in a retrograde manner to motor axons to stimulate presynaptic differentiation [Weatherbee et al. 2006; Kim et al. 2008; Zhang et al. 2008; Yamamoto et al. 2012]. In the absence of Lrp4, like in the absence of MuSK, mammalian motor axons fail to stop and differentiate and instead wander aimlessly across muscle [DeChiara et al. 1996; Weatherbee et al. 2006]. In zebrafish, Lrp4 may not have the same critical role for stimulating presynaptic differentiation, as motor axons display only a modest axon branching in the absence of Lrp4. Thus, Lrp4 appears to have acquired two new functions during vertebrate evolution: an effective activator of MuSK to stimulate muscle prepatternning and an essential retrograde signal to stimulate presynaptic differentiation. Because MuSK is required for accurate pathfinding and neuromuscular synapse formation in zebrafish, activation of MuSK by Wnts may lead to the production or presentation of a novel retrograde signal for differentiation of pioneering motor axons in zebrafish.

Motor axons within the main intramuscular nerve defasciculate and branch excessively in Wnt11 mutant mice but not in Wls conditionally mutant mice that are deficient in Wnt secretion from muscle. These findings indicate that the axon defasciculation and branching defects in Wnt11 mutant mice are not due to a loss of Wnt11 produced by muscle cells. Because Schwann cells express Wnt11 [Sienknecht and Fekete 2008] and because similar axon defasciculation and branching defects are evident in mutant mice that are deficient in the migration of Schwann cell precursors to the periphery [Morris et al. 1999; Woldeyesus et al. 1999; Yang et al. 2001], Wnt11 may be required for Schwann cell survival, migration, or signaling to motor axons.

What mechanisms underlie the change in the role for Lrp4 in muscle prepatternning during vertebrate evolution? The simplest explanation appears to be a shift in the timing of lrp4 expression, since lrp4 expression is not evident in zebrafish muscle when prepatternning is established, whereas lrp4 is expressed early in muscle development in mice. In addition, the association between Lrp4 and MuSK independently of Agrin may have increased during evolution, allowing Lrp4 to function as an effective ligand for MuSK. In this regard, although the extracellular region of Lrp4 is well conserved (80% identity) between mice and zebrafish, the extracellular region of MuSK is less well conserved (43% identity). Mammalian MuSK contains three Ig-like domains and a Fz-like domain [Burdens et al. 2013], whereas MuSK in all other vertebrate classes, including fish, contains an additional, kringle domain [Jennings et al. 1993; Valenzuela et al. 1995; Fu et al. 1999; Ip et al. 2000]. The addition of a kringle domain in zebrafish MuSK accounts for substantial sequence dissimilarity between the extracellular regions of zebrafish and mouse MuSK, as the sequences of the Ig-like and Fz-like domains are 55% identical. The function of the kringle domain is
not known, but it may impede binding between Lrp4 and MuSK, rendering prepatterning in nonmammalian vertebrates less sensitive to Lrp4 and necessitating a distinct, Wnt-dependent mechanism for stimulating MuSK prior to innervation. Because Lrp4 is required for synapse formation in zebrafish and mammals, potential interference by the kringle domain would presumably be overcome by Agrin binding to Lrp4.

The Fz-like domain is an ancient motif [Yan et al. 2014] that is present, together with a kringle domain, in a pair of mammalian receptor tyrosine kinases, Ror1/2, which have homologs in Drosophila and Caenorhabditis elegans. Although the function of the Drosophila homologs Dror [Wilson et al. 1993] and Dnkr [Oishi et al. 1997] are not known, CAM-1 and, in particular, the Fz-like domain in CAM-1 have an important role in neural development in C. elegans [Forrester et al. 1999; Kim and Forrester 2003; Green et al. 2007; Hayashi et al. 2009]. Thus, it is interesting to speculate that MuSK evolved from these ancestral invertebrate kinases but diverged so that the Ig-like domain and kinase activity rather than the Fz-like domain became central to MuSK function.

Materials and methods

Animals and histology

Mice were housed and maintained according to Institutional Animal Care and Use Committee [IACUC] guidelines. Hb9cre, Pax3cre, Sex-cre, Is12loxP-stop-loxP-DTA, Lrp4 mutant, MuSK mutant, Wnt4 mutant, Wnt11 mutant, and Wls conditionally mutant mice have been described previously [Stark et al. 1994; DeChiara et al. 1996; Yang et al. 2001; Majumdar et al. 2003; Lang et al. 2005; Weatherbee et al. 2006; Blitz et al. 2009]. In order to delete exons 9–12 in MuSK, we selected sgRNAs that were predicted to hybridize selectively to sequences in introns 8 and 12 of MuSK [http://crispr.mit.edu]. We tested three pairs of sgRNAs by transfecting mouse embryonic stem cells with DNA encoding the sgRNAs together with Cas9 and found that each sgRNA pair was effective in directing deletion of the desired sequence (Supplemental Fig. S1A). We found no evidence for mutations in these genes [Supplemental Fig. S1A].

Zebrafish strains and animal care

Zebrafish lprd184 and lprd185 alleles were generated in the TLF background, and all wild-type fish used were TLF strain. Homozygous mutants obtained by crossing heterozygous carriers were identified at 36 hpf based on swimming motility defects in response to light touch. Both lprd184 and lprd185 alleles showed identical prepatterning and neuromuscular phenotypes; only the results from the lprd184 allele are shown. All fish were raised and maintained as described previously [Mullins et al. 1994]. We performed all experiments involving fish according to animal protocols that were approved by the University of Pennsylvania IACUC.

Generation of TALEN mutant alleles

TALE nuclease plasmids were designed and engineered by the University of Utah Mutation Generation and Detection Core Facility and subcloned into pCS2TAL3-DDD and pCS2TAL3-RRR. The left TALEN was designed to target the sequence 5’-TCCCTCAGATGCGCCCGAT-3’. The right TALEN was designed to target the sequence 5’-ATGGCCCGTCTGATTGGACA-3’. Lrp4 TALEN mRNA was transcribed using the SP6 mMessage mMACHINE Kit [Ambion] and was diluted to 50 pg in 0.1 M KCl and microinjected into one-cell stage TLF embryos. Successful dsDNA breaks in lpr4 of G0 injected embryos were confirmed by PCR and high-resolution melt analysis. Homozygous F1 carrier alleles were identified and characterized by PCR followed by high-resolution melt analysis and sequencing [Dahlem et al. 2012].

Histology

Whole mounts of diaphragm muscles from P0, P21, and P60 mice were stained with Alexa 594-conjugated α-BGT [Invitrogen] to label AChRs and with antibodies to Neurofilament-L [Syntaptophysin] and Synapsin 1/2 [Syntaptophysin] to label motor axons and nerve terminals, respectively [Kim and Burden 2008]. Images were acquired with a Zeiss LSM 700 or 800 confocal microscope, and the number and size of AChR clusters as well as the density of synaptic AChRs were determined using Velocity 3D imaging software [Perkin Elmer], as described previously [Jaworski and Burden 2006; Fries et al. 2007]. The Wilcoxon-Mann-Whitney test was used to determine statistical significance and was conducted using GraphPad Prism 6.0 software.

Low-magnification images were captured on a Zeiss Axio Zoom.V16 fluorescence stereomicroscope. The distributions of prepatterned AChRs and synapses were determined by measuring the pixel value in 100-µm strips of the diaphragm muscle using Fiji software, as described previously [Kim and Burden 2008]. We used the Kruskal-Wallis test with Bonferroni correction to determine whether differences in median values were statistically significant (P < 0.05).

Zebrafish embryos at 19–26 hpf were anesthetized in 0.01% Tricaine, fixed in 4% paraformaldehyde with 1% DMSO diluted in phosphate-buffered saline [1× PBS at pH 7.4] for 3 h at room temperature, and washed with PBS. Embryos were permeabilized using 0.1% collagenase for 3–7 min at room temperature and then washed thoroughly with PBS. To label AChR clusters, embryos were incubated for 3 h at 4°C in 10 ng/mL Alexa fluor 594-conjugated α-BGT [Molecular Probes] diluted in incubation buffer (0.2% BSA, 0.5% Triton X-100 in 1× PBS) with 1% normal goat

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serum added. Motor axons were labeled with the znp-1 antibody (1:200) overnight at 4°C followed by incubation with goat anti-mouse secondary Alexa fluor 488-conjugated antibody (1:500). Stained embryos were immersed overnight and subsequently mounted in VectaShield mounting medium [Vector Laboratories]. Embryos were imaged in 1-µm sections using a 60× immersion objective on a Zeiss LSM 710 confocal microscope. Image stacks were compressed into maximum intensity projections in Fiji and converted to 16-bit images using MetaMorph [Molecular Devices]. AChR clusters were counted using the “count nuclei” function with minimum/maximum length set at 4/30 and minimum average intensity set at 15. The results were recorded in Graphpad Prism for statistical analysis.

In situ hybridization was performed using the RNAscope kit (Advanced Cell Diagnostics) in whole zebrafish embryos expressing Tg(smyhc1:mCherry-CAAX) as described (Gross-Thebing et al. 2014). Probes against Itp4 were designed and engineered by Advanced Cell Diagnostics. Embryos were imaged in 1-µm sections on a 60× immersion objective on an Olympus spinning disk confocal microscope. Image stacks were compressed into maximum intensity projections and processed using Adobe Photoshop to adjust brightness and contrast.

Cell culture
Skin and bone were removed from limbs of E17.5–E18.5 embryos, and the remaining tissue was dissociated and plated on collagen/laminin-coated tissue culture dishes (750 µg of collagen, 3.3 µg of laminin per milliliter), as described previously (Smith et al. 2001). After several days, the growth medium [80% DMEM/F12, 20% fetal bovine serum [Gibco], 1:500 primocin [InvivoGen], 5 pg/mL bFGF [Invitrogen]] was exchanged for differentiation medium [95% DMEM/F12, 5% heat-inactivated horse serum [Gibco], 1:500 primocin].

Several days after myotubes had formed, the cultures were treated for 8 h with 0.5 nM recombinant neural Agrin-B8 [R&D Systems] and stained with Alexa 594 a-BGT to label AChRs and with antibodies to myosin heavy chain [MyHC] [Sigma] to measure myotube surface area. Images were collected using a Zeiss LSM 800 confocal microscope, and the number and size of AChR clusters, normalized to MyHC-stained myotube surface area, were determined using Velocity 3D imaging software, as described previously (Friese et al. 2007).

Alternatively, myotube lysates were prepared as described previously (Friese et al. 2007), and MuSK was immunoprecipitated from cleared lysates with antibodies to the first Ig-like domain of MuSK (Huijbers et al. 2013). The antibody/MuSK complex was captured with protein A-agarose beads (Roche). Following several washes, bound proteins were eluted from the beads with SDS sample buffer, heated for 5 min to 95°C, resolved by SDS-PAGE, and transferred to PVDF membranes [Herbst and Burden 2000]. Membranes were blocked in Tris-buffered saline with 0.05% Tween-20 and 2% BSA and probed with rabbit antibodies to the N terminus [GTEKLPKAPVITTPLETVDA] of mammalian MuSK diluted in blocking buffer. Antibody binding to MuSK was visualized with secondary horseradish peroxidase conjugated-mouse anti-rabbit IgG [Jackson ImmunoResearch] and quantified using a ChemiDoc imaging system [Bio-Rad].

Electrophysiology
We recorded from diaphragm muscles of postnatal 4-wk-old mice using standard intracellular microelectrodes and measured the frequency and amplitude of spontaneous meps, as described previously [Jevsek et al. 2006; Friese et al. 2007]. We recorded from 18 to 23 muscle fibers from four mice of each genotype.

In addition, we stimulated the common peroneal nerve from P14 mice at low [10-Hz], medium [25-Hz], and high [50-Hz] frequencies and used an extracellular electrode to record the CMAP from the tibialis anterior muscle. We used the Wilcoxon-Mann-Whitney test to determine whether differences in the mean values were significant (P < 0.05).

Behavior
Forelimb and all limb muscle strength was determined in P60 male mice using a grip force tensiometer [Bioseb]. Mice held by their tails were allowed to grip a grid that was connected to the grip strength meter, and the mice were gently pulled horizontally until their grip was released. Five trials were conducted; forelimb and all limb tests were separated by a 20- to 30-min interval. We averaged the force [g] from the three maximum scores and normalized this value to body weight [g]. Motor function of P60 male mice was assessed on a RotaRod (AccuRotor four-channel, Omnitech Electronics, Inc.). Mice were placed on the RotaRod [3.0-cm rotating cylinder] rotating at 5.5 rpm, and the speed of rotation was increased linearly to 40 rpm over the course of 5 min. The time to fall from the rod was measured. Each mouse was subjected to three trials in 5-min intervals, and we recorded the longest latency to fall from the three trials.

We assessed motor fatigue in P60 male mice using an inverted wire hang test. Individual mice were placed on a wire, which was supported by two columns 27 cm apart, mounted 60 cm above a padded laboratory bench. After gently placing the forelimbs on the wire, the time to fall from the wire was measured. We recorded the longest latency to fall from three trials, separated by 15–30 min. We used the Kruskal-Wallis test to determine whether differences in the median values were significant (P < 0.05).

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References
Arber S, Burden SJ, Harris AJ. 2002. Patterning of skeletal muscle. Curr Opin Neurobiol 12: 100–103.
Barik A, Zhang B, Sohal GS, Xiong WC, Mei L. 2014. Crosstalk between Agrin and Wnt signaling pathways in development of vertebrate neuromuscular junction. Dev Neurobiol 74: 828–838.
Blitz E, Viukov S, Sharir A, Shwartz Y, Galloway JL, Pryce BA, Johnson RL, Tabin CJ, Schweitzer R, Zelzer E. 2009. Bone ridge patterning during musculoskeletal assembly is mediated through SCX regulation of Bmp4 at the tendon-skeleton junction. Dev Cell 17: 861–873.
Burden SJ, Yamoto N, Zhang W. 2013. The role of MuSK in synapse formation and neuromuscular disease. Cold Spring Harb Perspect Biol 5: a009167.

Dahlem TJ, Hoshijima K, Juryneec MJ, Gunther D, Starker CG, Locke AS, Weis AM, Voytas DF, Grunwald DJ. 2012. Simple methods for generating and detecting locus-specific mutations induced with TALENs in the zebrafish genome. PLoS Genet 8: e1002861.

DeChiara TM, Bowen DC, Valenzuela DM, Simmons MV, Poueymirou WT, Thomas S, Kinetz E, Compton DL, Rojas E, Park JS, et al. 1996. The receptor tyrosine kinase MuSK is required for neuromuscular junction formation in vivo. Cell 85: 501–512.

Flanagan-Steef H, Fox MA, Meyer D, Sanes JR. 2005. Neuromuscular synapses can form in vivo by incorporation of initially aneural postsynaptic specializations. Development 132: 4471–4481.

Forrester WC, Dell M, Perens E, Garriga G. 1999. A C. elegans Ror receptor tyrosine kinase regulates cell motility and asymmetric cell division. Nature 400: 881–885.

Friese MB, Blagden CS, Burden SJ. 2007. Synaptic differentiation is defective in mice lacking acetylcholine receptor β-subunit tyrosine phosphorylation. Development 134: 4167–4176.

Fu AK, Smith FD, Zhou H, Chu AH, Tsim KW, Peng BH, Ip NY. 1999. Xenopus muscle-specific kinase: molecular cloning and prominent expression in neural tissues during early embryonic development. Eur J Neurosci 11: 373–382.

Gofflot F, Hall M, Morriss-Ray GM. 1998. Genetic patterning of the posterior neuropore region of curly tail mouse embryos: deficiency of Wnt5a expression. Int J Dev Biol 42: 637–644.

Gordon LR, Gribble KD, Syrett CM, Granato M. 2012. Initiation of synapse formation by Wnt-induced MuSK endocytosis. Development 139: 1023–1033.

Green JL, Inoue T, Sternberg PW. 2007. The C. elegans ROR receptor tyrosine kinase, CAM-1, non-autonomously inhibits the Wnt pathway. Development 134: 4053–4062.

Gross-Thebing T, Paksa A, Raz E. 2014. Simultaneous high-resolution detection of multiple transcripts combined with localization of proteins in whole-mount embryos. BMC Biol 12: 55.

Hamblet NS, Lijam N, Ruiz-Lozano P, Wang J, Yang Y, Luo Z, Mei L, Chien KR, Sussman DJ, Wynshaw-Boris A. 2002. Dishevelled 2 is essential for cardiac outflow tract development, somite segmentation and neural tube closure. Development 129: 5827–5838.

Hayashi Y, Hirotsu T, lwata R, kage-Nakadai E, Kunitomo H, Ishihara T, Iino Y, Kubo T. 2009. A trophic role for Wnt–Ror kinase signaling during developmental pruning in Caenorhabditis elegans. Nat Neurosci 12: 981–987.

Henriquez JP, Webb A, Bence M, Bildsoe H, Sahoo M, Hughes SM, Salinas PC. 2008. Wnt signaling promotes AChR aggregation at the neuromuscular synapse in collaboration with agrin. Proc Natl Acad Sci 105: 18812–18817.

Herbst R, Burden SJ. 2000. The juxtaplamine membrane region of MuSK has a critical role in agrin-mediated signaling. EMBO J 19: 67–77.

Huijbers MG, Zhang W, Klooster R, Niks EH, Friesie MB, Strasheijrm KR, Thiessen PE, Vrolijk H, Plommp J, Vogela P, et al. 2013. MuSK IgG4 autoantibodies cause myasthenia gravis by inhibiting binding between MuSK and Lrp4. Proc Natl Acad Sci 110: 20783–20788.

Ips FC, Class DG, Gies DR, Cheung J, Lai KO, Fu AK, Yancopoulos GD, Ip NY. 2000. Cloning and characterization of muscle-specific kinase in chicken. Mol Cell Neurosci 16: 661–673.

Jaworski A, Burden SJ. 2006. Neuromuscular synapse formation in mice lacking motor neuron- and skeletal muscle-derived Neuregulin-1. J Neurosci 26: 655–661.

Jennings CG, Dyer SM, Burden SJ. 1993. Muscle-specific trk-related receptor with a kringle domain defines a distinct class of receptor tyrosine kinases. Proc Natl Acad Sci 90: 2895–2899.

Jevsek M, Jaworski A, Polo-Parada L, Kim N, Fan J, Landmesser LT, Burden SJ. 2006. CD24 is expressed by myofiber synaptic nuclei and regulates synaptic transmission. Proc Natl Acad Sci 103: 6374–6379.

Jing L, Lefebvre JL, Gordon LR, Granato M. 2009. Wnt signals organize synaptic prepattern and axon guidance through the zebrafish unlinked/MuSK receptor. Neuron 61: 721–733.

Kim N, Burden SJ. 2008. MuSK controls where motor axons grow and form synapses. Nat Neurosci 11: 19–27.

Kim C, Forrester WC. 2003. Functional analysis of the domains of the C. elegans Ror receptor tyrosine kinase CAM-1. Dev Biol 264: 376–390.

Kim YJ, Serpe M. 2013. Building a synapse: a complex matter. Fly (Austin) 7: 146–152.

Kim N, Stiegler AL, Cameron TO, Hallock PT, Gomez AM, Huang JH, Hubbard SR, Dustin ML, Burden SJ. 2008. Lrp4 is a receptor for Agrin and forms a complex with MuSK. Cell 135: 334–342.

Koles K, Budnik V. 2012. Wnt signaling in neuromuscular junction development. Cold Spring Harb Perspect Biol 4: a008045.

Konecny I, Cossins J, Waters P, Beeson D, Vincent A. 2013. MuSK myasthenia gravis IgG4 disrupts the interaction of LRP4 with MuSK but both IgG4 and IgG1-3 can disperse preformed agrin-independent AChR clusters. PLoS One 8: e80695.

Kummer TT, Misgeld T, Sanes JR. 2006. Assembly of the postsynaptic membrane at the neuromuscular junction: paradigm lost. Curr Opin Neurobiol 16: 74–82.

Landmesser L, Morris DG. 1975. The development of functional innervation in the hind limb of the chick embryo. J Physiol 249: 301–326.

Lang D, Lu MM, Huang L, Engleka KA, Zhang M, Chu EY, Lipner S, Skoulitchi A, Miller SE, Epstein JA. 2005. Pax3 functions at a nodal point in melanocyte stem cell differentiation. Nature 433: 884–887.

Lin W, Burgess RW, Dominguez B, Pfaff SL, Sanes JR, Lee KF. 2001. Distinct roles of nerve and muscle in postsynaptic differentiation of the neuromuscular synapse. Nature 410: 1057–1064.

Majumdar A, Vainio S, Kispert A, McMahon J, McMahon AP. 2003. Wnt1 and Ret/Cd47 pathways cooperate in regulating ureteric branching during metanephric kidney development. Development 130: 3175–3185.

Maslakowski P, Yancopoulos GD. 1998. The Wnt receptor CRD domain is also found in MuSK and related orphan receptor tyrosine kinases. Curr Biol 8: R407.

Messejant J, Dobbertin A, Girard E, Deleters P, Manuel M, Mangione A, Schmitt A, Le Denmat D, Molgo J, Zytnicki D, et al. 2015. MuSK frizzled-like domain is critical for mammalian neuromuscular junction formation and maintenance. J Neurosci 35: 4926–4941.

Morris JK, Lin W, Hauser C, Marchuk Y, Getman D, Lee KF. 1999. Rescue of the cardiac defect in ErbB2 mutant mice reveals essential roles of ErbB2 in peripheral nervous system development. Neuron 23: 273–283.

Mullins MC, Hammerschmidt M, Haftter P, Nusslein-Volhard C. 1994. Large-scale mutagenesis in the zebrafish: in search of...
genes controlling development in a vertebrate. *Curr Biol* **4**: 189–202.

Oishi I, Sugiyama S, Liu ZJ, Yamamura H, Nishida Y, Minami Y. 1997. A novel *Drosophila* receptor tyrosine kinase expressed specifically in the nervous system. Unique structural features and implication in developmental signaling. *J Biol Chem* **272**: 11196–11193.

Panzer JA, Song Y, Balice-Gordon RJ. 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.

Port F, Basler K. 2010. Wnt trafficking: new insights into Wnt maturation, secretion and spreading. *Traffic* **11**: 1265–1271.

Sienknecht UJ, Fekete DM. 2008. Comprehensive Wnt-related gene expression during cochlear duct development in chicken. *J Comp Neurol* **510**: 378–395.

Smith CL, Mittaud P, Prescott ED, Fuhrer C, Burden SJ. 2001. Src, Fyn, and Yes are not required for neuromuscular synapse formation but are necessary for stabilization of agrin-induced clusters of acetylcholine receptors. *J Neurosci* **21**: 3151–3160.

Stark K, Vainio S, Vassileva G, McMahon AP. 1994. Epithelial transformation of metanephric mesenchyme in the developing kidney regulated by Wnt-4. *Nature* **372**: 679–683.

Stiegler AL, Burden SJ, Hubbard SR. 2006. Crystal structure of the agrin-responsive immunoglobulin-like domains 1 and 2 of the receptor tyrosine kinase MuSK. *J Mol Biol* **364**: 424–433.

Yamaguchi TP, Bradley A, McMahon AP, Jones S. 1999. A Wnt5a pathway underlies outgrowth of multiple structures in the vertebrate embryo. *Development* **126**: 1211–1223.

Wilson C, Goberdhan DC, Steller H. 1993. Dror, a potential neurotrophic receptor gene, encodes a *Drosophila* homolog of the vertebrate Trk-related receptor tyrosine kinases. *Proc Natl Acad Sci* **90**: 7109–7113.

Yamaguchi TS, Britsch S, Riethmacher D, Xu L, Sonnenberg-Riethmacher E, Abou-Rebyeh F, Harvey R, Caroni P, Birchmeier C. 1999. Peripheral nervous system defects in erbB2 mutants following genetic rescue of heart development. *Genes Dev* **13**: 2538–2548.

Xu YK, Nusse R. 1998. The Frizzled CRD domain is conserved in diverse proteins including several receptor tyrosine kinases. *Curr Biol* **8**: R405–R406.

Yamaguchi TP, Bradley A, McMahon AP, Jones S. 1999. A Wnt5a pathway underlies outgrowth of multiple structures in the vertebrate embryo. *Development* **126**: 1211–1223.

Yan J, Jia H, Ma Z, Ye H, Zhou M, Su L, Liu J, Guo AY. 2014. The evolutionary analysis reveals domain fusion of proteins with Frizzled-like CRD domain. *Gene* **533**: 229–239.

Yang X, Arber S, William C, Li L, Tanabe Y, Jessell TM, Birchmeier C, Burden SJ. 2001. Patterning of muscle acetylcholine receptor gene expression in the absence of motor innervation. *Neuron* **30**: 399–410.

Yumoto N, Kim N, Burden SJ. 2012. Lrp4 is a retrograde signal for presynaptic differentiation at neuromuscular synapses. *Nature* **489**: 438–442.

Zhang B, Luo S, Wang Q, Suzuki T, Xiong WC, Mei L. 2008. LRP4 serves as a coreceptor of agrin. *Neuron* **60**: 285–297.

Zhang W, Coldefy AS, Hubbard SR, Burden SJ. 2011. Agrin binds to the N-terminal region of Lrp4 protein and stimulates association between Lrp4 and the first immunoglobulin-like domain in muscle-specific kinase (MuSK). *J Biol Chem* **286**: 40624–40630.

Zhang B, Liao C, Bates R, Yin Y, Xiong WC, Mei L. 2012. Wnt proteins regulate acetylcholine receptor clustering in muscle cells. *Mol Brain* **5**: 7.