

## Differential requirement for MuSK and dystroglycan in generating patterns of neuromuscular innervation

Julie L. Lefebvre, Lili Jing, Sara Becaficco, Clara Franzini-Armstrong, and Michael Granato

*PNAS* 2007;104;2483-2488; originally published online Feb 6, 2007;  
doi:10.1073/pnas.0610822104

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# Differential requirement for MuSK and dystroglycan in generating patterns of neuromuscular innervation

Julie L. Lefebvre\*, Lili Jing, Sara Becaficco, Clara Franzini-Armstrong, and Michael Granato†

Department of Cell and Developmental Biology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104-6058

Communicated by Joshua R. Sanes, Harvard University, Cambridge, MA, December 6, 2006 (received for review October 26, 2006)

Vertebrates display diverse patterns of neuromuscular innervation, but little is known about how such diversity is generated. In mammals, neuromuscular junctions form predominantly at equatorial locations, giving rise to a focal innervation pattern along a central endplate band. In addition, vertebrate striated muscles exhibit two nonfocal neuromuscular patterns, myoseptal and distributed innervation. Although agrin-MuSK-rapsyn signaling is essential for the focal innervation pattern, it is unknown whether the same genetic program also controls synaptogenesis at nonfocal innervation sites. Here we show that one of three transcripts generated by the zebrafish *unplugged* locus, *unplugged FL*, encodes the zebrafish MuSK ortholog. We demonstrate that UnpFL/MuSK is critical for the assembly of focal synapses in zebrafish and that it cooperates with dystroglycan in the formation of nonfocal myoseptal and distributed synapses. Our results provide the first genetic evidence that neuromuscular synapse formation can occur in the absence of MuSK and that the combinatorial function of UnpFL/MuSK and dystroglycan generates diverse patterns of vertebrate neuromuscular innervation.

muscle-specific kinase | neuromuscular junction | synaptogenesis

The formation of synapses requires coordinated localization of pre- and postsynaptic elements at prospective sites of innervation. In the developing mammalian muscle, motor axons grow together through the central region of the muscle and then extend individual branches that terminate onto myofibers at an equatorial position (1). Acetylcholine receptors (AChRs) aggregate in apposition to the presynaptic nerve terminals, and the result is a focal band of neuromuscular junctions (NMJs). Focal innervation patterns are most prevalent and best studied in mammals. However, patterns of nonfocal innervation, in which synapses form at multiple sites distributed along the length or at the myoseptal ends of muscle fibers, are widespread in vertebrates such as avians, amphibians, and teleost fish and are also present in mammals, e.g., in extraocular and laryngeal muscle (2–6). The diversity of innervation patterns present in vertebrates raises the question of whether these different types of NMJs develop in similar ways and under the same molecular controls.

Genetic and molecular studies in mice have established that the agrin-MuSK-rapsyn pathway controls neuromuscular synapse formation at focal sites of innervation, although recent studies suggest a more complex view in which the assigned roles of some of these core molecular players have been revised (reviewed in refs. 7 and 8). It remains unchallenged that postsynaptic differentiation is directed by the muscle-specific receptor tyrosine kinase, MuSK (9). Before receiving signals from the nerve, MuSK patterns the presumptive postsynaptic region by localizing AChRs and other postsynaptic proteins to the central endplate zone (10, 11). Upon release of nerve-derived agrin, MuSK recruits rapsyn, an obligate intracellular effector, which clusters AChRs beneath the nerve (12–16). Interestingly, agrin also binds one component of the DGC complex,  $\alpha$ -dystroglycan ( $\alpha$ -DG), with high affinity, and this interaction has been implicated in agrin-mediated AChR cluster maturation (17–19).

Most genetic studies on the role of MuSK have focused on selected groups of rodent muscle, mainly the diaphragm, which

display focal innervation patterns (9, 20). MuSK homologs have been identified in several nonmammalian vertebrates in which nonfocal innervation is more prevalent (21–24), but there are no genetic studies addressing whether the MuSK pathway is involved in the formation of nonfocal patterns of innervation. Here we examine the role of MuSK signaling in the formation of NMJs in the zebrafish because they exhibit focal and nonfocal innervation patterns. We have previously reported that the zebrafish *unplugged* gene encodes two MuSK homolog isoforms, *unplugged Full-Length* (UnpFL) and *Splice Variant 1* (UnpSV1), and that *unplugged* exerts its role in motor axon guidance through the SV1 isoform (Fig. 1A) (25). In contrast to the mouse MuSK knockout (9), *unplugged*-null mutants develop neuromuscular junctions and are viable (25). Here we show that the UnpFL isoform is the MuSK ortholog, and we provide the first genetic evidence that vertebrate NMJs can form in the absence of UnpFL/MuSK. Finally, we show that dystroglycans are critical in the formation of synapses at nonfocal sites of innervation. Based on our results, we propose a model by which combinatorial functions of UnpFL and dystroglycans generate diverse patterns of neuromuscular innervation.

## Results and Discussion

***unplugged* Is Essential for Focal Equatorial Synapses and AChR Prepotentiation.** The axial muscle of zebrafish exhibits three patterns of neuromuscular innervation: (i) focal innervation along the center of the myotomes, (ii) distributed innervation, and (iii) myoseptal innervation. We previously showed that *unplugged*-null mutants exhibit AChR clusters at distributed and myoseptal synaptic sites but did not examine AChRs at focal sites (25). Because the *unplugged* gene displays a high degree of sequence similarity to mammalian MuSKs, we wondered whether *unplugged* plays a role in the development of focal synapses, analogous to those absent in mice lacking MuSK. In wild-type embryos at 26 h postfertilization (hpf), dense AChR clusters form along the length of the dorsal and ventral primary motor axons (Fig. 1B). These synapses have an *en passant* morphology and are restricted to the center of muscle fibers, giving rise to a focal innervation pattern (26). *unplugged* mutant embryos lack these *en passant* synapses (Fig. 1C) (100% hemisegments affected), demonstrating that *unplugged* is essential for the formation of synapses in focal innervation.

In 17-hpf wild-type embryos, the appearance of early equatorial synapses is preceded by a diffuse band of small aneural AChR clusters located along the presumptive axonal path in the dorsal and ventral myotomes (Fig. 1D) (27). These AChR clusters are morphologically similar to those observed in prepatterned mammalian

Author contributions: J.L.L., C.F.-A., and M.G. designed research; J.L.L., L.J., S.B., C.F.-A., and M.G. performed research; J.L.L., L.J., S.B., C.F.-A., and M.G. analyzed data; and J.L.L. and M.G. wrote the paper.

The authors declare no conflict of interest.

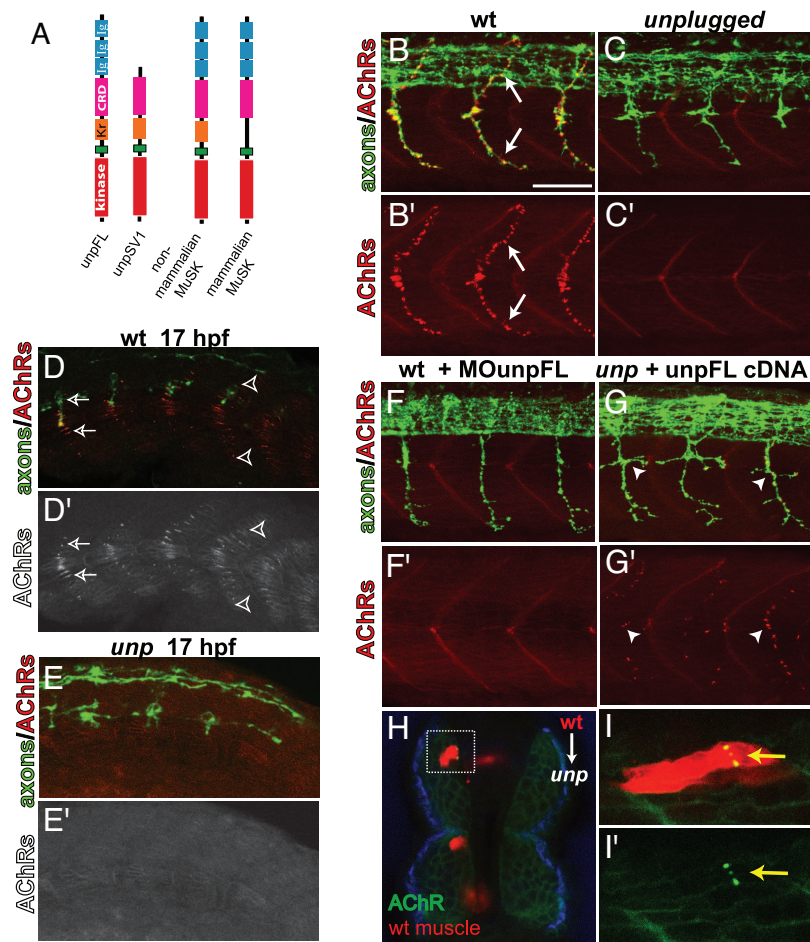
Abbreviations: AChR, acetylcholine receptor; NMJ, neuromuscular junction; hpf, hours postfertilization; MO, morpholino;  $\alpha$ -DG,  $\alpha$ -dystroglycan;  $\beta$ -DG,  $\beta$ -dystroglycan.

\*Present address: Harvard University, Cambridge, MA 02138.

†To whom correspondence should be addressed. E-mail: granatom@mail.med.upenn.edu.

This article contains supporting information online at [www.pnas.org/cgi/content/full/0610822104/DC1](http://www.pnas.org/cgi/content/full/0610822104/DC1).

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**Fig. 1.** UnpFL is essential for focal innervation. (A) Domain organization of UnpFL and UnpSV1, nonmammalian MuSK, and mammalian MuSK. (B and B') In wild-type embryos (lateral view), *en passant* equatorial AChR clusters (red,  $\alpha$ -BTX) are apposed to primary motor axons (green, znp-1/SV2, arrows). (C and C') In *unplugged* mutants, equatorial AChR clusters are absent. (D and D') Before the arrival of growth cones (caudal segments, right), AChRs are prepatterned in a central band (open arrowheads). As growth cones approach (rostral somites, left), they colocalize with discrete AChR clusters (open arrows). (E and E') In *unplugged* mutants, AChR prepatterning is absent. (F and F') Injection of MO against UnpFL abolishes AChR clusters. (G and G') Mosaic expression of UnpFL in *unplugged* muscle restores AChR clustering (arrowhead). (H) Cross-section of chimeric *unplugged* embryo with wild-type muscle clones (red) and AChR clusters (yellow, dashed box). (I and I') Lateral view of same clones. (Scale bar: 50  $\mu$ m.)

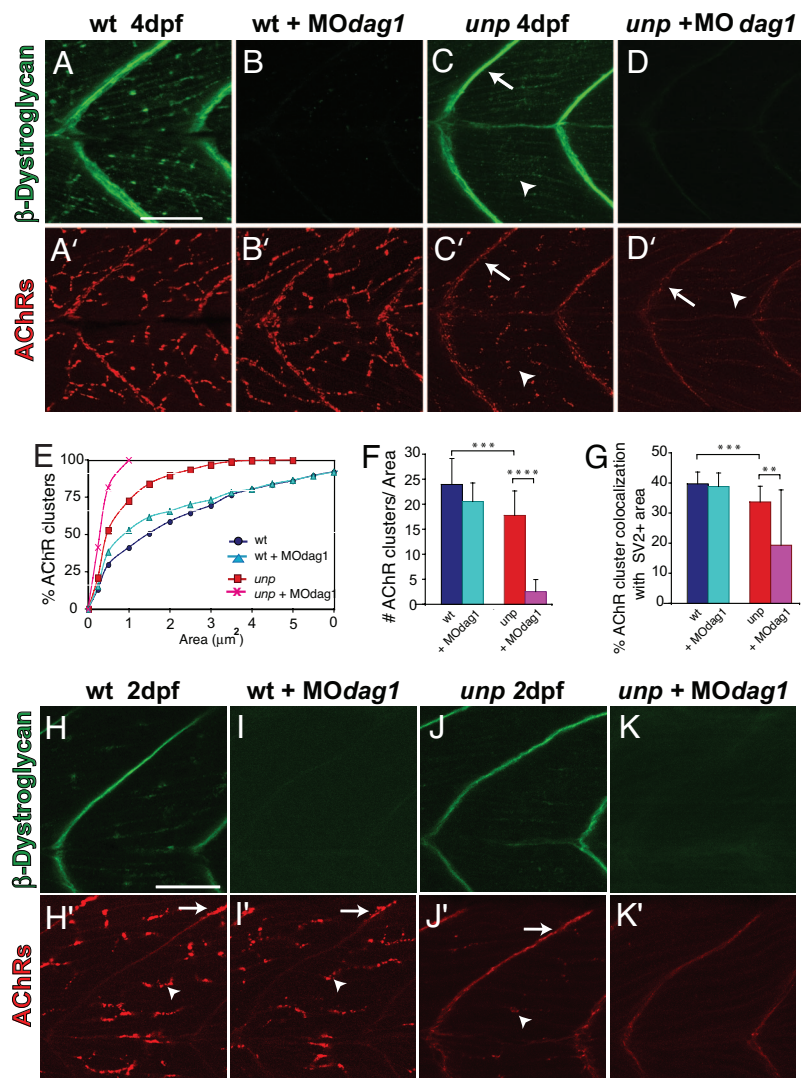
endplate bands, where MuSK initiates clustering of AChRs before signals from the nerve growth cone (10, 11). As motor growth cones extend into the myotome, AChR clusters become restricted along the axon while aneural clusters disperse (27, 28). In *unplugged* mutant embryos, AChR prepatterning is completely absent (Fig. 1E). Thus, similar to mammalian MuSK, *unplugged* is essential for both the formation of focal NMJs and initiation of the AChR prepattern.

**UnpFL Is the Zebrafish MuSK Ortholog.** The *unplugged* locus generates three splice variants, of which the UnpFL encodes a protein isoform most similar to mammalian MuSK (25) (Fig. 1A). To determine whether the UnpFL variant is critical for AChR clustering, we reduced UnpFL expression by using antisense oligonucleotide morpholinos (MOs) previously shown to block expression of functional UnpFL protein (25). Analysis of MO-injected embryos at 26 hpf revealed that knockdown of UnpFL resulted in either a strong reduction or a complete absence of AChR clusters (Fig. 1F) [reduction in 45.2% and absence in 54.8% of hemisegments ( $n = 520$ ); see *Materials and Methods* for details]. Furthermore, expression of myc-tagged UnpFL cDNA in myotomal cells in *unplugged* mutant embryos was sufficient to rescue AChR clustering (Fig. 1G) ( $n = 292$  UnpFL-myc-positive hemisegments displaying AChR clusters in 62 *unplugged* embryos examined). These AChR clusters were consistently apposed to motor axons, even along aberrant axonal projections. In contrast, injection of UnpSV1 cDNA into *unplugged* mutants rarely resulted in AChR clusters, and those that formed were of abnormal morphology and present only on few fibers ( $n = 11$  UnpSV1-GFP-positive hemisegments displayed AChR clusters in 62 *unplugged* embryos). Finally, analyses of

chimeric embryos, in which labeled wild-type cells were transplanted into *unplugged* host embryos, revealed that only wild-type-derived medial myofibers (i.e., those apposed to motor axons) formed AChR clusters ( $n = 114$  wild-type medial muscle clones with AChR clusters in 50 chimeric embryos) (Fig. 1H and I). Thus, like mammalian MuSK (23), *unplugged* functions cell-autonomously in muscle to initiate synapse formation. Taken together, these data demonstrate that UnpFL is a zebrafish MuSK ortholog and induces neuromuscular synapse formation.

The *unplugged* gene plays distinct roles in motor axon guidance and neuromuscular synapse formation, mediated by two receptor tyrosine kinase isoforms, UnpSV1 and UnpFL, respectively. The two isoforms differ in their ectodomain modules and expression profiles. UnpSV1 is expressed only transiently and acts cell-nonautonomously in adaxial cells, which initially border the future axonal path (25, 29, 30). Furthermore, the UnpSV1 ectodomain lacks the Ig domains required for agrin responsiveness (31) and signals independent of rapsyn to provide guidance information for advancing growth cones in a contact-independent manner (25, 30). In contrast, UnpFL is expressed slightly later (25) and throughout the myotome (unpublished data), where it acts cell-autonomously to direct the formation of focal synapses with motor axons. After synaptogenesis, UnpFL mRNA and protein expression are maintained, and both are concentrated at mature neuromuscular synapses (25). Unlike UnpSV1, the UnpFL ectodomain contains the Ig domains required for agrin responsiveness, and functions through *twitch once*/rapsyn as agrin morphants and *twitch once* mutants both lack focal NMJs (32, 33). Thus, UnpFL-mediated AChR clustering likely employs the agrin-MuSK-rapsyn signaling pathway. Together, these data suggest that UnpSV1 and UnpFL





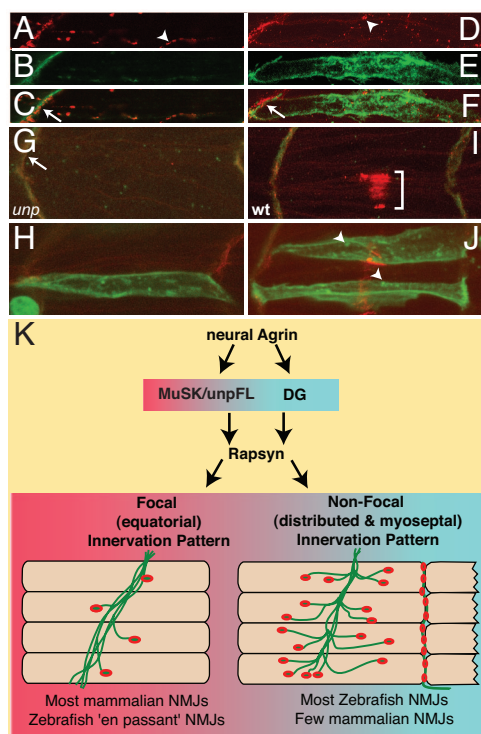
muscle fiber morphology and integrity [supporting information (SI) Fig. 5 A–I]. Reduction of dystroglycan protein levels in *dag1* morphant larvae persisted at least until 96 hpf (SI Fig. 5J), enabling us to examine the role of dystroglycans in NMJ formation.

**Absence of UnpFL/MuSK Reveals a Critical Role for Dystroglycans in the Formation of Distributed and Myoseptal Synapses.** To examine the role of dystroglycans, we injected *dag1*-MO into wild-type and *unplugged* larvae. To quantify the relative effects of dystroglycan reduction on AChR aggregation, we measured the area, number, and synaptic localization of AChR clusters present at distributed synaptic sites (three selected fields per larvae;  $n = 8$  larvae). In 96-hpf wild-type *dag1*-MO-injected larvae, AChR clusters were present along muscle fibers and the myosepta (Fig. 3*A*, *A'*, *B*, and *B'*), and the number of clusters (Fig. 3*F*) and the percent localization of AChR clusters to presynaptic SV2-positive sites (Fig. 3*G*) were similar to those in uninjected wild-type larvae (see [SI Table 1](#)) (cluster number/area,  $P = 0.992$ ; percentage of colocalization,  $P = 0.562$ ; Mann–Whitney test). Only the AChR cluster area in *dag1* morphants showed a slight reduction in cluster area distribution compared with uninjected wild type (Fig. 3*E*, Kolmogorov–Smirnov test). Thus, in the presence of UnpFL/MuSK, removal of dystroglycans does not impair AChR cluster formation at distributed and at myoseptal synaptic sites.

In contrast, removal of dystroglycans in *unplugged* mutants

interfered with AChR clustering. Compared with the distributed AChR clusters present along uninjected *unplugged* muscle fibers (Fig. 3C'), the number and size of these AChR aggregates in *dag1*-MO-injected *unplugged* larvae were significantly reduced or almost absent (Fig. 3D'). Similarly, AChR localization was also markedly reduced along the myosepta when compared with uninjected *unplugged* larvae (Fig. 3D', arrow). Quantification of distributed AChR clusters revealed that dystroglycan knockdown in *unplugged* mutants resulted in a significant reduction in AChR area (Fig. 3E, Kolmogorov–Smirnov test) and a 6-fold reduction in the number of distributed clusters (Fig. 3F) compared with uninjected *unplugged* mutant larvae (Fig. 3G) ( $P < 0.0001$ , Mann–Whitney test) (see [SI Table 1](#)). Thus, removal of dystroglycans in *unplugged* mutants further reduced or even eliminated AChR clustering at distributed synaptic sites. Among the remaining AChR clusters in *dag1*-MO-injected *unplugged* larvae, few colocalized with SV2-positive terminals compared with uninjected *unplugged* ( $P < 0.01$ , Mann–Whitney test). Thus, at nonfocal synaptic sites dystroglycans direct an alternative AChR clustering pathway, which is revealed only in the absence of UnpFL/MuSK signaling.

To distinguish whether the reduction of AChR clusters results from impaired AChR cluster maintenance or from a defect in cluster formation, we examined distributed AChR clusters as they first form. In both uninjected and *dag1*-MO injected wild-type embryos at 48 hpf, neural AChR clusters are present along the



**Fig. 4.** At focal synapses dystroglycan fails to induce AChR clustering. (A–C) Endogenous  $\beta$ -DG (green) and AChRs clusters (red) in wild type. Forced expression of *dystroglycan-myc* in *dag1*-MO-injected *unplugged* mutant larvae restores  $\beta$ -DG localization and clustering of AChRs at distributed (arrowhead) and myoseptal (arrow) sites (D–F). (G–J) High-magnification confocal images of 28-hpf axial muscle pioneers stained for  $\beta$ -DG (green) and AChRs (red). *unplugged* mutants retain myoseptal AChR localization (arrow) but lack focal AChRs (G), even when dystroglycan is overexpressed (H). Wild-type muscle pioneers display large AChR clusters (I, bracket) lacking dystroglycan. Forced dystroglycan expression in muscle pioneers results in aggregates (J, arrowheads), which fail to colocalize with AChR clusters. (K) Proposed model: upon release of neural agrin, *unplugged*/MuSK recruits rapsyn, leading to focal innervation. At sites of distributed and myoseptal innervation, *unplugged*/MuSK and dystroglycan, upon activation by neural agrin, cooperate to recruit rapsyn and direct AChR clustering.

vertical myosepta and distributed along muscle fibers (Fig. 3H' and I'). In *unplugged* mutants, the first small AChR clusters emerge at distributed and myoseptal synaptic sites, consistent with the initiation of distributed and myoseptal innervation (Fig. 3J'). In *dag1*-MO-injected *unplugged* mutants, AChRs were only diffusely localized along the myosepta and along muscle fibers but no AChR clusters were detectable, strongly suggesting that dystroglycan is critical in their aggregation (Fig. 3K') ( $n = 40$  embryos; four individual experiments). We cannot exclude the possibility that dystroglycan acts to stabilize nascent AChR clusters, but the absence of AChR clusters at the earliest stages of synaptogenesis strongly argues that dystroglycan is sufficient for synapse formation in nonfocal innervation. Together, these genetic analyses demonstrate that dystroglycans do not require the function of *unplugged*/MuSK to induce postsynaptic differentiation. Although UnpFL/MuSK is required for the assembly of focal synapses, UnpFL/MuSK signaling cooperates with dystroglycans to direct synapse formation in distributed and myoseptal innervation, resulting in the generation of diverse patterns of neuromuscular innervation.

Finally, we asked whether dystroglycans can compensate for the absence of *unplugged* in the formation of focal synapses. Because  $\beta$ -DG expression is undetectable at focal innervation sites, we used a muscle-specific  $\alpha$ -actin promoter (42, 43) to force expression of Myc-tagged full-length dystroglycan (*dag1*, giving rise to  $\alpha$ -DG and

$\beta$ -DG after posttranslational cleavage). We first ensured that this construct produces functional dystroglycan protein, as injection of this plasmid lacking the MO target sequence into *dag1*-MO-injected *unplugged* embryos resulted in  $\beta$ -DG expression. Moreover, at 96 hpf, AChR clustering and colocalization with dystroglycan at distributed and myoseptal innervation sites was restored, demonstrating that the construct produces functional dystroglycan (Fig. 4A–F). In contrast, analysis of 28-hpf embryos injected with the same construct revealed that in *unplugged* muscle fibers dystroglycan fails to restore AChR clusters at focal synapses (Fig. 4G and H). Furthermore, forced expression of dystroglycan in wild-type muscle fibers did not lead to colocalization of dystroglycan and AChR clusters at focal sites of innervation (Fig. 4I and J). Thus, focal synapse assembly requires only *unplugged*/MuSK signaling, completely independent of dystroglycan. Second, *unplugged*/MuSK is not sufficient to recruit dystroglycan to AChR clusters, but additional, yet unknown, mechanisms exist to control the selective localization of dystroglycan to distributed and myoseptal synapses.

The presence of morphologically intact neuromuscular synapses in *unplugged*-null mutants is the first genetic evidence that fully functional vertebrate neuromuscular synapses can form in the complete absence of MuSK. Recent studies have challenged some of the widely held paradigms on the molecular mechanisms underlying neuromuscular synaptogenesis (reviewed in ref. 8). For example, cell type-specific Neuregulin-1 or ErbB receptor-deficient mice and other double mutant combinations have redefined the roles of Neuregulin ErbB signaling and the role of presynaptic agrin during this process (44–46). Our results that functional neuromuscular synapses can form in the absence of *unplugged*/MuSK challenge the conventional view that vertebrate NMJ formation requires MuSK, at least at nonfocal sites of innervation, and suggest the existence of other genetic pathways that direct neuromuscular synapse formation.

The development of NMJs at nonfocal synaptic sites in the complete absence of *unplugged*/MuSK signaling reveals a previously unappreciated role for dystroglycans in synapse formation. Genetic studies in mice have shown that dystroglycans are required for later aspects of AChR cluster maturation rather than formation, and that dystroglycans are not sufficient to compensate for the absence of MuSK (9, 41, 47, 48). Here, we demonstrate that dystroglycans are sufficient to direct neuromuscular synapse formation at distributed and myoseptal sites of innervation (Fig. 4K). Although we cannot exclude the possibility that this alternative pathway is only engaged when challenged by the absence of MuSK signaling, we propose that, for synapse assembly at nonfocal innervation sites, this pathway occurs concurrently or even cooperates with MuSK signaling. Interestingly, some ligands and downstream effectors may be shared between MuSK and dystroglycan. For example, agrin, which enhances MuSK signaling, could potentially initiate or promote dystroglycan-mediated AChR clustering.  $\alpha$ -DG is known to bind agrin and laminins, and these interactions have been functionally implicated in AChR clustering *in vitro* (18, 37, 49, 50). Similarly, the MuSK effector rapsyn is required for AChR clustering in all patterns of innervation (32). Thus, rapsyn likely participates in dystroglycan-directed AChR clustering by way of its direct interactions with  $\beta$ -DG and AChRs (12, 38, 51). Although elucidation of the precise signaling pathway remains, our results provide clear genetic evidence that dystroglycans play a critical role in the formation of vertebrate neuromuscular synapses.

What is the biological significance of dystroglycan's ability to direct synapse formation at distributed and myoseptal synaptic sites? Patterns of distributed and/or myoseptal innervation are widespread in fish and amphibian species using near synchronous and unilateral activation of body wall muscles to generate rapid, undulating movements, as well as in birds (2–4). Patterns of distributed innervation are also present in some mammalian muscles, most notably in laryngeal as well as in extraocular muscle, one of the fastest mammalian muscles (5, 6). We propose that dystro-

glycans control an evolutionary more primordial postsynaptic differentiation program to provide massive (distributed) and fast muscle activation. The requirement for MuSK signaling may have evolved with progressive positioning of innervation sites to the center of the muscle, to ultimately provide focal and monosynaptic input to individual muscle fibers, providing mammals with superior control over individual muscles fibers. In mammals, where focal innervation predominates, MuSK signaling superimposed the primordial “dystroglycan program,” resulting in the equatorial pattern of focal neuromuscular synapses (Fig. 4K).

Our studies demonstrate that UnpFL/MuSK is critical for synapse development at focal innervation sites but only plays a limited role at nonfocal synaptic sites. This distinction raises the interesting possibility that *unplugged*/MuSK signaling not only serves postsynaptic differentiation, but that it may pattern muscle fibers to confine structural and signaling components to a central region. Polarization of muscle cells in such a manner may be a prerequisite for synapse formation; by ensuring the focal enrichment of postsynaptic components, synapses can be rapidly assembled at a defined innervation site (10, 11, 52). In addition to patterning the presumptive innervation site, *unplugged*/MuSK signaling may also pattern other components along the central muscle cell surface, for example, by modifying the extracellular matrix to guide growth cones along their intended axonal path (25). Interestingly, components of the planar cell polarity pathway with known roles in regulating the organization and dynamics of the cytoskeleton, such as APC, Dishevelled, and small GTPases Rac and Rho, have been identified as downstream effectors of MuSK and agrin-mediated AChR clustering (53–56). Although the roles of these factors in vertebrate NMJ development have yet to be examined *in vivo*, they are involved in multiple morphogenetic processes and neuronal con-

nectivity (57–60). The presence of a *frizzled*-like cysteine-rich domain in MuSKs implicates Wnt signaling in MuSK-induced postsynaptic differentiation, and future studies may reveal a Wnt-related MuSK pathway.

## Materials and Methods

**Zebrafish Strains and Breeding.** Wild-type and *unplugged* mutant strains were maintained in a Tu or TL genetic background and raised as described (61). For all experiments we used *unplugged*<sup>thr307</sup> and *unplugged*<sup>lbb72</sup>-null alleles (25).

**Antisense MO Oligonucleotide Injections.** Approximately 5–6 ng of *unplugged* FL MO (25) and/or  $\alpha$ -DG and  $\beta$ -DG MO (40) was injected into one-cell embryos. Dag-1 morphants exhibiting trunk defects and muscular dysmorphogenesis (40) were discarded.

**Chimeric Embryos.** Chimeric embryos were generated by performing cell transplantations of rhodamine-labeled wild-type donor cells into *unplugged* mutant hosts as previously described in ref. 29.

**Electron Microscopy.** At 4–5 days after fertilization, larvae were fixed and processed as described previously (26). Synaptic profiles were quantified by the presence of a presynaptic bouton morphology, characterized by clustered synaptic vesicles and pre- and postsynaptic membrane densities.

**Additional Details.** See *SI Materials and Methods*.

We thank laboratory members for helpful discussions and advice on the manuscript. This work was supported by grants from the National Science Foundation and the National Institutes of Health (to M.G.).

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