Crosstalk Between Agrin and Wnt Signaling Pathways in Development of Vertebrate Neuromuscular Junction

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ABSTRACT: **Neuromuscular junction (NMJ) is a cholinergic synapse where motor neurons elicit muscle contraction. Agrin and its coreceptors LRP4 and MuSK are critical for vertebrate NMJ formation. This paper reviews recent evidence for Wnts and Wnt signaling molecules in NMJ formation including a possible retrograde mechanism by muscle β-catenin. We also present data that Wnt3a, 7a, 8a and 10b could inhibit agrin-mediated AChR clustering. Together with the stimulating effect of Wnt9a, 9b, 10b, 11 and 16 on AChR clustering in the absence of agrin, these results suggest diverse roles for Wnt ligands in NMJ development.** © 2014 Wiley Periodicals, Inc. Develop Neurobiol 74: 828–838, 2014

Keywords: **NMJ; WNT; LRP4; musk; aneural clusters**

INTRODUCTION

The neuromuscular junction (NMJ) is a synapse between the axon of a motor neuron and a skeletal muscle fiber. Because it is large and easily accessible, we know more about the NMJ than any other type of synapse. This peripheral synapse is a highly specialized structure, consisting of three main components: the presynaptic nerve terminal, the postsynaptic muscle membrane and Schwann cells that wrap the synapse. The nerve terminal contains round synaptic vesicles, some of which are docked at active zones. The postsynaptic membrane exhibits elaborated in-foldings called junctional folds, the top of which are packed with acetylcholine receptors (AChRs). In between pre- and postsynaptic membranes are synaptic clefts that contain synaptic basal lamina, where proteins critical for NMJ formation and/or maintenance are enriched, including neural Agrin, acetylcholinesterase, heparan sulfate proteoglycans, and β2 laminin. Motor neurons and muscle cells develop independently, but their mutual interactions contribute to NMJ formation (Ruegg and Bixby, 1998; Sohal, 1995; Wu Korkut and Budnik, 2009; Zhang et al., 2009, 2010). Prior to the arrival of motor axon growth cones, muscle surface express AChRs in small solitary, aneural clusters; after innervation, new AChR clusters are induced at the site of the nerve contact.

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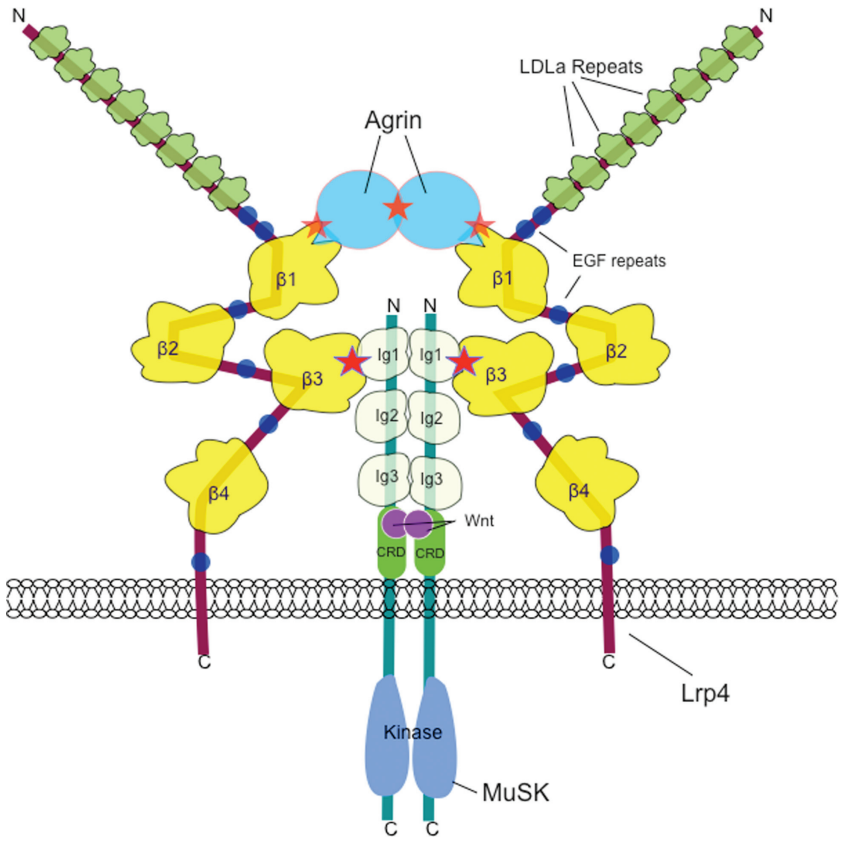

Figure 1 Agrin-Lrp4-Musk complex. Two Agrin-Lrp4 binary complexes associate with each other with a noncrystallographic two-fold symmetry (Zong and Jin, 2012; Zong et al., 2012). MuSK dimerization is important for MuSK activation (Stiegler et al., 2006). Agrin interacts with Lrp4 at the first β1 propeller domain (Zong et al., 2012). Lrp4 and MuSK interact through the third β3 propeller of Lrp4 and the Ig1 domain of MuSK. Wnts may interact with the CRD domain of MuSK. Red stars indicate the sites of interaction.

Agrin-Lrp4-MuSK Signaling

Agrin is a glycoprotein that plays an essential role in NMJ formation. It is synthesized in motor neurons, transported anterogradely to nerve terminals and released into synaptic clefts (Nitkin et al., 1987; McMahan, 1990; Reist et al., 1992; Ruegg et al., 1992; Tsim et al., 1992; Campagna et al., 1995; Ruegg and Bixby, 1998). Agrin is also synthesized in skeletal muscles, but muscle Agrin lacks the 8-amino acid insert in the C-terminus and is >5000-fold less active (Gesemann et al., 1995). Agrin binds to its receptor Lrp4 (low-density lipoprotein receptor-related protein 4) to activate the receptor tyrosine kinase MuSK (Kim et al., 2008; Zhang et al., 2008) (Figs. 1 and 2). Lrp4 is a member of the LDLR family and has a large and complex extracellular region, a transmembrane domain, and a short intracellular C-terminal region. The extracellular domain is composed of eight LDLa (LDL class A) repeats and four homologous YWTD motif-containing β-propeller (β1-4) domains which are separated by EGF-like modules (Herz and Willnow, 1994) (Fig. 1). MuSK has an extracellular domain that contains three Ig domains and one cysteine rich domain (CRD), a transmembrane domain, and an intracellular region where the kinase domain is located (Masiakowski and Carroll, 1992; Masiakowski and Yancopoulos, 1998; Barik et al., 2012) (Fig. 1). Mutant mice lacking Agrin, Lrp4, or MuSK do not form NMJs (DeChiara et al., 1996; Gautam et al., 1996; Glass et al., 1996; Weatherbee et al., 2006), indicating critical roles of these proteins in NMJ formation. Signaling downstream of MuSK remains unclear. Nevertheless, mutation of Dok7, a protein that interacts with MuSK, or Rapsyn, a protein that associates with AChRs, prevents NMJ formation (Sealock et al., 1984; Froehner, 1990; Gautam et al., 1995; Apel

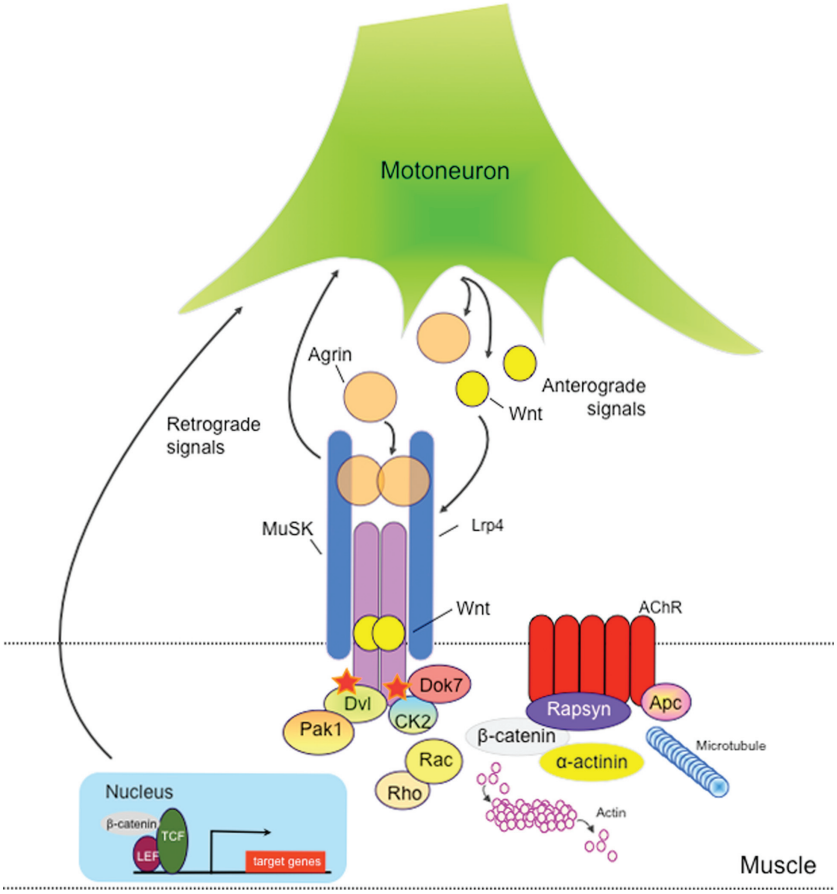

Figure 2 Agrin and Wnt signaling in NMJ formation. See text for details.

et al., 1997; Okada and Shiraishi, 2006; Hamuro et al., 2008).

Questions

When either Lrp4 or MuSK is mutated, muscle cells do not form aneural AChR clusters, and thus muscle fibers are not pre-patterned. These muscle cells do not form AChR clusters in response to Agrin. On the other hand, however, Agrin is not necessary for pre-existing AChR clusters or muscle pre-patterning. These observations raise the following questions. Do Lrp4 and MuSK control muscle pre-patterning in an autonomous manner, i.e., without a ligand? Or, are they regulated by ligands that are yet to be identified? Recent evidence suggests that Wnt may regulate vertebrate NMJ formation by directly interacting with Lrp4 and MuSK.

Wnt Signaling

Wnts are secreted glycoproteins that are conserved among metazoans (MacDonald et al., 2009). They are critical for establishment of body plan including gastrulation and formation of the anterior–posterior axis (Logan and Nusse, 2004). In neural development, Wnts regulate axon pathfinding, dendritic development and synaptogenesis (Hall et al., 2000; Speese and Budnik, 2007; Ciani and Salinas, 2008; Salinas and Zou, 2008; Henriquez et al., 2008; Korkut and Budnik, 2009; Sahores et al., 2010). There are multiple Wnt isoforms: 5 in worms, 7 in flies, 15 in zebrafish, and 19 in mice and humans. They transduce signals through Frizzled receptors that have 3 homologs in worms, 5 in flies, 12 in fish and 11 in mammals and co-receptors such as low-density lipoprotein receptor-related protein 5 and 6 (Lrp5 and Lrp6) (He et al., 2004).

In canonical pathway, upon Wnt binding to Frizzled and LRP5/6, Dvl is recruited, LRP6 undergoes Frizzled/Dvl-dependent phosphorylation to recruit the Axin complex and inhibit GSK3 in the complex. This leads to inhibition of β-catenin phosphorylation and its accumulation in the cytoplasm (Logan and Nusse, 2004; Gordon and Nusse, 2006; Clevers and Nusse, 2012; Kim et al., 2013). β-catenin translocates to the nucleus to regulate gene

expression through association with lymphoid enhancer factor/T cell factor (LEF/TCF) transcription factors. In the planar cell polarity pathway (PCP), Dvl activates GSK3β which phosphorylates microtubule-associated proteins (MAPs), such as MAP1B, Tau, MAP2 to increase microtubule stability. Dvl also activates Dvl-associated activator of morphogenesis 1 (Daam1) and regulates actin cytoskeleton via small GTPases like Rho in a manner independent of GSK3β (Simons and Mlodzik, 2008). In the calcium pathway, Dvl activates PLC, which hydrolyzes PIP2 to produce IP3 and DAG. Subsequent increase in intracellular calcium, due to activation of IP3 receptor, activates calcium/calmodulin-dependent protein kinase II (CamKII) and PKC. Recently, in Drosophila, Frizzled was shown to be internalized upon Wnt binding and cleaved, and the resulting C-terminal fragment translocates to the nucleus for gene regulation (Mathew et al., 2005; Speese and Budnik, 2007).

Wnt signaling has been shown to regulate NMJ formation in invertebrates. It inhibits NMJ formation in C. elegans (Klassen and Shen, 2007). In Drosophila, Wnt promotes NMJ formation (Packard et al., 2002; Korkut et al., 2009; Korkut and Budnik, 2009; Koles et al., 2012). This review focuses on roles of Wnts in vertebrate NMJ assembly.

Wnts in AChR Clustering In Vitro

When mouse MuSK was cloned, its cysteine-rich domain (CRD) was found to be homologous to Frizzled's CRD domain that binds to Wnt (Valenzuela et al., 1995; Glass et al., 1996). Little was known then about the function of the CRD in AChR clustering or NMJ formation. The first hint that Wnt signaling may regulate vertebrate NMJ formation came when MuSK was found to interact with Dvl (Luo et al., 2002) (Fig. 2). Suppression of Dvl expression or disruption of Dvl-MuSK interaction inhibits Agrin- and neuron-induced AChR clustering. Inhibition of Dvl by a dominant-negative mutant in muscles reduces not only amplitudes of spontaneous synaptic currents, but also their frequency, at neuromuscular synapses in culture. This result raises the possibility that Dvl signaling may be important for both post- and presynaptic differentiation. In addition, adenomatous polyposis coli (APC), an adaptor downstream of Dvl in the canonical pathway, was shown to regulate AChR clustering (Wang, 2003) (Fig. 2). In the latter model, Agrin promotes APC interaction and co-clustering with AChR β subunit. AChR cluster numbers as well as the APC-AChR interactions were reduced in muscle cells that overexpress an APC fragment that is necessary for APC-AChR association, indicating that this interaction is involved for AChR clustering. Because APC is known to interact with actin and microtubules, Wang et al. proposed that APC may link AChRs to the cytoskeleton and thus localizing them to the NMJ (Wang et al., 2003). Finally, in vitro studies suggest that β-catenin regulates AChR clustering by bridging AChR and Rapsyn with α-actinin-associated cytoskeleton (Zhang et al., 2007; Dobbins et al., 2008) (Fig. 2).

Eight Wnts (Wnt2, Wnt3a, Wnt 4, Wnt6, Wnt7a, Wnt7b, Wnt9a, and Wnt11) can bind to the extracellular domain of MuSK (Strochlic et al., 2012; Zhang et al., 2012) (data not shown). The CRD domain of MuSK is sufficient to interact with Wnts; deletion of the CRD significantly attenuates the binding activity (Zhang et al., 2012). These results are in agreement with the observation that the CRD of unplugged, the zebrafish ortholog of MuSK, binds to Wnts (Zhang et al., 2004). However, CRD domain deletion does not abolish Wnt binding to mouse MuSK, suggesting involvement of other domains in MuSK for Wnt interaction (Zhang et al., 2012). Expression of Wnt4 in myotubes or treatment with soluble Wnts (Wnt9a, Wnt9b, Wnt10b, Wnt11, or Wnt16) increase the number of AChR clusters, in the absence of exogenous, neuronal Agrin (Strochlic et al., 2012; Zhang et al., 2012) (Fig. 4). The effect of soluble Wnts has the following features. First, it is concentration-dependent and saturable, with EC50 values of $\sim$0.5 n*M*, indicating Wnts act by activating a high-affinity receptor. Second, the maximal response of Wnts is about 50% of that of Agrin, suggesting that they are less efficient than Agrin. Third, Wnt induction of AChR clusters is abolished in MuSK-/- muscle cells, and can be rescued by expression of wild type MuSK, but not by MuSK mutant lacking CRD domain. Together, these results provide evidence that Wnts promote AChR clustering by directly interacting with MuSK (Zhang et al., 2012).

Wnt regulation of mammalian NMJ formation appears complex. Besides MuSK, several Wnts including Wnt7a, Wnt9a, and Wnt11 could also directly interact with Lrp4 (Zhang et al., 2012). In addition, Wnts may regulate Agrin-induced AChR clustering. For example, Wnt3 has no effect on AChR clusters in the absence of Agrin, but potentiates Agrin-induced AChR clustering (Henriquez et al., 2008). On the other hand, Wnt3a, Wnt7a, Wnt8a, and Wnt10b inhibit Agrin-induced formation of AChR clusters although they have little effect on AChR clusters in the absence of Agrin (Figs. 3 and 4) (Wang et al., 2008).

In Vivo Evidence for Wnt Regulation of NMJ Formation

In vivo evidence for Wnt regulation of NMJ formation is weak, but encouraging. First, at the level of

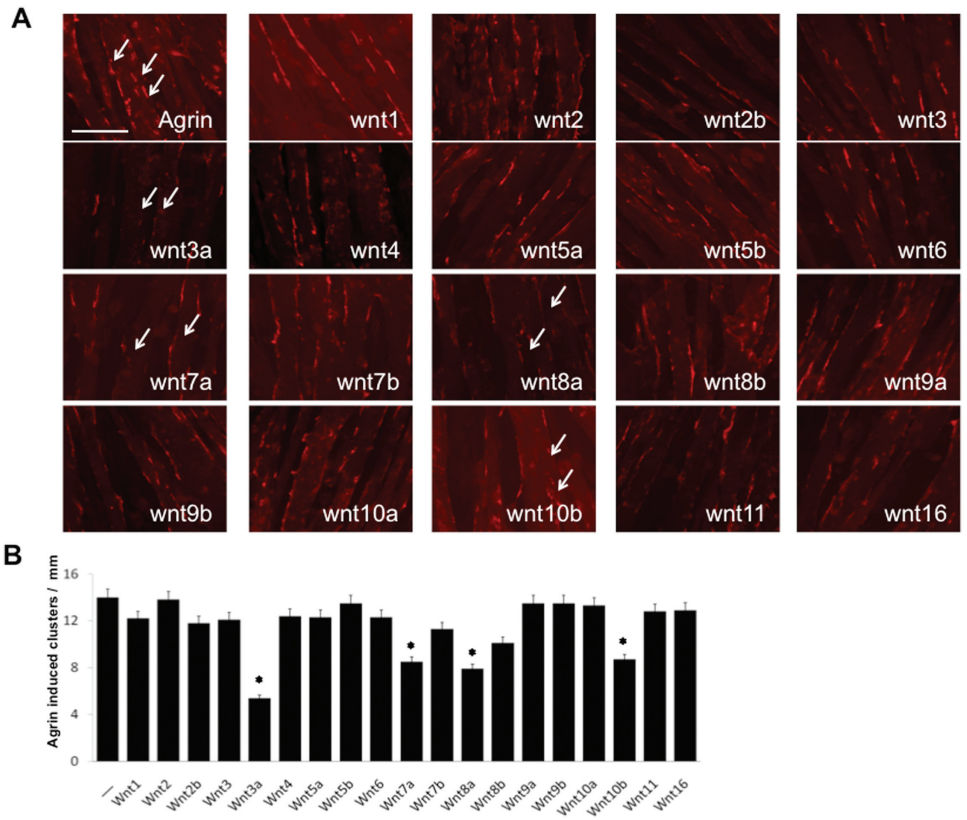

Figure 3 Inhibitory effects of Wnts on Agrin-induced AChR clustering (A) C2C12 myotubes were stimulated with conditioned media containing Wnt and Agrin for 16 h. AChR clusters were visualized by R-BTX staining and indicated by arrows. (B) Quantification data of A. AChR clusters greater than 4 μm in length were quantified. *$p < 0.01$, Student's t test. Scale bar 10 μm.

ligands, implantation of cells over-expressing Wnt3 and Sfrp1 (a Wnt antagonist) in chick wing increases and decreases, respectively, AChR clusters in dorsal and ventral muscle masses (Henriquez et al., 2008), suggesting that endogenous Wnts may regulate NMJ formation. A recent study showed that Wnt4 mutation reduces pre-patterned AChR clusters in mice (Strochlic et al., 2012). In zebrafish, adaxial muscles adjacent to the notochord form aneural AChR clusters prior to innervation by motor axons. As they migrate to the outer layers, fast muscle fibers move in to occupy the vacant spaces (Wu et al., 2010). Motor axons innervate fast muscle fibers where aneural AChR clusters used to be. Granato and colleagues showed that the SV1 isoform of unplugged is crucial for AChR pre-patterning and motor axon guidance (Zhang and Granato, 2000; Zhang et al., 2004). AChR prepatterning involves Wnt11r and Wnt4a; only the combined ablation of both Wnts causes complete loss of aneural AChR clusters (Gordon et al., 2012). Wnt11r, whose mRNA is expressed in the lateral mesoderm, may act in a paracrine mode. Whereas Wnt4a, expressed in the adaxial muscles may operate in an autocrine fashion. Wnt11r is thought to act by binding directly to the CRD of unplugged. However, it remains unclear whether muscle-derived Wnt4a acts similarly. Regardless, activation of unplugged appears to initiate Dvl-dependent, non-canonical or PCP signaling in muscle fibers to restrict growth cone guidance and aneural AChR clusters to the central region of muscle fibers (Jing et al., 2009, 2010). These studies provide in vivo evidence that Wnt regulates the formation of aneural AChR clusters by interacting directly with MuSK. Intriguingly, adaxial muscle prepatterning is dispensable for the formation of neural synapses onto fast twitch muscle fibers, but crucial for motor axonal guidance.

Despite strong in vitro evidence and the observation that the canonical Wnt signaling is active in mice skeletal muscles (Wu, 2012; Kuroda et al., 2013), genetic evidence remains weak for the role of Wnt signaling in NMJ formation. This may be due to the functional redundancy stemming from the multiple isoforms of Wnt and its signaling components.

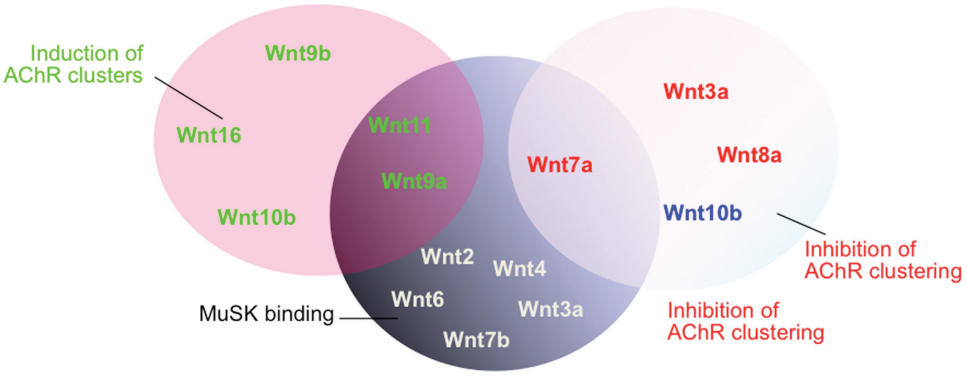

Figure 4 Venn diagram of Wnts for AChR clustering in the absence or presence of Agrin and for binding to MuSK. Wnt10b highlighted in blue behaves in an opposite manner in the presence or absence of Agrin.

For example, mice without Wnt11, an isoform most homologous to Wnt11r in zebrafish, were able to form functional and morphologically normal NMJs (Banerjee et al., 2011). One doable experiment in mice is to determine whether the CRD in MuSK is required for mouse NMJ formation and in what aspects. However, the answers may not be as straightforward because LRP4 may serve as a receptor for Wnts and consequently regulate MuSK activity.

Potential Mechanisms of Wnt Regulation of NMJ Formation

Signaling Initiated by Wnt Binding to MuSK and Lrp4. Wnts (Wnt4, Wnt9a, and Wnt11) directly interact with the CRD of MuSK, which leads to its dimerization and activation (Strochlic et al., 2012; Zhang et al., 2012). Intriguingly, Wnt-induced AChR clustering (in the absence of Agrin) requires Lrp4 (Zhang et al., 2012). Among Wnts, Lrp4, and MuSK, three interactions have been demonstrated: between Wnt and MuSK, between Wnt and Lrp4; and between Lrp4 and MuSK. It is likely that Lrp4 is necessary for forming the Wnt-Lrp4-MuSK complex. Regardless, the dose-response curve of Agrin in the absence and presence of Wnts superimpose with each other, suggesting that the AChR cluster-inducing effects by Wnts and Agrin are not additive. Therefore, Wnts may act via similar pathways that are activated by Agrin. For example, Wnt3 regulation of Agrin-induced clustering does not require ROCK, but Rac1 (Henriquez et al., 2008), a Rho GTPase that is necessary for Agrin-induced clustering (Weston et al., 2000; Weston et al., 2003; Weston et al., 2007).

Dvl appears to play an important role in Wnt regulation of NMJ formation. In zebrafish and in myotubes in culture, Wnts initiate Dvl-dependent, noncanonical pathways to form AChR clusters (Henriquez et al., 2008; Jing et al., 2009; Jing et al., 2010; Banerjee et al., 2011). When expressed in adaxial muscle cells, the dominant negative mutant also attenuates AChR prepatterning (Jing et al., 2009; Jing et al., 2010; Banerjee et al., 2011). Upon Agrin stimulation, MuSK in mammalian muscles becomes rapidly endocytosed, which is required for Agrin-induced AChR clustering (Zhu et al., 2007). Interestingly, Wnt11a and Wnt4a were shown to stimulate MuSK translocation from muscle surface to recycling endosomes (Gordon et al., 2012). This transition appears necessary for AChR accumulation at the NMJ. Interestingly, PCP pathway components translocate to the recycling endosomes in a MuSK-dependent manner and are in turn necessary for MuSK translocation to endosomes, AChR localization and axonal guidance. It is likely that Wnt-induced trafficking of the MuSK receptor to endosomes initiates a signaling cascade that align pre- with postsynaptic elements. It will be interesting to know whether zebrafish Lrp4 has similar roles as its mammalian counterpart.

MuSK Binding-Independent Signaling. Of the six Wnts that induce AChR clusters in the absence of Agrin, only three (Wnt4, Wnt9a, and Wnt11) bind to MuSK. Similarly, of the four Wnts that inhibit Agrin-induced AChR clusters, only Wnt7a interacts with MuSK (Figs. 3 and 4). How do these Wnts contribute to aneural AChR clusters or NMJ formation? It remains to be determined whether Lrp4 serves as a receptor for these negative Wnts. On the other hand, they could bind to Frizzled and Lrp5/6 to activate classic Wnt pathways that regulate AChR clustering and NMJ formation. For example, Wnt3a was shown to disperse AChR clusters by repressing the

expression of Rapsyn, a critical protein for AChR clustering (Wang et al., 2008). This effect was prevented by DKK1, an antagonist of the Wnt canonical pathway and could be rescued by forced expression of β-catenin or Rapsyn. These observations suggest that the Wnt β-catenin canonical pathway may negatively regulate postsynaptic differentiation at the NMJ.

Potential Sources of Wnts for NMJ Formation

If Wnts are critical for aneural AChR cluster formation or muscle fiber prepatterning, they should be expressed in developing motor neurons, muscles or cells adjacent to the NMJ. Quantitative RT-PCR analysis indicates that this is the case for many Wnts; in particular, Wnt2, Wnt4, Wnt9, and Wnt11 are expressed at levels significantly higher than other Wnts in skeletal muscles (Strochlic et al., 2012; Zhang et al., 2012). In zebrafish, Wnt11r is expressed in cells adjacent to adaxial slow muscle cells (Jing et al., 2009). The counterparts of the adaxial close cells in mice are unknown. It is possible that muscle precursor cells from somites may be primed by Wnt during migration (Cossu and Borello, 1999). Wnt3 is present in lateral motoneurons of the spinal cord during the period of motoneuron-muscle innervation (Henriquez et al., 2008). Such Wnts may not be involved in muscle prepatterning, which occurs prior to the arrival of motor axons.

Potential Functions of Wnt Signaling Proteins

In vitro studies suggest that several proteins critical for Wnt signaling regulate Agrin-induced AChR clusters. They include Dvl and casein kinase 2 (CK2) that interact with MuSK, Apc that interacts with AChR β-subunit, and β-catenin that interacts with Rapsyn (Luo et al., 2002; Wang, 2003; Zhang et al.; Cheusova et al., 2006). With exception of β-catenin and CK2, the roles of these proteins in NMJ formation have not been investigated in loss-of-function mutant mice. Nevertheless, in vitro studies suggest that they regulate AChR clustering by previously unappreciated mechanisms.

Dvl and Apc. As an adaptor protein downstream of Frizzled, Dvl is implicated in most, if not all, Wnt signaling pathways. It interacts directly with MuSK and Pak1 (Luo et al., 2002). Mice have three isoforms (Dvl1, 2, and 3) with functional redundancy. Lethality of double mutants makes it difficult to determine whether Dvl is critical for NMJ formation in vivo. Knockdown of all three isoforms attenuates Agrin-induced clustering in cultured myotubes (Luo et al., 2002). Apc is a component of the signaling complex that controls β-catenin stability and thus Wnt canonical pathway (Nathke, 2004; Sansom et al., 2004; Segditsas and Tomlinson, 2006). In addition, it regulates cytoskeleton by associating with microtubules, microfilaments, and intermediate filaments (Etienne-Manneville et al., 2005; Barth et al., 2008). At the NMJ, it interacts with the cytoplasmic domain of the β subunit of AChR. This interaction is increased by Agrin and required for Agrin-induced AChR clustering (Wang, 2003). Whether Dvl and Apc are critical for NMJ formation in mice remains unknown. However, AChR clusters do form in mice whose β-catenin levels are increased or diminished (despite reduced density and larger in size, see below) (Li et al., 2008; Liu et al., 2012; Wu et al., 2012). Interacting with MuSK and Pak1 at the same time, Dvl is thought to serve as an adapter protein for Agrin signaling (Luo et al., 2002). On the other hand, considering recent findings of the role of microtubules in AChR clustering (Schmidt et al., 2012), Apc may regulate AChR clustering by bridging the AChR to the cytoskeleton.

CK2. CK2 is localized at the NMJ and interacts with and phosphorylates MuSK on serine residues (Cheusava et al., 2006). CK2 knockdown or pharmacological inhibition reduces Agrin-induced AChR clusters in cultured muscles. It seems that serine phosphorylation of MuSK does not alter MuSK activity but destabilizes AChR aggregates. Muscle-specific CK2b knockout mice form NMJs, but become myasthenic with impaired NMJ structure and function.

Muscle β-catenin for Pre- and Postsynaptic Differentiation. In vitro studies suggest that β-catenin associates with the AChR complex via direct interaction with Rapsyn (Zhang et al., 2007). Agrin stimulation increases the association of β-catenin with surface AChRs. Suppression of β-catenin expression reduces AChR clusters in Agrin-stimulated muscle cells. When β-catenin is ablated in muscle cells, AChR clusters increase in size and are distributed in distributed in a wider central region (Li et al., 2008). Agrin-induced clustering does not require the transcription activity of TCF/LEF1 factors (Zhang et al., 2007). It is likely that β-catenin contributes to clustering by regulating cytoskeleton in a manner dependent on α-catenin.

Muscle-specific mutation of β-catenin increases the central area where AChR clusters are distributed (Li et al., 2008). In addition, β-catenin mutation causes presynaptic deficits including mislocation of phrenic nerve branches and reduced synaptic

vesicles. Interestingly, some of these phenotypes were also observed in muscle-specific β-catenin gain-of-function mice (Liu et al., 2012; Wu et al., 2012). These observations suggest first that β-catenin may play a role in deciding where to form aneural as well as nerve-induced AChR clusters. Second, muscle β-catenin directs a retrograde signal necessary for presynaptic differentiation (Fig. 2). Third, there is an intricate balance of levels or signaling of β-catenin in muscle fibers; either increase or decrease of β-catenin activity impairs NMJ formation. The question is how this balance of β-catenin is regulated in vivo. Similar ACh release deficits were observed in cultured frog neuromuscular synapses where Dvl function in myocytes was inhibited (Luo et al., 2002), suggesting that β-catenin may be downstream of Dvl. Whether it is regulated by Wnts remains unclear. Interestingly, mice lacking Wnt4 reveal innervation defects including over-growth of motor axons and bypassing AChR clusters (Strochlic et al., 2012).

In summary, emerging evidence supports diverse roles for Wnt ligands and signaling molecules in NMJ development in vertebrates. Wnts may act as ligands for MuSK and Lrp4 to stimulate pathways that are MuSK-specific, which may be an underlying mechanism of aneural AChR cluster formation or muscle pre-patterning. Alternatively, Wnts could regulate Agrin-induced AChR clustering by initiating Wnt pathways. In addition, proteins that are well characterized for Wnt signaling have previously unappreciated functions in AChR clustering and NMJ formation.

METHODS

Constructs

Constructs used were described in details before (Zhang et al., 2012). In short, Wnt cDNAs were generated by PCR and subcloned into pFlag-CMV1 downstream of an artificial signal peptide sequence and a Flag epitope. Templates for PCR were purchased from Open Biosystems (catalog number in parentheses): Wnt2 (4162686), Wnt2b (8734025), Wnt3 (5726751), Wnt3a (40007188), Wnt5a (3487288), Wnt5b (6438917), Wnt7a (6415801), Wnt8a (40129440), Wnt8b (40056929), Wnt9a (30435371), Wnt9b (5588904), Wnt10a (4921327), Wnt10b (7868324), Wnt11 (40129997), and Wnt16 (40105502). Dr. Xi He generously provided expression constructs of Wnt1, Wnt4, Wnt6, and Wnt7b.

Cell Culture and Transfection

Cells were cultured and maintained (HEK293 cells and mouse C2C12 muscle cells) as described previously (Luo et al., 2008; Zhang et al., 2008). They were transfected with PEI (polyethylenimine, Sigma, 408727), as described previously (Boussif et al., 1995) with modification. Expression of the Flag-tagged Wnts was confirmed by western blotting with anti-Flag antibody (Zhang et al., 2012).

Recombinant Protein Production and Purification

Agrin was generated and prepared as previously described (Luo et al., 2002, Zhang et al., 2007, Zhang et al., 2012). HEK293 cells were transfected with plasmids encoding Flag-tagged Wnts. Twenty-four hours after transfection, cells were switched to Dulbecco's Modified Eagle Medium supplemented with 0.05% of fetal bovine serum, and conditioned media were collected and cleaned by centrifugation (1000 rpm, 10 min at room temperature). Control conditioned medium was collected from nontransfected HEK293 cells.

AChR Cluster Assays

AChR clusters in C2C12 myotubes were quantified as described previously with modification (Luo et al., 2002, Zhang et al., 2007, 2012).

Statistical Analysis

Two-tailed Student's t test was used to compare data between two groups using PRISM program. Differences were considered significant $p < 0.05$. Values and error bars in figures denote mean ± SD.

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REFERENCES

Apel ED, Glass DJ, Moscoso LM, Yancopoulos GD, Sanes JR. 1997. Rapsyn is required for MuSK signaling and recruits synaptic components to a MuSK-containing scaffold. Neuron 18:623–635.

Banerjee S, Gordon L, Donn TM, Berti C, Moens CB, Burden SJ, Granato M. 2011. A novel role for MuSK and non-canonical Wnt signaling during segmental neural crest cell migration. Development 138:3287–3296.

Barik A. Xiong W-C, Mei L. 2012. MuSK: A Kinase Critical for the Formation and Maintenance of the Neuromuscular Junction. In: Mukai H (eds), Protein Kinase Technologies: Neuromethods. Humana Press. 203–217.

Barth AI, Caro-Gonzalez HY, Nelson WJ. 2008. Role of adenomatous polyposis coli (APC) and microtubules in directional cell migration and neuronal polarization. Semin Cell Dev Biol 19:245–251.

Campagna JA, Ruegg MA, Bixby JL. 1995. Agrin is a differentiation-inducing "stop signal" for motoneurons in vitro. Neuron 15:1365–1374.

Cheusova T, Khan MA, Schubert SW, Gavin AC, Buchou T, Jacob G, Sticht H, Allende J, Boldyreff B, Brenner

HR, Hashemolhosseini S. 2006. Casein kinase 2-dependent serine phosphorylation of MuSK regulates acetylcholine receptor aggregation at the neuromuscular junction. Genes Dev 20:1800–1816.

Ciani L, Salinas PC. 2008. From neuronal activity to the actin cytoskeleton: A role for CaMKKs and betaPIX in spine morphogenesis. Neuron 57:3–4.

Clevers H, Nusse R. 2012. Wnt/beta-catenin signaling and disease. Cell 149:1192–1205.

Cossu G, Borello U. 1999. Wnt signaling and the activation of myogenesis in mammals. EMBO J 18:6867–6872.

DeChiara TM, Bowen DC, Valenzuela DM, Simmons MV, Poueymirou WT, Thomas S, Kinetz E, Compton DL, Rojas E, Park JS, Smith C, DiStefano PS, Glass DJ, Burden SJ, Yancopoulos GD. 1996. The receptor tyrosine kinase MuSK is required for neuromuscular junction formation in vivo. Cell 85:501–512.

Dobbins GC, Luo S, Yang Z, Xiong WC, Mei L. 2008. alpha-Actinin interacts with Rapsyn in Agrin-stimulated AChR clustering. Mol Brain 1:18.

Etienne-Manneville S, Manneville JB, Nicholls S, Ferenczi MA, Hall A. 2005. Cdc42 and Par6-PKCzeta regulate the spatially localized association of Dlg1 and APC to control cell polarization. J Cell Biol 170:895–901.

Froehner SC, Scotland PB, Patrick J. 1990. The postsynaptic 43K protein clusters muscle nicotinic acetylcholine receptors in Xenopus oocytes. Neuron 5:403–410.

Gautam M, Noakes PG, Moscoso L, Rupp F, Scheller RH, Merlie JP, Sanes JR. 1996. Defective neuromuscular synaptogenesis in Agrin-deficient mutant mice. Cell 85:525–535.

Gautam M, Noakes PG, Mudd J, Nichol M, Chu GC, Sanes JR, Merlie JP. 1995. Failure of postsynaptic specialization to develop at neuromuscular junctions of Rapsyn-deficient mice. Nature 377:232–236.

Glass DJ, Bowen DC, Stitt TN, Radziejewski C, Bruno J, Ryan TE, Gies DR. et al. 1996. Agrin acts via a MuSK receptor complex. Cell 85:513–523.

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

Gordon MD, Nusse R. 2006. Wnt signaling: multiple pathways, multiple receptors, and multiple transcription factors. J Biol Chem 281:22429–22433.

Hall AC, Lucas FR, Salinas PC. 2000. Axonal remodeling and synaptic differentiation in the cerebellum is regulated by WNT-7a signaling. Cell 100:525–535.

Hamuro J, Higuchi O, Okada K, Ueno M, Iemura S, Natsume T, Spearman H, et al. 2008. Mutations causing DOK7 congenital myasthenia ablate functional motifs in Dok-7. J Biol Chem 283:5518–5524.

Hart M, Concordet JP, Lassot I, Albert I, del los Santos R, Durand H, Perret C, et al. 1999. The F-box protein beta-TrCP associates with phosphorylated beta-catenin and regulates its activity in the cell. Curr Biol 9:207–210.

He X, Semenov M, Tamai K, Zeng X. 2004. LDL receptor-related proteins 5 and 6 in Wnt/beta-catenin signaling: Arrows point the way. Development 131: 1663–1677.

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

Herz J, Willnow TE. 1994. Functions of the LDL receptor gene family. Ann NY Acad Sci 737:14–19.

Jing L, Gordon LR, Shtibin E, Granato M. 2010. Temporal and spatial requirements of unplugged/MuSK function during zebrafish neuromuscular development. PLoS One 5:e8843.

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

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

Kim SE, Huang H, Zhao M, Zhang X, Zhang A, Semonov MV, MacDonald BT, 2013. "Wnt stabilization of beta-catenin reveals principles for morphogen receptor-scaffold assemblies." Science 340:867–870.

Klassen MP, Shen K. 2007. Wnt signaling positions neuromuscular connectivity by inhibiting synapse formation in *C. elegans*. Cell 130:704–716.

Koles K, Nunnari J, Korkut C, Barria R, Brewer C, Li Y, Leszyk J, et al. 2012. Mechanism of evenness interrupted (Evi)-exosome release at synaptic boutons. J Biol Chem 287:16820–16834.

Korkut C, Ataman B, Ramachandran P, Ashley J, Barria R, Gherbesi N, Budnik V. 2009. Trans-synaptic transmission of vesicular Wnt signals through Evi/Wntless. Cell 139: 393–404.

Korkut C, Budnik V. 2009. WNTs tune up the neuromuscular junction. Nat Rev Neurosci 10:627–634.

Kuroda K, Kuang S, Taketo MM, Rudnicki MA. 2013. Canonical Wnt signaling induces BMP-4 to specify slow myofibrogenesis of fetal myoblasts. Skelet Muscle 3:5.

Li XM, Dong XP, Luo SW, Zhang B, Lee DH, Ting AK, Neiswender H, et al. 2008. Retrograde regulation of motoneuron differentiation by muscle beta-catenin. Nat Neurosci 11:262–268.

Liu CY, Zha ZY, Zhou X, Zhang H, Huang W, Zhao D, Li T, et al. 2010. The hippo tumor pathway promotes TAZ degradation by phosphorylating a phosphodegron and recruiting the SCF{beta}-TrCP E3 ligase. J Biol Chem 285:37159–37169.

Liu Y, Sugiura Y, Wu F, Mi W, Taketo MM, Cannon S, Carroll T, et al. 2012. beta-Catenin stabilization in skeletal muscles, but not in motor neurons, leads to aberrant motor innervation of the muscle during neuromuscular development in mice. Dev Biol 366:255–267.

Logan CY, Nusse R. 2004. The Wnt signaling pathway in development and disease. Annu Rev Cell Dev Biol 20: 781–810.

Luo ZG, Wang Q, Zhou JZ, Wang J, Luo Z, Liu M, He X, et al. 2002. Regulation of AChR clustering by

Dishevelled interacting with MuSK and PAK1. Neuron 35:489–505.

MacDonald BT, Tamai K, He X. 2009. Wnt/beta-catenin signaling: Components, mechanisms, and diseases. Dev Cell 17:9–26.

Masiakowski P, Carroll RD. 1992. A novel family of cell surface receptors with tyrosine kinase-like domain. J Biol Chem 267:26181–26190.

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

Mathew D, Ataman B, Chen J, Zhang Y, Cumberledge S, Budnik V. 2005. Wingless signaling at synapses is through cleavage and nuclear import of receptor DFrizzled2. Science 310:1344–1347.

McMahan UJ. 1990. The Agrin hypothesis. Cold Spring Harb Symp Quant Biol 55:407–418.

Nathke I. 2004. APC at a glance. J Cell Sci 117:4873–4875.

Nitkin RM, Smith MA, Magill C, Fallon JR, Yao YM, Wallace BG, McMahan UJ. 1987. Identification of Agrin, a synaptic organizing protein from Torpedo electric organ. J Cell Biol 105:2471–2478.

Okada K, Inoue A, Okada M, Murata Y, Kakuta S, Jigami T, Kubo S, et al. 2006. The muscle protein Dok-7 is essential for neuromuscular synaptogenesis. Science 312.

Packard M, Koo ES, Gorczyca M, Sharpe J, Cumberledge S, Budnik V. 2002. The Drosophila Wnt, wingless, provides an essential signal for pre- and postsynaptic differentiation. Cell 111:319–330.

Reist NE, Werle MJ, McMahan UJ. 1992. Agrin released by motor neurons induces the aggregation of acetylcholine receptors at neuromuscular junctions. Neuron 8:865–868.

Ruegg MA, Bixby JL. 1998. Agrin orchestrates synaptic differentiation at the vertebrate neuromuscular junction. Trends Neurosci 21:22–27.

Ruegg MA, Tsim KW, Horton SE, Kroger S, Escher G, Gensch EM, McMahan UJ. 1992. The Agrin gene codes for a family of basal lamina proteins that differ in function and distribution. Neuron 8:691–699.

Sahores M, Gibb A, Salinas PC. 2010. Frizzled-5, a receptor for the synaptic organizer Wnt7a, regulates activity-mediated synaptogenesis. Development 137:2215–2225.

Salinas PC, Zou Y. 2008. Wnt signaling in neural circuit assembly. Annu Rev Neurosci 31:339–358.

Sansom OJ, Reed KR, Hayes AJ, Ireland H, Brinkmann H, Newton IP, Batlle E, et al. 2004. Loss of Apc in vivo immediately perturbs Wnt signaling, differentiation, and migration. Genes Dev 18:1385–1390.

Sealock R, Wray BE, Froehner SC. 1984. Ultrastructural localization of the Mr 43,000 protein and the acetylcholine receptor in Torpedo postsynaptic membranes using monoclonal antibodies. J Cell Biol 98:2239–2244.

Segditsas S, Tomlinson I. 2006. Colorectal cancer and genetic alterations in the Wnt pathway. Oncogene 25: 7531–7537.

Simons M, Mlodzik M. 2008. Planar cell polarity signaling: From fly development to human disease. Annu Rev Genet 42:517–540.

Sohal GS. 1995. Sixth Annual Stuart Reiner Memorial Lecture: Embryonic development of nerve and muscle. Muscle Nerve 18:2–14.

Speese SD, Budnik V. 2007. Wnts: Up-and-coming at the synapse. Trends Neurosci 30:268–275.

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.

Strochlic L, Falk J, Goillot E, Sigoillot S, Bourgeois F, Delers P, Rouviere J, et al. 2012. Wnt4 participates in the formation of vertebrate neuromuscular junction. PLoS One 7:e29976.

Tsim KW, Ruegg MA, Escher G, Kroger S, McMahan UJ. 1992. cDNA that encodes active Agrin. Neuron 8:677–689.

Valenzuela DM, Stitt TN, DiStefano PS, Rojas E, Mattsson K, Compton DL, Nunez L, 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.

Wang J, Jing Z, Zhang L, Zhou G, Braun J, Yao Y, Wang ZZ. 2003. Regulation of acetylcholine receptor clustering by the tumor suppressor APC. Nat Neurosci 6:1017–1018.

Wang J, Ruan NJ, Qian L, Lei WL, Chen F, Luo ZG. 2008. Wnt/beta-catenin signaling suppresses Rapsyn expression and inhibits acetylcholine receptor clustering at the neuromuscular junction. J Biol Chem 283:21668–21675.

Weatherbee SD, Anderson KV, Niswander LA. 2006. LDL-receptor-related protein 4 is crucial for formation of the neuromuscular junction. Development 133:4993–5000.

Weston C, Gordon C, Teressa G, Hod E, Ren XD, Prives J. 2003. Cooperative regulation by Rac and Rho of Agrin-induced acetylcholine receptor clustering in muscle cells. J Biol Chem 278:6450–6455.

Weston C, Yee B, Hod E, Prives J. 2000. Agrin-induced acetylcholine receptor clustering is mediated by the small guanosine triphosphatases Rac and Cdc42. J Cell Biol 150:205–212.

Weston CA, Teressa G, Weeks BS, Prives J. 2007. Agrin and laminin induce acetylcholine receptor clustering by convergent, Rho GTPase-dependent signaling pathways. J Cell Sci 120:868–875.

Wu H, Lu Y, Barik A, Joseph A, Taketo MM, Xiong WC, Mei L. 2012. beta-Catenin gain of function in muscles impairs neuromuscular junction formation. Development 139:2392–2404.

Wu H, Xiong WC, Mei L. 2010. To build a synapse: Signaling pathways in neuromuscular junction assembly. Development 137:1017–1033.

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

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

Zhang B, Luo S, Dong XP, Zhang X, Liu C, Luo Z, Xiong WC, Mei L. 2007. Beta-catenin regulates acetylcholine receptor clustering in muscle cells through interaction with Rapsyn. J Neurosci 27:3968–3973.

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 B, Luo S, Dong XP, Zhang X, Liu C, Luo Z, Xiong WC, et al. Beta-catenin regulates acetylcholine receptor clustering in muscle cells through interaction with Rapsyn. J Neurosci 27:3968–3973.

Zhang B, Xiong WC, Mei L. 2009. Get ready to Wnt: Prepatterning in neuromuscular junction formation. Dev Cell 16:325–327.

Zhang J, Granato M. 2000. The zebrafish unplugged gene controls motor axon pathway selection. Development 127:2099–2111.

Zhang J, Lefebvre JL, Zhao S, Granato M. 2004. Zebrafish unplugged reveals a role for muscle-specific kinase homologs in axonal pathway choice. Nat Neurosci 7: 1303–1309.

Zong Y, Jin R. 2012. Structural mechanisms of the Agrin-LRP4-MuSK signaling pathway in neuromuscular junction differentiation. Cell Mol Life Sci 70: 3077–3088.

Zong Y, Zhang B, Gu S, Lee K, Zhou J, Yao G, Figueiredo D, et al. 2012. Structural basis of Agrin-LRP4-MuSK signaling. Genes Dev 26:247–258.