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The University of Osaka

博士論文

筋特異的キナーゼの活性化に必要な二量体構造と

その構造基盤の解明

Structural Basis for the Dimeric Activation Mechanism of
Muscle-Specific Kinase

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Abstract

Muscle-specific kinase (MuSK) is a single-pass transmembrane receptor tyrosine kinase essential for neuromuscular junction (NMJ) formation and maintenance. Ligand-induced MuSK dimerization activates signaling pathways that promote clustering of acetylcholine receptors (AChRs) at the NMJ, a process required for normal muscle contraction. Dysregulation of MuSK signaling reduces AChR density and causes neuromuscular disorders, yet the structural basis of MuSK activation remains poorly understood.

Physiological activation of MuSK depends on Agrin, a motor neuron-derived protein that activates MuSK indirectly through formation of the Agrin-LRP4-MuSK (AML) complex. This indirect mechanism complicates structural analysis of the active MuSK dimer. Here, we define the extracellular structural requirements for signaling-competent MuSK dimerization by combining domain-level functional analysis with artificial dimerization strategies.

Functional analyses of MuSK extracellular domain deletion mutants revealed that the contiguous Ig1-Ig2 domains are essential for Agrin-dependent activation. Introduction of flexibility between Ig1 and Ig2 abolished AChR clustering despite preserved autophosphorylation, indicating that Ig1 and Ig2 function as a rigid tandem unit to enforce a productive dimer geometry.

To further probe orientation dependence, we engineered artificial MuSK dimerizers based on a high-affinity MuSK-binding peptide, L1. Two L1-fused Fc constructs with opposite fusion orientations displayed comparable MuSK-binding affinity and dimerization capacity; however, only Fc-L1 functioned as a MuSK agonist. Structural modeling suggested that Fc-L1 aligns two MuSK molecules in an Ig1-proximal configuration resembling that induced by Agrin.

Together, these findings demonstrate that MuSK activation requires not merely dimerization but a specific extracellular orientation, with Ig1 domain proximity being critical for downstream signaling.

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List of Abbreviations

• Base abbreviations

A	Adenine
C	Cytosine
G	Guanine
T	Thymine

• Amino acid abbreviations table

1-Letter	3-Letter	Amino acid
A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic Acid
E	Glu	Glutamic Acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine

AChR	Acetylcholine receptor
AML complex	Agrin–MuSK–LRP4 complex
BTX / α-BTX	α -Bungarotoxin
C2C12	Mouse myoblast cell line
DMEM	Dulbecco's Modified Eagle Medium
DOK7	Docking Protein 7
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
Fc	Fragment crystallizable region of IgG
Fz domain	Frizzled-like domain
Ig domain	Immunoglobulin-like domain
IP	Immunoprecipitation
JM	Juxtamembrane domain
KO	Knock out
LgBiT	Large subunit of NanoBiT luciferase
LRP4	Low-density lipoprotein receptor–related protein 4
MAP tag	MAP epitope tag
MG	Myasthenia gravis
MSD	Meso Scale Discovery
MuSK	Muscle-specific kinase
NanoBiT	NanoLuc Binary Technology
NEAA	Non-essential amino acids
NMJ	Neuromuscular junction
NZ-1	Anti-PA-tag monoclonal antibody
PA tag	PA epitope tag
PBS	Phosphate-buffered saline
PMab-1	Anti-MAP-tag monoclonal antibody
RTK	Receptor tyrosine kinase
SDS–PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SEC	Size-exclusion chromatography
SmBiT	Small subunit of NanoBiT luciferase
SPR	Surface plasmon resonance
TBS	Tris-buffered saline
TKD	Tyrosine kinase domain
TM	Transmembrane domain
WT	Wild-type

1. General Introduction

Receptor tyrosine kinases (RTKs) constitute a large family of single-pass transmembrane proteins that mediate a wide variety of signaling pathways in multicellular organisms^{1,2}. Many RTKs are activated through ligand-induced dimerization or oligomerization events occurring at the cell surface. Upon ligand binding, two receptor molecules are brought into close proximity in a defined orientation, which promotes trans-autophosphorylation of their intracellular kinase domains and triggers downstream signaling cascades^{1,3}. Because RTK-mediated signaling regulates fundamental processes such as cell proliferation, differentiation, and survival, dysregulation of RTK activity leads to a broad spectrum of human diseases^{1,4}. Conversely, aberrant hyperactivation of RTKs is also a hallmark of oncogenic transformation⁵. These facts show that RTKs generally require strictly controlled dimeric architectures-both in distance and orientation-to achieve their biologically meaningful activated states⁶.

Among well-characterized RTKs whose activation mechanisms have been structurally defined, the epidermal growth factor receptor (EGFR) is a representative example. EGFR consists of four extracellular domains (domains I-IV), a transmembrane (TM) helix, a juxtamembrane (JM) domain, a tyrosine kinase domain, and a C-terminal regulatory tail (Fig. 1a). The extracellular region of EGFR dynamically transitions between a tethered and an extended conformation⁷. In the absence of its ligand EGF, the tethered form is favored, whereas interaction with EGF stabilizes the extended conformation⁷. In this activated state, EGFR dimerizes via the dimerization arm located opposite to the ligand-binding site⁸. This dimerization constrains the relative orientations of the TM and JM domains, ultimately enabling formation of an asymmetric active dimer of the intracellular kinase domains, leading to trans-autophosphorylation^{9,10}. Phosphorylated tyrosine residues within EGFR recruit adaptor proteins such as Shc, Grb2, and SOS, thereby activating downstream pathways including the MAPK, Akt, and mTOR cascades (Fig. 1b)¹¹. Consequently, EGFR activation induces diverse cellular responses, including proliferation and differentiation. Structural studies have revealed many of these conformational states, and it is now well-established that ligand-induced EGFR activation is highly regulated; mere physical proximity of two EGFR molecules is insufficient to elicit kinase activation⁹. Moreover, even when autophosphorylation occurs, some dimeric arrangements fail to activate downstream signaling such as Akt, underscoring the importance of precise receptor geometry¹².

(a)



(b)

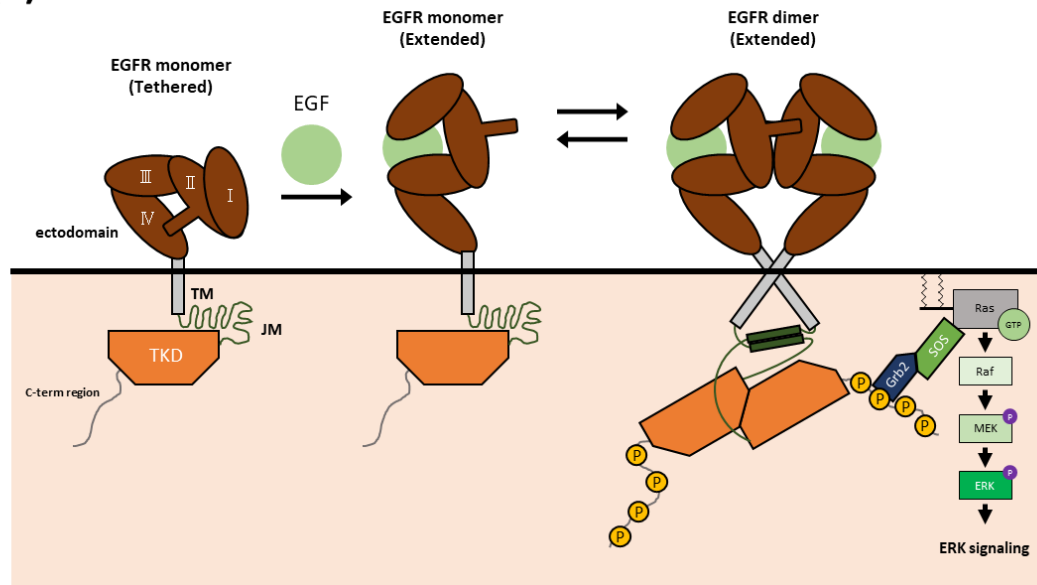


Fig.1 : Activation mechanism of the epidermal growth factor receptor (EGFR)

- (a) Domain organization of EGFR. EGFR consists of four extracellular domains (I–IV), a transmembrane (TM) segment, a juxtamembrane (JM) region, a tyrosine kinase domain (TKD), and a C-terminal regulatory tail.
- (b) Schematic of EGFR activation. In the absence of ligand, the extracellular region predominantly adopts a tethered conformation. Binding of EGF stabilizes the extended conformation and exposes the dimerization interface, leading to formation of an extracellular dimer. This, in turn, promotes formation of an asymmetric active dimer of the TKDs and induces autophosphorylation of tyrosine residues. Phosphorylated tyrosines recruit adaptor proteins such as Grb2, which in turn recruits SOS, resulting in activation of Ras-GTP, stimulation of the Erk signaling pathway, and ultimately induction of cellular responses such as proliferation.

Among RTKs, I focused on muscle-specific kinase (MuSK). MuSK is a single-pass transmembrane receptor expressed on skeletal muscle cells, composed of four extracellular domains (Ig1, Ig2, Ig3, and Fz), a transmembrane segment, a juxtamembrane (JM) region, and an intracellular tyrosine kinase domain (TKD) (Fig. 2a)¹³. Unlike many RTKs that are activated by a single ligand, MuSK activation requires two distinct components: the neuron-derived protein Agrin and its muscle-resident co-receptor LRP4^{14,15}. These three molecules assemble into Agrin-MuSK-LRP4 (AML) complex (Fig. 2b)¹⁶⁻¹⁸. AML complex is thought to form higher-order assemblies containing two molecules each of Agrin, MuSK, and LRP4^{14,17,19}, within which MuSK undergoes dimerization, leading to autophosphorylation of MuSK intracellular region^{20,21}.

Autophosphorylation of MuSK begins at Tyr553 located in the JM region²². Phosphorylated Tyr553 (pTyr553) is specifically recognized by DOK7, a cytoplasmic adaptor expressed in skeletal muscle^{23,24}. Interaction between two DOK7 molecules and two MuSK molecules induces formation of MuSK-DOK7 complex. Formation of this complex induces phosphorylation of tyrosine residues within MuSK kinase domain, resulting in full activation of the receptor²⁵. Activated MuSK triggers AChR clustering signal, which is essential for the formation and maintenance of neuromuscular junction (NMJ)^{26,27}.

NMJ is a specialized synaptic junction that mediates signal transmission from motor neurons to muscle fibers^{28,29}. Upon activation by Agrin and LRP4, MuSK induces clustering of acetylcholine receptor (AChR) at the postsynaptic membrane^{14,22}. These clustered AChR serve as ligand-gated sodium ion channels that respond to acetylcholine released from motor neurons, initiating depolarization of the muscle membrane and ultimately driving muscle contraction once the threshold is reached³⁰. Thus, proper localization of AChR to the NMJ is indispensable for normal muscle contraction³¹.

Impaired MuSK signaling decreases synaptic AChR density and is implicated in a range of neuromuscular disorders³¹. A prominent example is myasthenia gravis (MG)³², an autoimmune disease caused by the production of autoantibodies targeting key proteins required for NMJ maintenance³². Among these targets is MuSK; anti-MuSK autoantibodies can interfere with assembly of the Agrin-LRP4MuSK signaling complex and thereby attenuate MuSK activation (Fig. 2c)^{33,34}. Consequently, AChR clustering at the postsynaptic membrane is disrupted, resulting in compromised neuromuscular transmission and muscle weakness³¹. Because neuromuscular diseases frequently impair muscle strength and severely affect patients' quality of life (QOL), understanding the molecular basis of MuSK activation is of great clinical importance. Nevertheless, despite its central role in NMJ formation, the activation mechanism of MuSK remains incompletely understood²⁹.

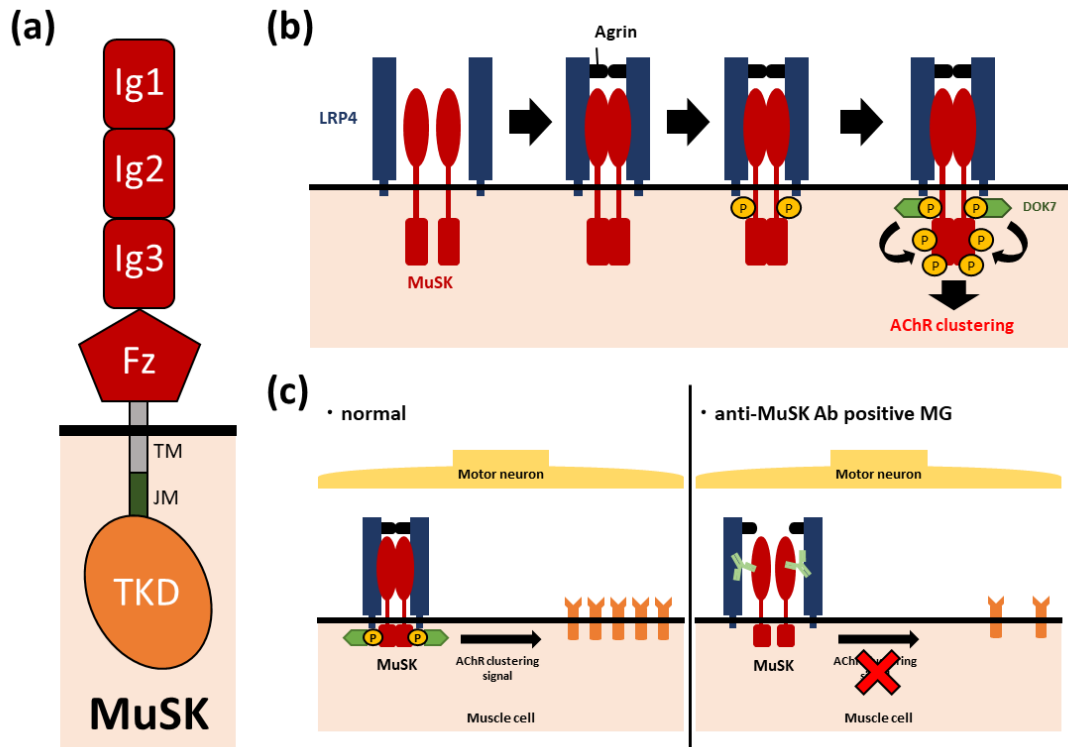


Fig.2 : Activation of muscle-specific kinase (MuSK) and its dysfunction in neuromuscular disease

- (a) Domain organization of MuSK. MuSK comprises four extracellular domains (Ig1, Ig2, Ig3, and Fz), a transmembrane (TM) segment, a juxtamembrane (JM) region, and a tyrosine kinase domain (TKD).
- (b) Schematic of MuSK activation. MuSK forms a complex with Agrin and its co-receptor LRP4 (AML complex), which induces MuSK dimerization. Autophosphorylation of Tyr553 in the JM region (pTyr553) is followed by recruitment of the adaptor protein DOK7, resulting in formation of a 2:2 MuSK–DOK7 complex. This complex promotes phosphorylation of the TKD and triggers the downstream signal that induces AChR clustering.
- (c) Mechanism by which anti-MuSK autoantibodies cause myasthenia gravis. Autoantibodies against MuSK block formation of the AML complex and suppress AChR clustering signals at the neuromuscular junction (NMJ). As a consequence, the number of AChRs at the NMJ decreases, impairing neuromuscular transmission and leading to muscle weakness and fatigability.

AChR clustering can also be induced by agonistic anti-MuSK antibodies, several of which have been developed as therapeutic candidates³⁵⁻⁴¹. However, not all anti-MuSK antibodies function as agonists^{33,34,42}, implying-as in many other RTKs-that MuSK activation requires a

highly specific dimeric arrangement rather than simple crosslinking. At present, the relative orientation of the two MuSK molecules within the AML complex remains unknown, and it is unclear what dimeric geometry is required to trigger downstream signaling²⁹. Identification of an activation-competent MuSK dimer configuration would greatly facilitate rational design of MuSK agonists.

In this study, I sought to elucidate how two MuSK molecules must be arranged to induce downstream AChR clustering signaling. In Chapter 2, I examined which extracellular domains of MuSK are essential for Agrin–LRP4–dependent activation and how each domain contributes to the process. In Chapter 3, I utilized a high-affinity MuSK-binding peptide—whose interaction mode with MuSK was previously revealed by crystallography—to engineer peptide-based MuSK agonists and investigated how these artificial dimerizers bring two MuSK molecules together. In Chapter 4, I performed functional analyses of this MuSK-binding peptide. Finally, based on these results, I discuss the structural requirements for activation-competent MuSK dimers and the contributions of individual extracellular domains to MuSK-mediated signaling. The results of this study demonstrate that, in both Agrin-dependent and Agrin-independent activation, specific orientations of two MuSK molecules are required for inducing AChR clustering signal. Furthermore, all four extracellular domains in MuSK must be brought together in appropriate relative orientations in order to induce downstream signaling.

2. Dissection of MuSK functional domain

2.1. Introduction

As described in Chapter 1, activation of MuSK requires formation of the Agrin–MuSK–LRP4 (AML) complex at the muscle cell surface. However, how this complex is assembled and how its assembly leads to MuSK activation remain incompletely understood⁴³. Biochemical studies suggest that MuSK does not directly interact with Agrin; rather, LRP4 engages both MuSK and Agrin to mediate AML complex formation (Fig. 3a)^{16,17}. The Agrin–LRP4 interaction has been elucidated by crystallography and subsequent biochemical validation (Fig. 3b)¹⁹. In contrast, MuSK-mediated interactions and the dimeric arrangement of MuSK within the AML complex remain poorly defined.

The extracellular region of MuSK comprises three immunoglobulin-like domains (Ig1–Ig3) followed by a Frizzled-like cysteine-rich domain (Fz)¹³. Among these, the Ig1 domain is known to be indispensable for MuSK activation⁴⁴. Ig1 is thought to contribute to MuSK activation through two distinct roles. First, Ig1 domain functions as a homotypic interaction module that enables dimerization of MuSK within the AML complex⁴⁵. A previously reported crystal structure of the MuSK Ig1–Ig2 fragment suggested that MuSK forms a dimer through a hydrophobic interface involving residues such as M48 and L83 located in Ig1 (Fig. 3c), and mutational studies have shown that substitution of either of these residues with arginine abolishes Agrin-dependent MuSK phosphorylation⁴⁵.

Second, the Ig1 domain has also been reported to mediate the interaction with LRP4 during AML complex formation¹⁷. A recent electron microscopy study visualized a ternary complex containing one molecule each of Agrin, MuSK, and LRP4, in which the Ig1 domain of MuSK engages the β 3 domain of LRP4 (Fig. 3d)¹⁸. Biochemical studies further support the importance of the β 3 domain in MuSK–LRP4 binding, indicating that the Ig1– β 3 interaction plays a central role in MuSK activation¹⁷. Nevertheless, whether this interaction is strictly required for MuSK activation has not been experimentally verified using MuSK mutants. To clarify these issues, functional analyses at both the domain and residue levels are required⁴³.

Although previous studies have established that Ig1 is essential for MuSK function, this does not imply that the remaining extracellular domains are dispensable. Indeed, a MuSK variant lacking all extracellular domains except Ig1 fails to induce Agrin-dependent AChR clustering⁴⁴. What remains unclear is which domain(s) outside Ig1 contributes to MuSK activation and through what mechanisms.

In this chapter, I first established a cell-based experimental system that allows

quantitative evaluation of MuSK activation using MuSK-knockout C2C12 cells complemented with MuSK mutants. Using this system, I assessed how various domain-deleted or point-mutated MuSK constructs affect MuSK autophosphorylation and AChR clustering. These analyses revealed several features of the activation-competent MuSK dimer. First, both Ig1 and Ig2 domains were essential for Agrin-dependent AChR clustering. Moreover, structural and functional domain analyses allowed identification of key residues within Ig1 that are critical for activation and further suggested a mechanistic role for Ig2 in facilitating AChR clustering. Importantly, some MuSK mutants exhibited robust autophosphorylation yet completely failed to induce AChR clustering, demonstrating that MuSK autophosphorylation alone is insufficient for downstream signaling, and that MuSK must be recruited into a specific dimeric orientation to trigger AChR clustering. These results collectively underscore that MuSK activation is a geometrically constrained process requiring both domain composition and proper extracellular alignment.

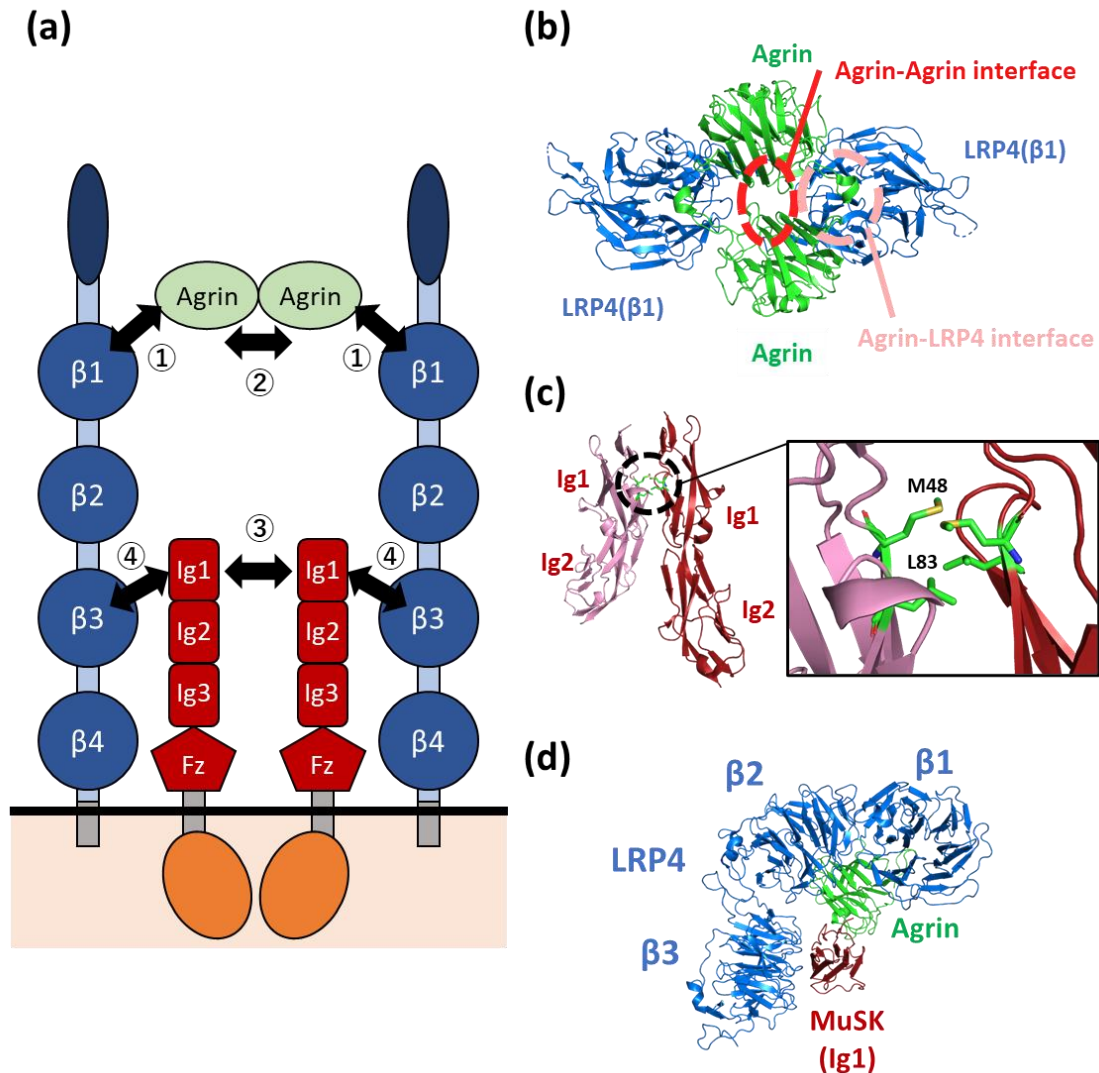


Fig.3 : Overview of the Agrin-MuSK-LRP4 (AML) complex and related structural information

- Schematic representation of known interactions within the AML complex. Four types of interactions have been reported: (1) Agrin-LRP4 interaction, (2) Agrin-Agrin dimerization, (3) MuSK-MuSK dimerization, and (4) MuSK-LRP4 interaction.
- Crystal structure of the Agrin-LRP4 $\beta 1$ -domain complex (PDB ID: 3V64). Two Agrin molecules bind one LRP4 $\beta 1$ domain each, forming a 2:2 complex.
- Crystal structure of the MuSK Ig1-Ig2 fragment (PDB ID: 2IEP). In the crystal, two MuSK molecules form a dimer through an interface that includes residues M48 and L83 in Ig1.
- Cryo-EM structure of a ternary complex containing one molecule each of Agrin, LRP4, and MuSK (PDB ID: 8S9P). In this structure, the MuSK Ig1 domain interacts with the $\beta 3$ domain of LRP4.

2.2. Result

2.2.1. AChR clustering assay using the C2C12 cell line

To functionally analyze MuSK mutants, it was necessary to establish an experimental system capable of quantitatively evaluating MuSK activation in a cellular context. Because the final output of MuSK signaling is the induction of AChR clustering, such an assay requires a muscle-derived cell line that endogenously expresses the necessary machinery, including LRP4 and AChR. However, wild-type muscle cells naturally express endogenous MuSK on their plasma membrane, making it impossible to assess the activity of exogenously introduced MuSK mutants without interference. Thus, to evaluate mutant function, endogenous MuSK must be knocked out and replaced with the mutant constructs.

C2C12 cells, a widely used mouse myoblast cell line, represent an appropriate model system⁴⁶. Upon differentiation into myotubes, C2C12 cells upregulate MuSK, LRP4, and AChR expression, and respond robustly to Agrin stimulation by inducing AChR clustering. Before proceeding, I confirmed that Agrin treatment indeed induced MuSK phosphorylation and AChR clustering in differentiated C2C12 myotubes.

For Agrin stimulation, I used a C-terminal Agrin fragment (AgrinC50), consisting of ~50 kDa of the human Agrin C-terminus (Fig. 4a)⁴⁷. Previous studies indicate that this region is both necessary and sufficient to induce AChR clustering, and therefore I adopted the same construction. Purified AgrinC50 (Fig. 4b) induced dose-dependent increases in MuSK phosphorylation and AChR clustering in C2C12 myotubes (Fig. 4c–e). Based on these results, AgrinC50 was used as the standard stimulus for all subsequent assays.

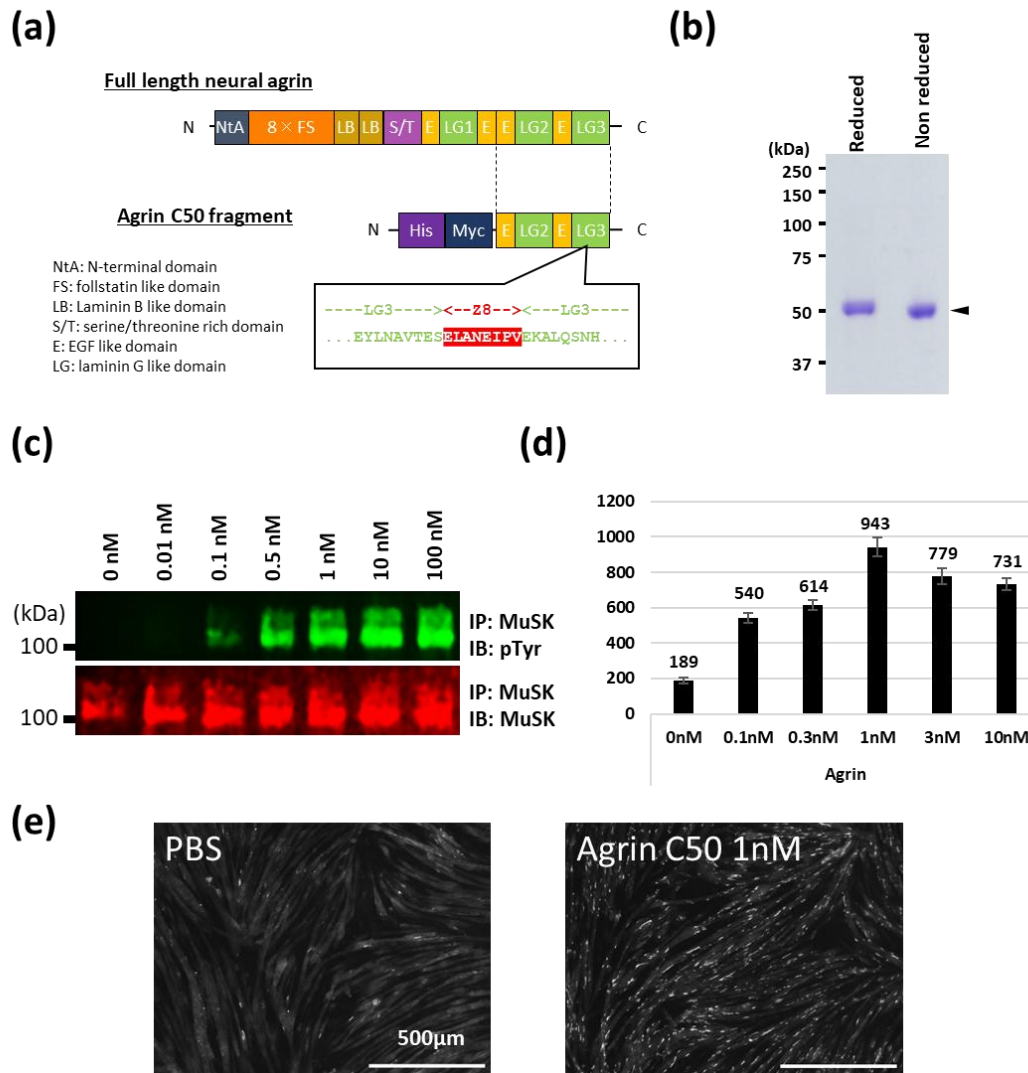


Fig.4 : Activation of MuSK by the AgrinC50 fragment

- Domain organization of full-length Agrin and the AgrinC50 fragment used in this study.
- SDS-PAGE analysis of purified AgrinC50 (2 µg per lane).
- MuSK phosphorylation in C2C12 myotubes upon AgrinC50 treatment. The lower panel shows total MuSK levels, and the upper panel shows phosphotyrosine signals.
- Quantification of AChR clustering induced by AgrinC50. For each condition, 35 fields were imaged and the number of AChR clusters per field was counted and averaged.
- Representative images of AChR clustering in C2C12 myotubes treated with 1 nM AgrinC50. Each white dot corresponds to a cluster of AChRs.

2.2.2. Establishment of MuSK knockout (KO) C2C12 cell line

Having validated C2C12 myotubes as a suitable system for assessing MuSK activation, I next established a MuSK KO C2C12 line. CRISPR/Cas9-mediated disruption of MuSK has been reported previously⁴⁸, and I followed a similar strategy.

I used the lentiCRISPR v2 vector (Addgene #52691), which encodes both Cas9 and the guide RNA (Fig. 5a). The same guide RNA sequence from the prior report was used. After transfection of C2C12 cells with the plasmid, puromycin selection enriched for transfected cells. Surviving cells were subsequently subjected to limiting dilution cloning, and individual clones were screened for loss of MuSK expression.

Two complementary methods were used for validation. First, undifferentiated myoblasts were analyzed by flow cytometry using two independent anti-MuSK antibodies; KO clones exhibited no detectable shift relative to unstained controls (Fig. 5b). Second, differentiated myotubes from candidate clones were lysed and subjected to anti-MuSK immunoprecipitation followed by Western blotting, which confirmed complete absence of MuSK (Fig. 5c).

To verify functional knockout, myotubes derived from the MuSK KO line were treated with AgrinC50 and examined for AChR clustering. As expected, no clustering was observed (Fig. 5d). Thus, this MuSK KO C2C12 line was used in all following experiments.

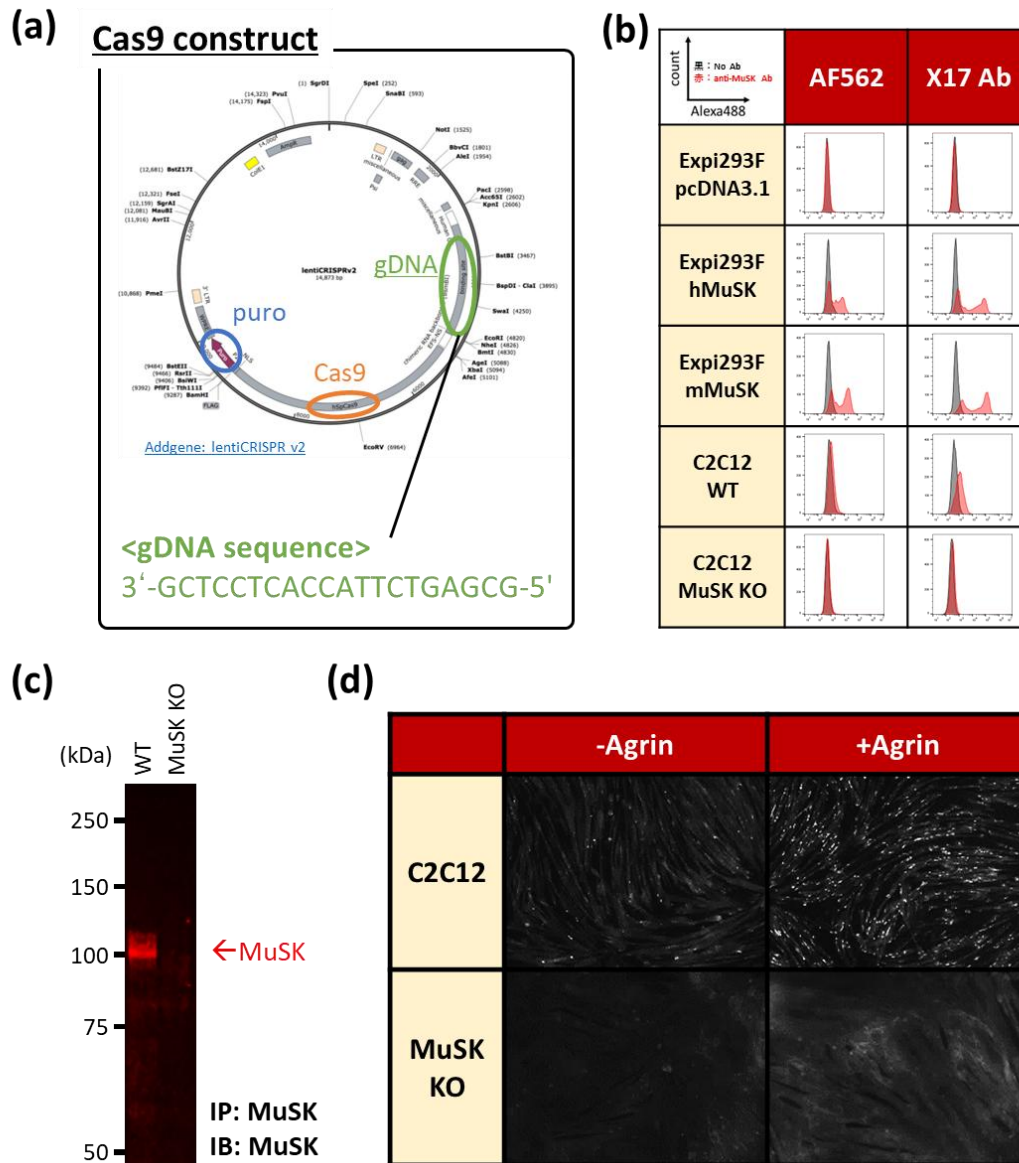


Fig.5 : Establishment of a MuSK knockout (mMuSK KO) C2C12 cell line

- (a) Schematic of the Cas9 construct and guide RNA sequence used to generate the mMuSK KO C2C12 line.
- (b) Flow cytometric analysis of MuSK expression in mMuSK KO C2C12 myotubes. Histograms show unstained cells (gray) and cells stained with different anti-MuSK antibodies (red). No peak shift is detected in the KO line for either antibody, indicating loss of MuSK expression.
- (c) Confirmation of MuSK knockout by immunoprecipitation–Western blot. Cell lysates from wild-type and mMuSK KO C2C12 myotubes were subjected to IP with the anti-MuSK antibody X17, followed by immunoblotting with an anti-rMuSK antibody (which cross-reacts with mMuSK). A MuSK band is detected only in wild-type myotubes.
- (d) Evaluation of AChR clustering in mMuSK KO C2C12 myotubes upon AgrinC50 treatment. In contrast to wild-type C2C12 myotubes, no AgrinC50-induced AChR clustering is observed in the KO line, confirming functional loss of MuSK.

2.2.3. Expression and functional rescue of human MuSK in MuSK KO C2C12 cell

With the MuSK KO cell line established, I next asked whether transient expression of human MuSK (hMuSK) could restore Agrin-dependent MuSK signaling.

I generated an expression construct encoding hMuSK(WT) fused at the N-terminus to a MAP tag⁴⁹ (MAP-hMuSK(WT); Fig. 6a). Transfection was performed in the myoblast state (30–50% confluency), followed by differentiation into myotubes. Immunoprecipitation of MuSK confirmed expression exclusively in MAP-hMuSK-transfected cells (Fig. 6b).

To evaluate functional rescue, MuSK autophosphorylation and AChR clustering were measured following Agrin stimulation. Autophosphorylation of MuSK increased following Agrin addition and peaked at 4 h (Fig. 6c). Furthermore, Agrin-dependent AChR clustering—absent in MuSK KO myotubes—was restored in MAP-hMuSK(WT)-expressing myotubes (Fig. 6d).

The distribution pattern of AChR clusters differed somewhat from wild-type C2C12 myotubes, likely due to variable expression levels of transiently introduced hMuSK among cells.

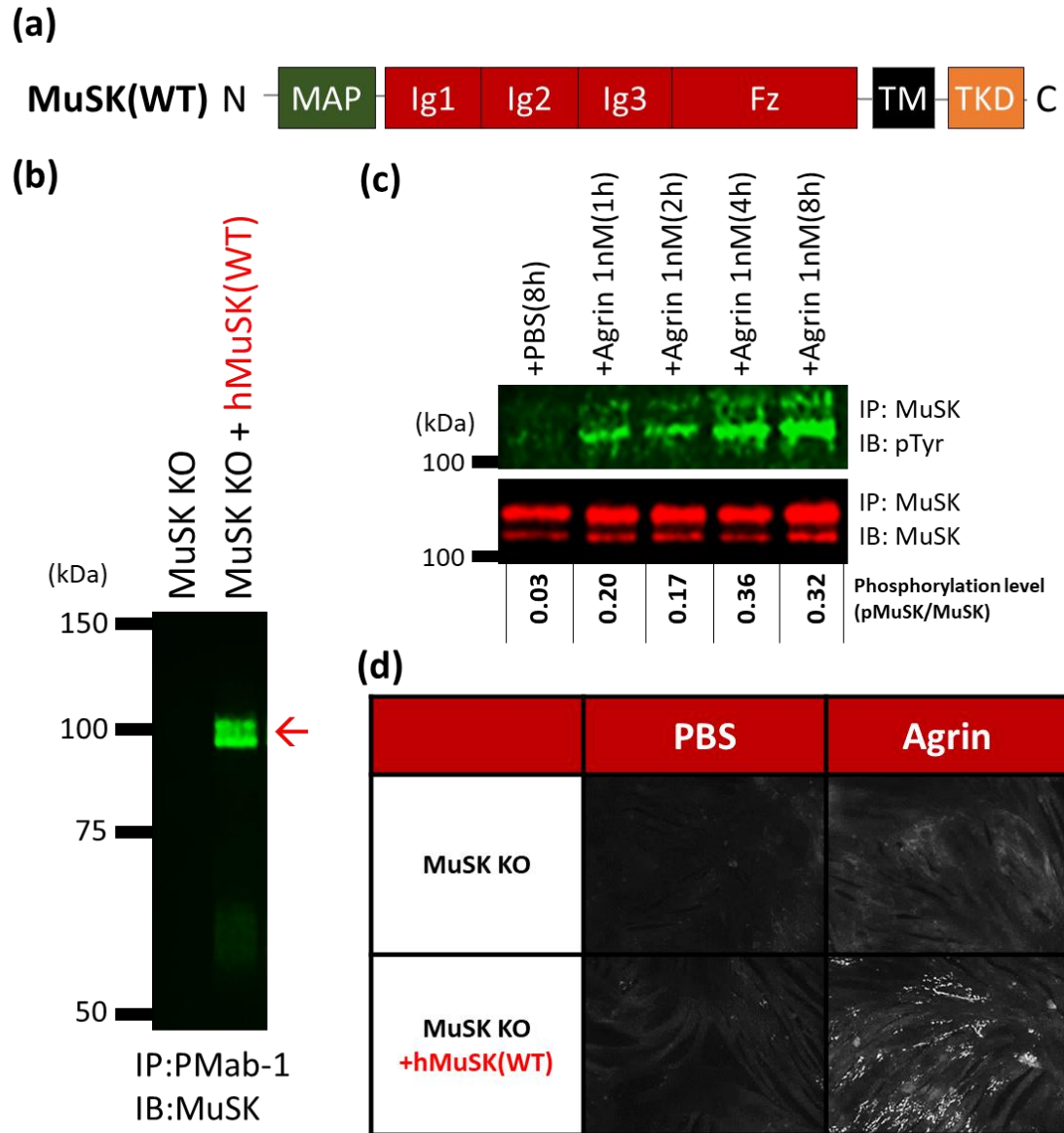


Fig.6 : Rescue of AChR clustering in mMuSK KO C2C12 cells by transient expression of hMuSK

- (a) Schematic of the hMuSK(WT) construct used for transient expression.
- (b) Expression of hMuSK(WT) in mMuSK KO C2C12 cells confirmed by IP–Western blot. Cells were transfected in the myoblast state and differentiated into myotubes before lysis and analysis.
- (c) Time course of hMuSK(WT) phosphorylation in response to 10 nM AgrinC50. The upper panel shows phosphotyrosine signals, and the lower panel shows total MuSK levels. Quantified phosphorylation levels are shown as the ratio of phosphotyrosine signal to total MuSK signal for each time point.
- (d) Restoration of AgrinC50-induced AChR clustering in mMuSK KO C2C12 myotubes by transient expression of hMuSK(WT).

2.2.4. Ig1 domain is essential for Agrin-dependent induction of AChR clustering

Having established that transient expression of human MuSK (hMuSK) in MuSK-knockout (KO) C2C12 myotubes restores Agrin-dependent AChR clustering, I next performed functional domain analyses using a series of MuSK mutants. As an initial validation of the assay, I revisited a MuSK construct lacking the Ig1 domain, which has been reported to be indispensable for MuSK activation, to confirm that our system reproduced the expected phenotype. The construction design is shown in Fig. 7a.

Upon transient expression of wild-type MuSK and the Ig1-deleted construct in Expi293F cells, flow cytometry indicated comparable cell-surface expression levels (Fig. 7b). I then expressed these constructs in MuSK KO C2C12 myotubes and quantified Agrin-induced MuSK phosphorylation and AChR clustering. Notably, MuSK(Ig23Fz) exhibited clear Agrin-dependent tyrosine phosphorylation of the intracellular region, comparable to wild-type MuSK (Fig. 7c). However, MuSK(Ig23Fz) completely failed to restore Agrin-dependent AChR clustering (Fig. 7d-e).

Thus, Agrin can elicit robust MuSK phosphorylation even in the absence of Ig1, yet this phosphorylation is not sufficient to drive the downstream cellular output. Consistent with prior reports, these results confirm that the Ig1 domain is essential for Agrin-dependent AChR clustering. Accordingly, in subsequent analyses, I prioritized AChR clustering as the primary functional readout for MuSK activation rather than relying solely on autophosphorylation.

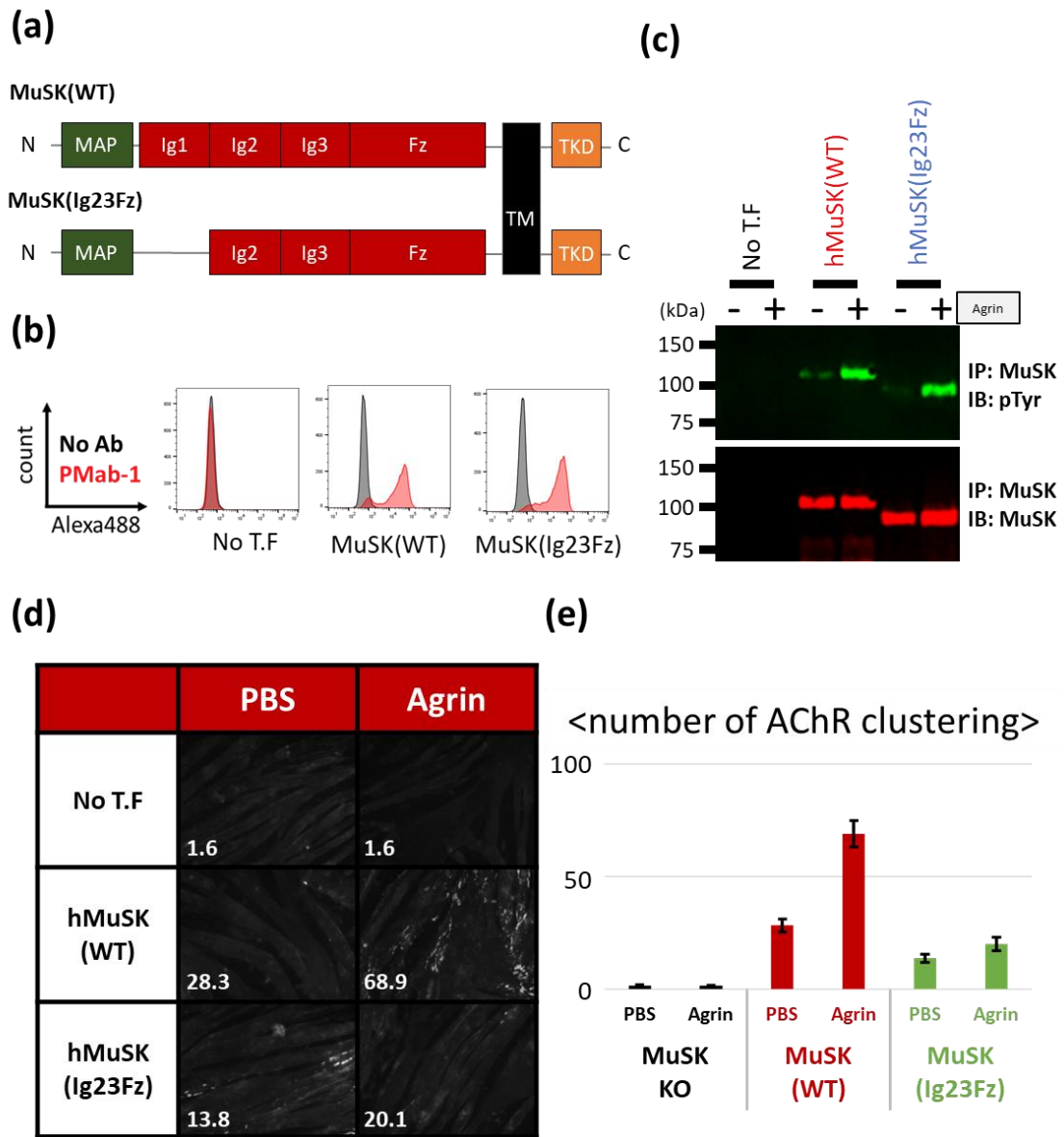


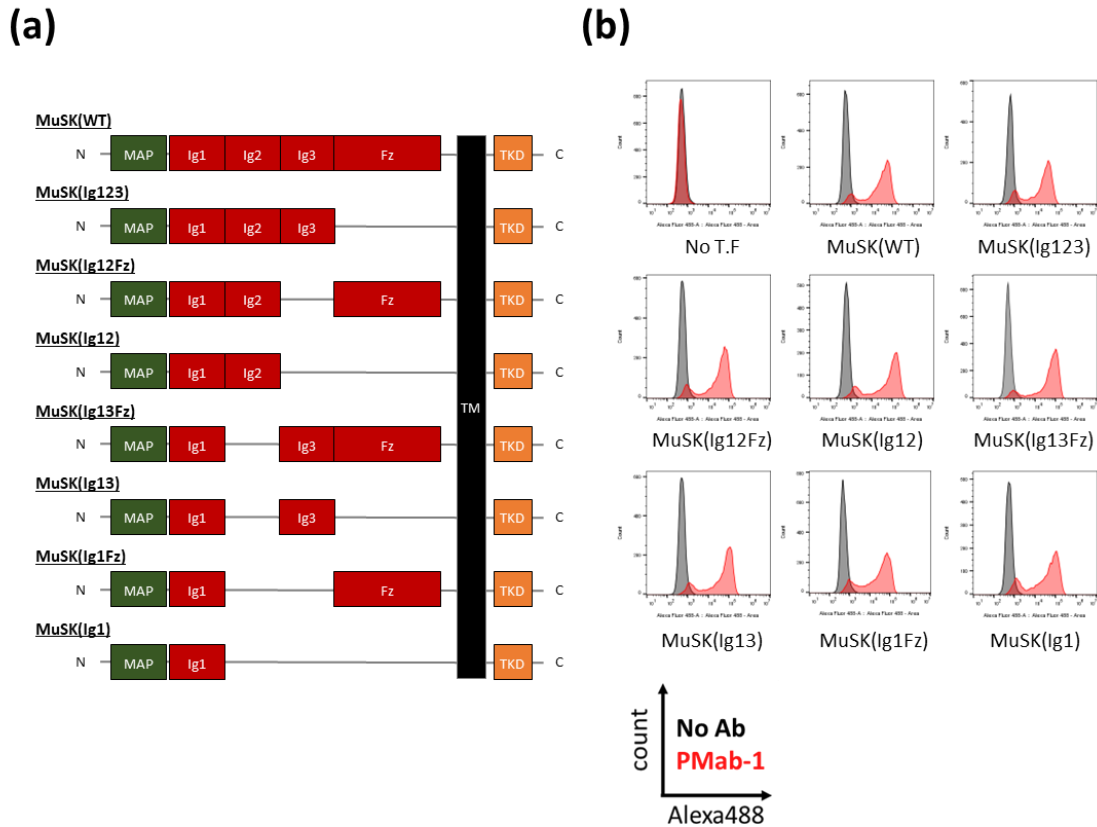
Fig.7 : Effect of Ig1 domain deletion on MuSK activation

- (a) Schematic of the constructs used: MAP-tagged hMuSK(WT) and hMuSK(Ig23Fz).
- (b) Flow cytometric analysis of expression levels of hMuSK(WT) and hMuSK(Ig23Fz) upon transient expression in Expi293F cells.
- (c) Autophosphorylation of hMuSK(Ig23Fz) in response to AgrinC50.
- (d) Representative images showing AChR clustering in mMuSK KO C2C12 myotubes expressing hMuSK(WT) or hMuSK(Ig23Fz) and stimulated with AgrinC50. The numbers in each panel indicate the average number of AChR clusters per field (XX fields analyzed per condition).

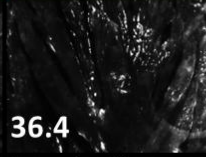
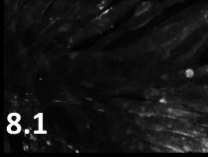
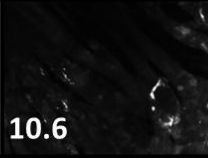
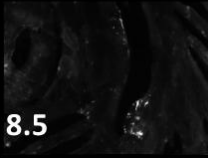
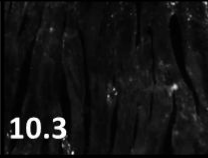
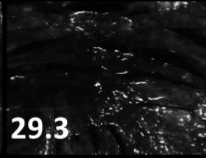
2.2.5. Ig2 domain is also essential for Agrin-dependent induction of AChR clustering

Consistent with previous reports, I confirmed that the Ig1 domain is indispensable for Agrin-dependent AChR clustering. I examined whether other extracellular domains also contribute to this response. I generated a panel of domain-deletion mutants (Fig. 8a) and confirmed by flow cytometry that all constructs were expressed at same levels comparable to MuSK(WT) in Expi293F cells (Fig. 8b).

These mutants were then transiently expressed in MuSK KO C2C12 myotubes, and their ability to restore Agrin-dependent AChR clustering was evaluated (Fig. 8c–d). Mutants retaining both Ig1 and Ig2 domains (MuSK(Ig12), MuSK(Ig123), and MuSK(Ig12Fz)) restored clustering to levels similar to MuSK(WT), and MuSK(Ig12) and MuSK(Ig12Fz) were nearly indistinguishable from wild-type. By contrast, deletion of Ig2 abolished Agrin-dependent clustering, indicating that Ig2 is also required, together with Ig1, to induce AChR clustering.



(c)

	PBS	Agrin		PBS	Agrin
MuSK (WT)	 10.1	 36.4	MuSK (Ig13Fz)	 12.1	 8.1
MuSK (Ig123)	 10.6	 22.3	MuSK (Ig13)	 10.7	 8.5
MuSK (Ig12Fz)	 10.1	 37.2	MuSK (Ig1Fz)	 8.0	 9.1
MuSK (Ig12)	 10.3	 29.3	MuSK (Ig1)	 10.0	 7.9

(d)

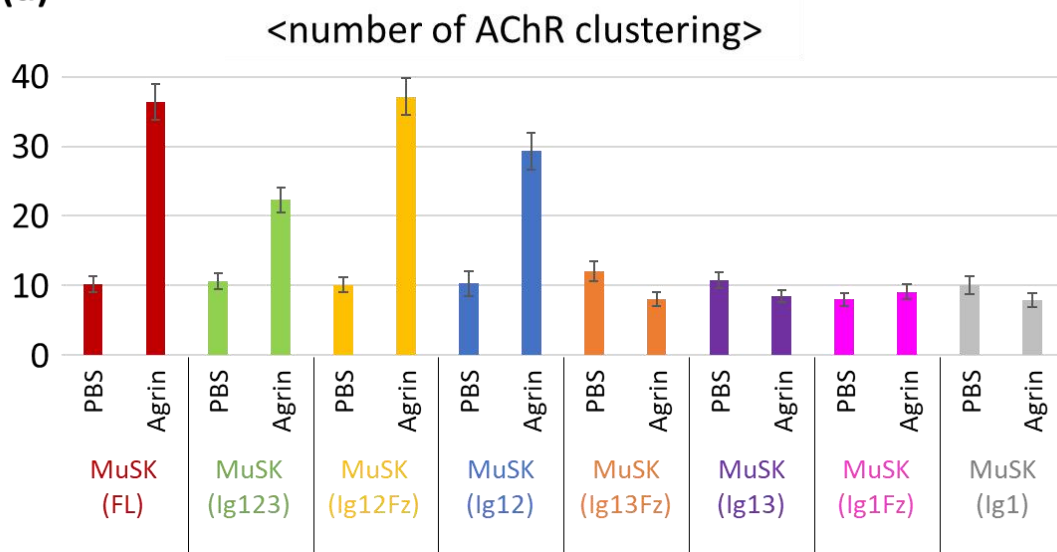


Fig.8 : Effect of deletions outside Ig1 on MuSK function

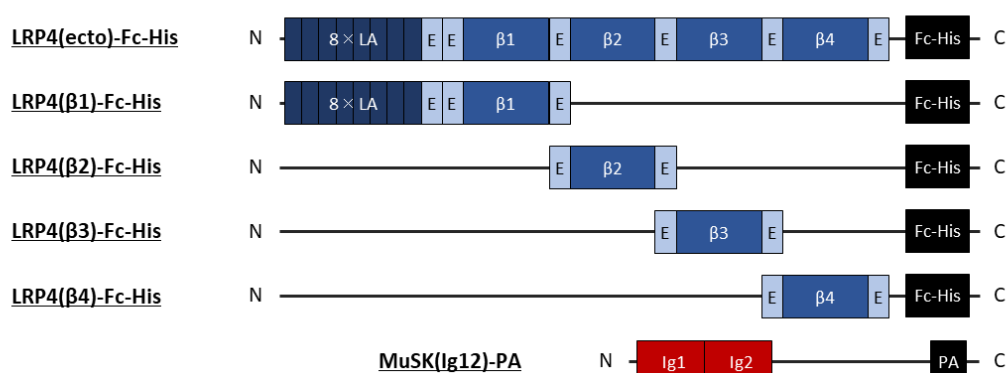
- Schematic of MuSK constructs used for domain-deletion analysis. All constructs carry an N-terminal MAP tag, as in hMuSK(WT).
- Expression levels of each construct upon transient expression in Expi293F cells, assessed by flow cytometry.
- Representative images of AChR clustering in mMuSK KO C2C12 myotubes expressing MuSK deletion constructs and stimulated with AgrinC50. The numbers in each panel indicate the average number of AChR clusters per field (60 fields analyzed per condition).
- Quantification of AChR clustering under each condition. Bars represent mean values; error bars indicate SEM.

2.2.6. MuSK interacts with the third β -propeller domain of LRP4

Because both Ig1 and Ig2 were found to be indispensable for Agrin-dependent AChR clustering, I next investigated how the Ig1–Ig2 tandem contributes to MuSK activation at the level of LRP4 engagement.

I first examined which β -propeller domain of LRP4 is recognized by the activation-competent MuSK(Ig12) fragment. Five LRP4 constructs were generated, comprising the full extracellular region and four fragments each containing a single β -propeller domain (Fig. 9a). All constructs were expressed at detectable levels, and pull-down assays revealed that MuSK(Ig12) specifically bound the third β -propeller ($\beta 3$) domain of LRP4, whereas no interaction was detected with the other β -propeller domains (Fig. 9b). These results identify $\beta 3$ domain of LRP4 as the principal binding site for MuSK Ig1–Ig2 region.

(a)



(b)

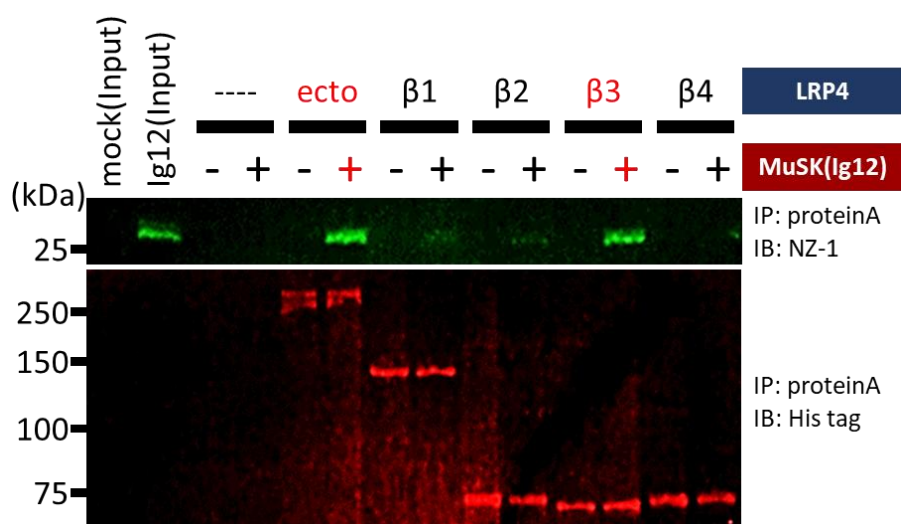


Fig9, LRP4-binding properties of MuSK Ig1–Ig2 constructs

(a) Domain organization of LRP4 constructs (full-length ectodomain and individual β -propeller domains) and the MuSK(Ig12) fragment used for binding assays.

(b) Binding of MuSK(Ig12) to each LRP4 β -propeller domain, assessed by IP-Western.

2.2.7. Ig1 domain has binding ability against LRP4- β 3 domain

Having established that MuSK(Ig12) interacts specifically with the β 3 domain of LRP4, I next sought to determine which component of the Ig1-Ig2 tandem mediates this interaction.

To this end, MuSK fragments containing either Ig1 alone or Ig2 alone were prepared and tested for binding to the LRP4 β 3 domain (Fig. 10a). This result by IP-Western showed that only Ig1-containing fragment exhibited detectable binding, whereas the Ig2-only fragment showed no interaction (Fig. 10b).

These findings demonstrate that Ig1 domain is both necessary and sufficient for LRP4 binding, while Ig2 domain does not directly participate in the MuSK-LRP4 interface. Together with the functional data presented in Section 2.2.5, these results suggest that Ig2 domain contributes to MuSK activation not by ligand recognition, but by enforcing the appropriate structural context required for productive downstream signaling.

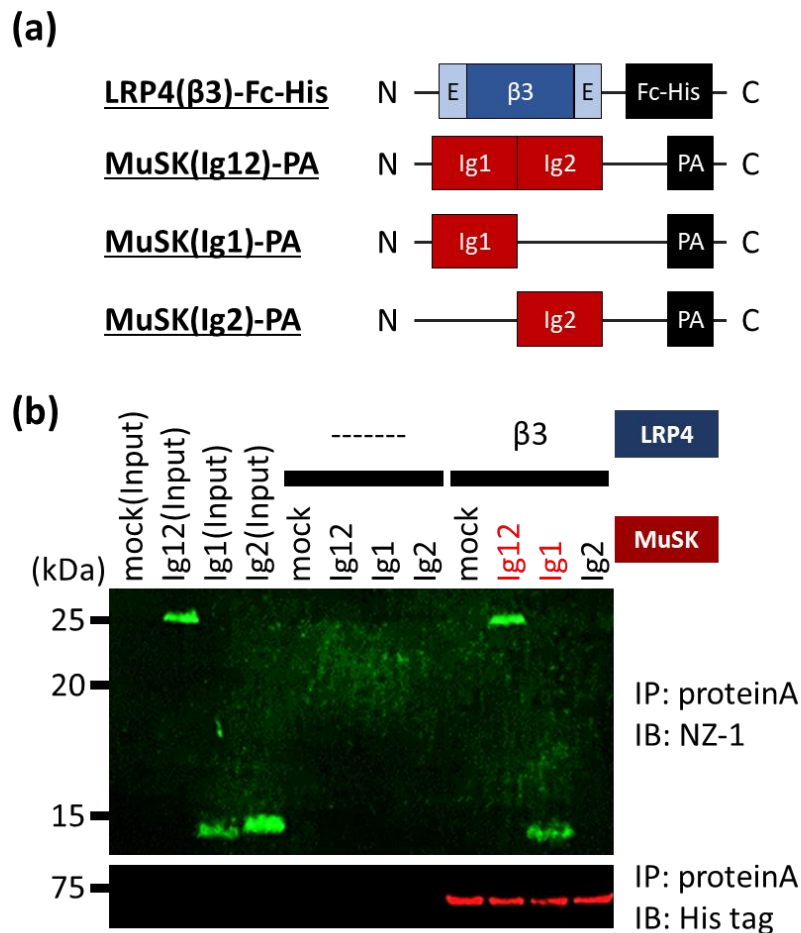


Fig10, LRP4-binding properties of MuSK Ig1-Ig2 constructs

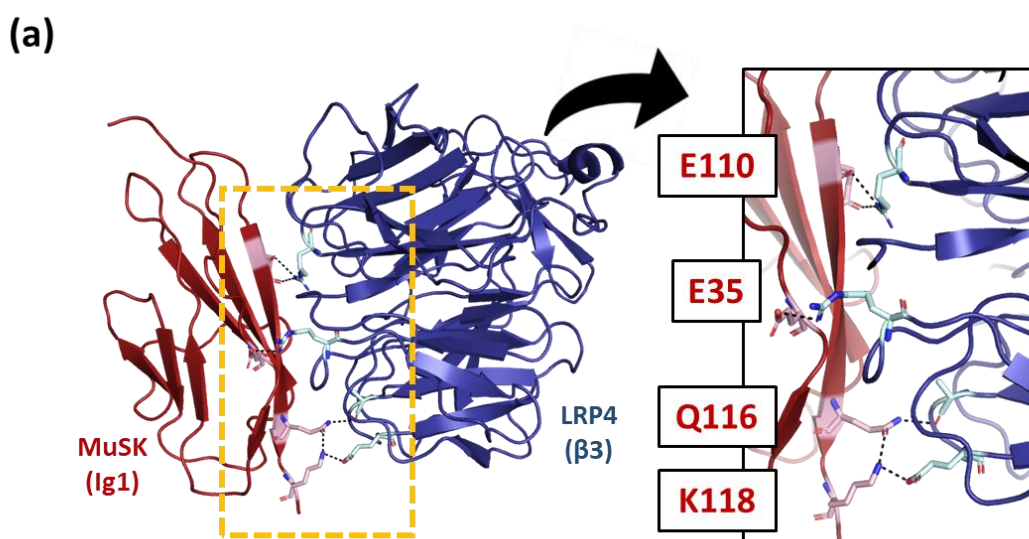
- (a) Domain organization of LRP4 β 3 and MuSK variants used to dissect the binding interface.
 (b) Binding of MuSK(Ig12), MuSK(Ig1), and MuSK(Ig2) to the LRP4 third β -propeller domain (β 3), assessed by IP-Western.

2.2.8. Identification of critical Ig1 residues required for AChR clustering (I)

Building on the finding that the Ig1 domain of MuSK specifically interacts with the third β -propeller domain of LRP4 (Section 2.2.7), I next sought to identify the Ig1 residues that are critical for MuSK activation within the Agrin–MuSK–LRP4 (AML) complex¹⁸. Guided by previously reported structural information, I focused on residues predicted to stabilize the MuSK–LRP4 interface and thereby promote formation of an activation-competent MuSK dimer.

Electron microscopy analysis of the Agrin–LRP4–MuSK ternary complex has identified several Ig1 residues that engage LRP4 through side-chain-mediated hydrogen bonds. Among these, four residues—E35, E110, Q116, and K118—form hydrogen bonds with the β 3 domain of LRP4 (Fig. 11a). To evaluate their functional importance, I generated alanine-substituted mutants at each residue and examined their ability to support Agrin-dependent AChR clustering.

Flow cytometric analysis confirmed surface expression of all mutant constructs, with only minor reductions observed for some variants relative to MuSK(WT) (Fig. 11b). Functional analysis revealed that substitution of E110, Q116, or K118 completely abolished Agrin-induced AChR clustering, whereas mutation of E35 had no effect (Fig. 11c-d). These results identify E110, Q116, and K118 as critical determinants of MuSK activation, likely by stabilizing the MuSK–LRP4 interface required for productive dimer assembly within the AML complex.



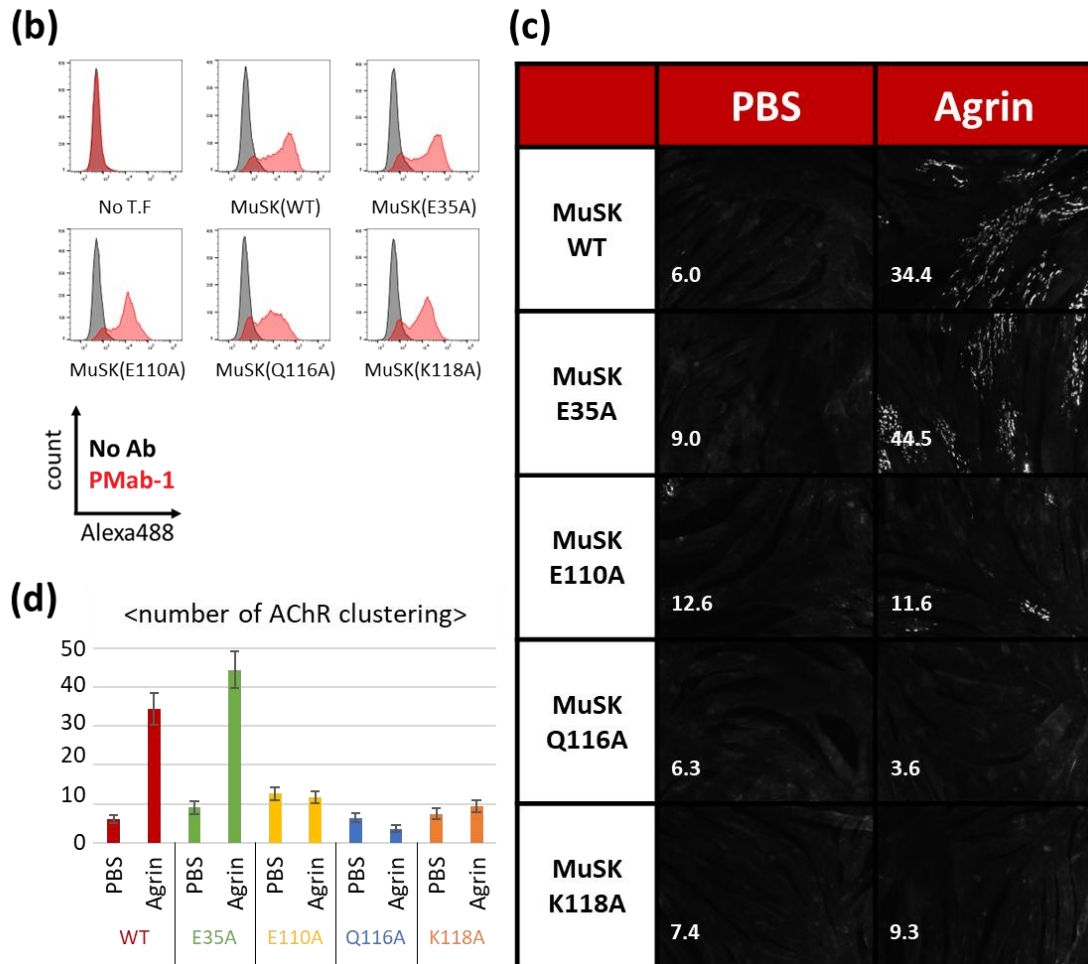


Fig.11 : Interaction between MuSK Ig1 domain and LRP4 β 3 domain

- (a) The left panel shows an overview of the MuSK Ig1 domain and LRP4 β 3 domain in complex (PDB ID: 8S9P). The region enclosed by the yellow box is enlarged on the right, highlighting the side-chain interactions. Four MuSK residues—E35, E110, Q116, and K118—form hydrogen bonds with LRP4 via their side chains.
- (b) Expression of alanine-substituted Ig1 mutants. Each mutant was generated in the MAP-hMuSK(WT) background. Expression was assessed by staining with the anti-MAP antibody PMab-1 (red), and compared with unstained controls (gray histograms). Rightward peak shifts indicate successful expression.
- (c) Agrin-dependent AChR clustering in mMuSK KO C2C12 myotubes expressing hMuSK(WT) or alanine-substituted Ig1 mutants. The numbers in each panel indicate the average number of clusters per field (50 fields analyzed per condition).
- (d) Quantification of AChR clustering under each condition. Bars represent mean values; error bars indicate SEM.

2.2.9. Identification of critical Ig1 residues required for AChR clustering (II)

In addition to its role in LRP4 binding, Ig1 domain has been proposed to mediate homotypic MuSK–MuSK interactions required for dimer formation. Structural and mutational analyses have identified a putative dimerization interface centered on residues M48 and L83 within Ig1 (Fig. 12a)⁴⁵. Substitution of either residue with arginine (M48R or L83R) was previously shown to reduce or abolish Agrin-dependent autophosphorylation. However, the impact of these mutations on AChR clustering signal—the physiological output of MuSK signaling—had not been examined.

At section 2.2.4, MuSK autophosphorylation does not necessarily correlate with AChR clustering, I investigated whether these Ig1 dimerization mutants retain any ability to induce AChR clustering. Flow cytometric analysis confirmed that both M48R and L83R were expressed at levels comparable to wild-type MuSK (Fig. 12b), excluding differences in expression as a confounding factor.

Functional analysis in MuSK KO C2C12 myotubes revealed that neither M48R nor L83R induced detectable AChR clustering following Agrin stimulation (Fig. 12c-d). Importantly, even the M48R mutants, which retains weak residual autophosphorylation, was completely inactive in terms of AChR clustering. These results demonstrate that the M48/L83-containing interface in Ig1 domain is essential not merely for MuSK dimerization per se, but for formation of a properly oriented MuSK dimer capable of transmitting downstream signaling.

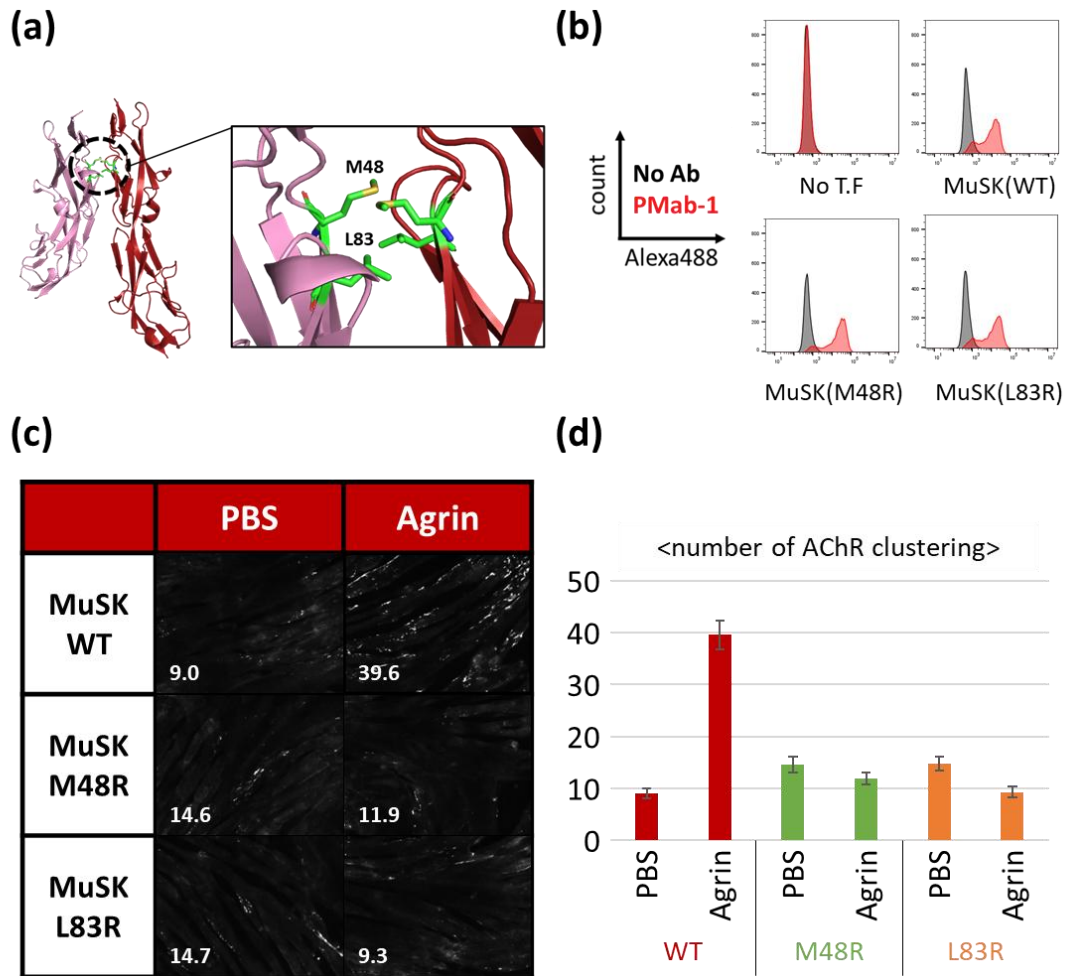


Fig. 12 : Effects of Ig1 mutations at the MuSK–MuSK dimer interface on MuSK activity

- (a) Structural details of the MuSK Ig1 dimer interface, highlighting residues implicated in dimer formation.
- (b) Expression of M48R and L83R mutants in the MAP–hMuSK(WT) background. Expression was confirmed by PMab-1 staining (red) compared with unstained controls (gray).
- (c) Agrin-dependent AChR clustering in mMuSK KO C2C12 myotubes expressing hMuSK(WT) or M48R/L83R mutants. The numbers in each panel indicate the average number of clusters per field (50 fields analyzed per condition).
- (d) Quantification of AChR clustering under each condition. Bars represent mean values; error bars indicate SEM.

2.2.10. The rigid architecture of the Ig1–Ig2 tandem is crucial for MuSK activation

Our domain-deletion analyses revealed that both the Ig1 and Ig2 domains are indispensable for Agrin-dependent induction of AChR clustering (Section 2.2.5). In contrast, binding assays demonstrated that only the Ig1 domain mediates direct interaction with the $\beta 3$ domain of LRP4, whereas Ig2 does not contribute to LRP4 binding (Section 2.2.6). This apparent discrepancy prompted a key mechanistic question: what functional role does the Ig2 domain play in MuSK activation ?

Insight into this question comes from the crystal structure of the Ig1–Ig2 tandem⁴⁵. Structural analysis shows that the Ig1 and Ig2 domains are not connected by a flexible linker but instead form a rigid unit stabilized by a continuous β -strand and multiple interdomain side-chain interactions (Fig. 13a). This architecture imposes a fixed relative orientation between the two domains and has been proposed to constrain the geometry of MuSK dimers formed within the Agrin–LRP4–MuSK complex.

To test the functional importance of this rigidity, I engineered a MuSK variant in which a flexible GS linker (GGGS) was inserted between Ig1 and Ig2 domain, thereby disrupting the native interdomain constraint (Fig. 13b). This GS-linker mutant was expressed at levels comparable to wild-type MuSK, as confirmed by flow cytometry (Fig. 13c). Functional analysis in MuSK KO C2C12 myotubes revealed a striking phenotype: although Agrin stimulation induced robust autophosphorylation of the GS-linker mutant, AChR clustering was completely abolished (Fig. 13d–f).

These results demonstrate that the rigid Ig1–Ig2 architecture is dispensable for initial MuSK autophosphorylation but is essential for coupling receptor activation to downstream AChR clustering. I therefore conclude that the Ig1–Ig2 tandem functions as a structurally constrained scaffold that enforces the precise dimeric geometry required for productive MuSK signaling, independently of direct LRP4 binding by Ig2.

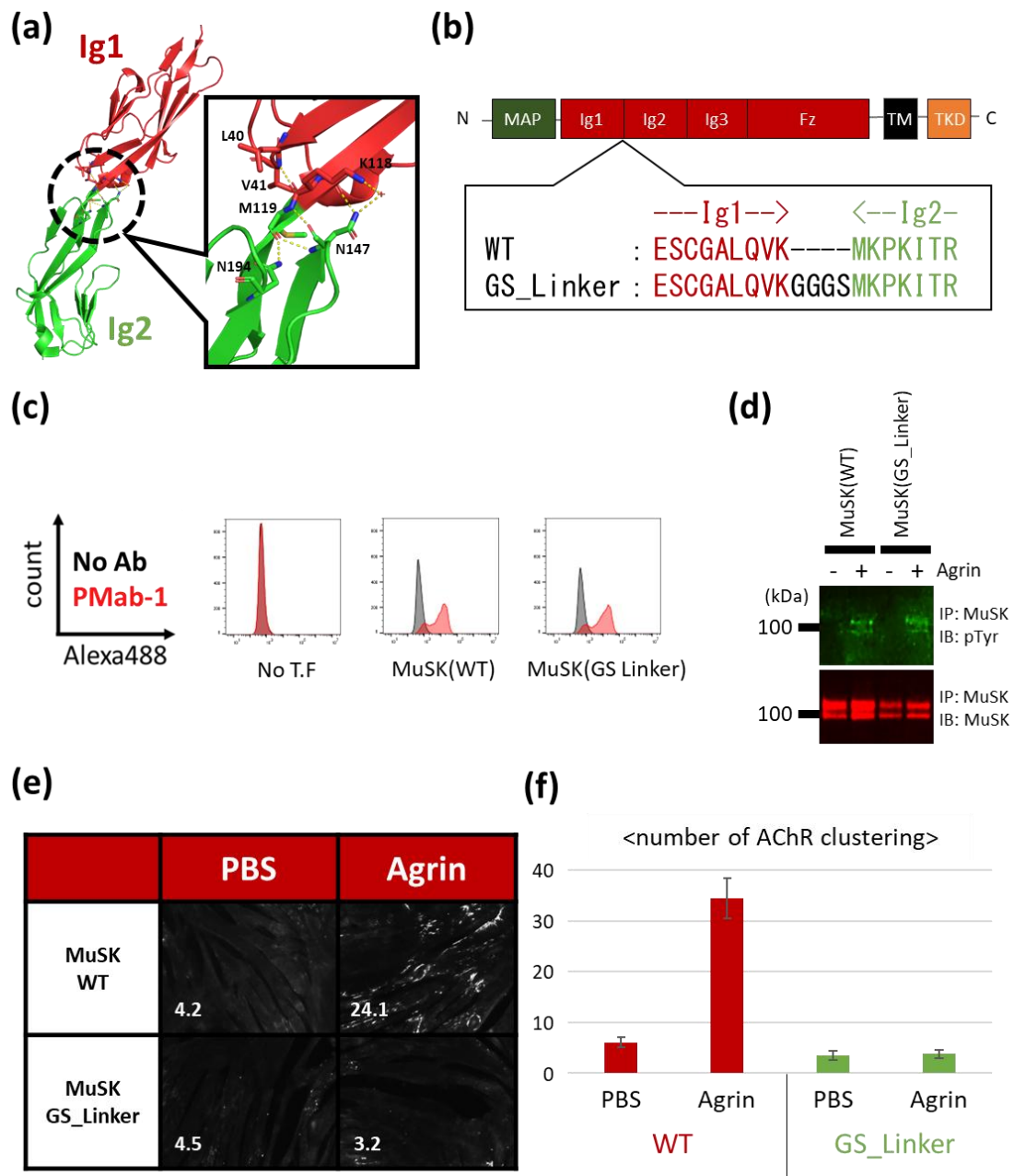


Fig.13 : Importance of the rigid Ig1–Ig2 architecture for MuSK activation

- (a) Structural interactions between the Ig1 and Ig2 domains of MuSK. No flexible linker is present between the two domains; instead, a continuous β -strand spans the boundary. Zoomed view (dotted box) highlights multiple interdomain contacts, including interactions involving residues K118–N147.
- (b) Schematic of MuSK mutants used to disrupt the Ig1–Ig2 interface. All constructs carry an N-terminal MAP tag.
- (c) Expression levels of each construct upon transient expression in Expi293F cells.
- (d) AgrinC50-induced phosphorylation of each construct expressed in mMuSK KO C2C12 myotubes.
- (e) AChR clustering activity of each construct expressed in mMuSK KO C2C12 myotubes. The numbers in each panel indicate the average number of clusters per field (50 fields analyzed per condition).
- (f) Quantification of AChR clustering under each condition. Bars represent mean values; error bars indicate SEM.

2.2.11. Ig1-Ig2 is not required for Agrin-independent AChR clustering

Although the Ig1-Ig2 tandem structure was shown to be indispensable for Agrin-dependent activation of MuSK, it remained unclear whether this requirement reflects a direct signaling function of these domains or instead their role in enforcing the correct extracellular dimer geometry within the AML complex. To distinguish between these possibilities, I tested whether MuSK constructs lacking Ig1-Ig2 could still induce downstream signaling when activated by an exogenous agonist that enforces dimerization independently of Agrin-LRP4.

As such an agonist, I employed the monoclonal antibody X17, which recognizes the MuSK Fz domain and has been reported to induce robust AChR clustering⁴⁰. I first confirmed that X17 induces AChR clustering in wild-type C2C12 myotubes at levels comparable to those induced by AgrinC50 (Fig. 14a).

I then generated a MuSK construct consisting solely of the Fz-containing extracellular region (MuSK(Fz)) (Fig. 14b) and expressed this construct, together with full-length hMuSK(WT), in MuSK KO C2C12 myotubes. Both constructs were expressed at comparable levels, as confirmed by flow cytometry (Fig. 14c). As expected, Agrin stimulation induced AChR clustering only in myotubes expressing full-length hMuSK(WT). Strikingly, however, X17 induced robust AChR clustering not only through hMuSK(WT) but also through hMuSK(Fz), with comparable efficiency (Fig. 14d-e).

These results demonstrate that Agrin-independent activation of MuSK does not require the Ig1-Ig2 tandem. Even in the absence of Ig1-Ig2, MuSK can trigger downstream AChR clustering provided that two receptor molecules are brought together in an appropriate orientation by an agonistic antibody. Thus, the essential role of Ig1-Ig2 in Agrin-dependent signaling is not to directly transmit intracellular signals, but to impose the precise extracellular dimer geometry required for productive activation within the physiological AML complex.

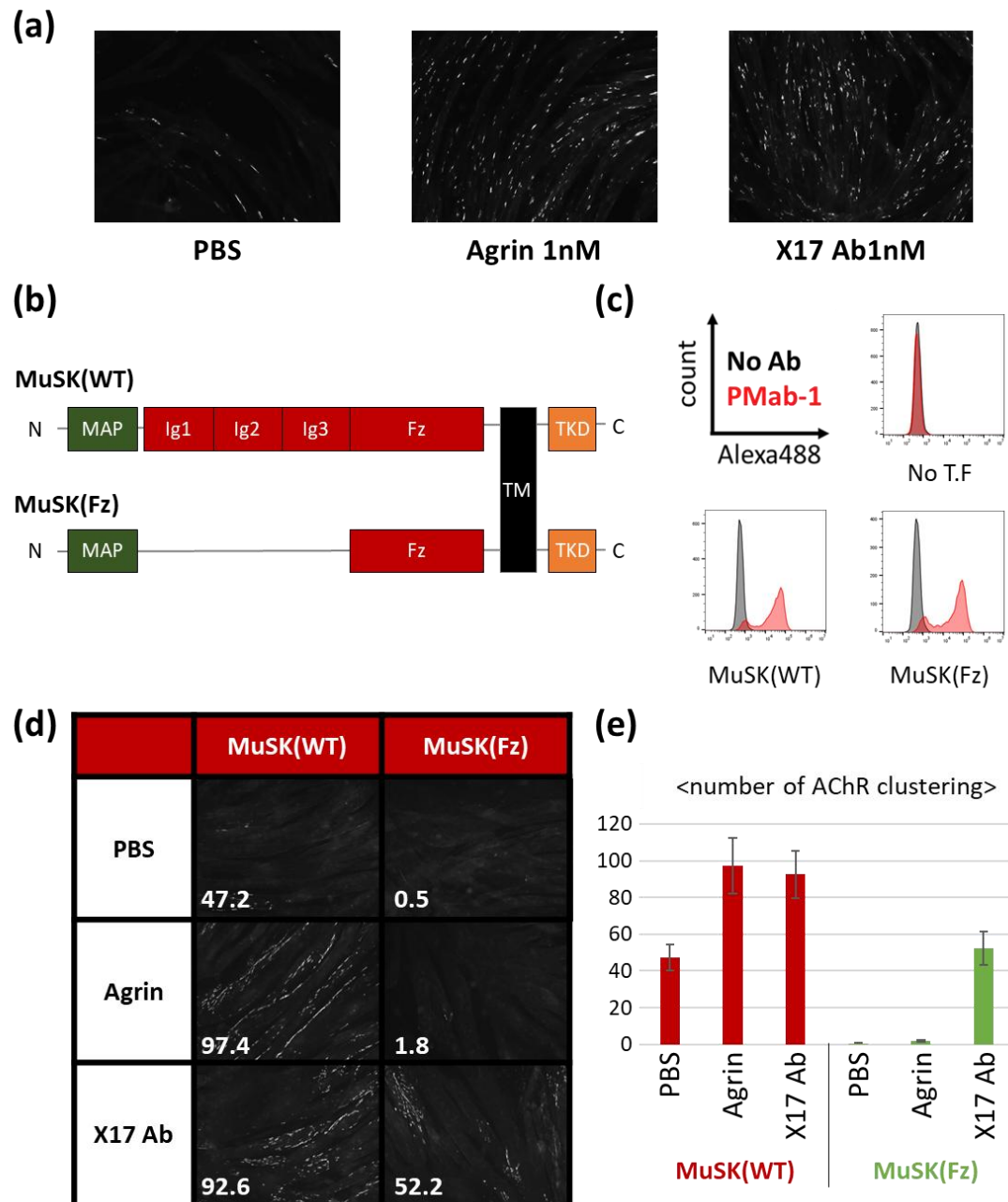


Fig.14 : Agonist activity of Fc-L1 toward hMuSK(Ig3Fz)

- (a) AChR clustering in wild-type C2C12 myotubes upon X17 antibody treatment. X17 induces AChR clustering in a manner similar to AgrinC50.
- (b) Schematic of the hMuSK(Fz) construct.
- (c) Expression levels of hMuSK(WT) and hMuSK(Fz) upon transient expression in Expi293F cells.
- (d) AChR clustering induced by 100 nM Fc-L1 in mMuSK KO C2C12 myotubes expressing hMuSK(WT) or hMuSK(Fz). The numbers in each panel indicate the average number of clusters per field (25 fields analyzed per condition).
- (e) Quantification of AChR clustering under each condition. Error bars indicate SEM.

2.2.12. Identification of an additional LRP4-binding domain in MuSK

The results above established that Ig1 is essential for both LRP4 binding and Agrin-dependent AChR clustering. However, MuSK(Ig23Fz), which lacks Ig1, still underwent Agrin-induced autophosphorylation (Fig. 7c). Together with the finding that Ig2 does not bind LRP4, this raised the possibility that another extracellular domain of MuSK may mediate a weak, Agrin-independent interaction with LRP4, forming a nonproductive MuSK dimer that supports autophosphorylation but fails to induce downstream signaling.

To test this hypothesis, I performed co-expression and co-immunoprecipitation assays using full-length LRP4 and MuSK variants. For this analysis, four MuSK constructs were generated, each containing only a single extracellular domain—Ig1, Ig2, Ig3, or Fz (Fig. 15a). All MuSK variants were expressed at comparable levels. Strikingly, only MuSK(Ig1) and MuSK(Ig3) co-precipitated with LRP4, whereas MuSK(Ig2) and MuSK(Fz) showed no detectable binding (Fig. 15b). These findings indicate that MuSK possesses two LRP4-binding domains: a high-affinity interaction mediated by Ig1 and a secondary, interaction mediated by Ig3.

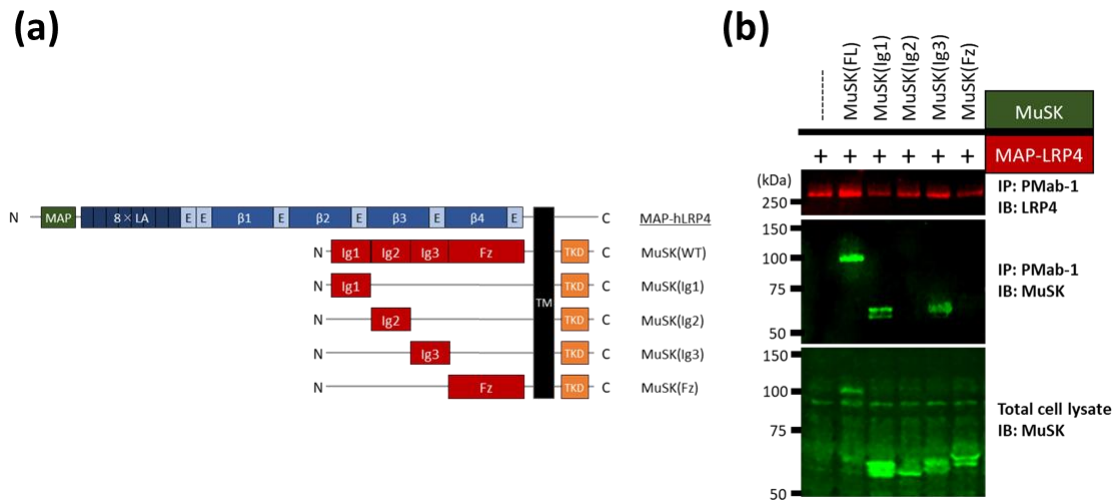


Fig.15 : Identification of LRP4-binding domains within MuSK

- Schematic of MuSK and LRP4 constructs used in co-expression assays. LRP4 carries an N-terminal MAP tag.
- Co-immunoprecipitation analysis of LRP4 binding to MuSK constructs. MuSK and LRP4 were co-expressed in Expi293F cells, and LRP4 was immunoprecipitated via its MAP tag. The top panel shows expression of LRP4, the middle panel shows co-precipitated MuSK, and the bottom panel shows total MuSK expression in cell lysates. Ig1 and Ig3 domains exhibit detectable binding to LRP4.

2.3. Discussion

2.3.1. Summary

In this chapter, I performed a functional domain analysis of MuSK by systematically analyzing a series of MuSK mutants. To enable accurate evaluation of mutant activity, I first established MuSK-knockout C2C12 cell line and demonstrated that re-expression of wild-type MuSK restored both MuSK autophosphorylation and AChR clustering. Using this system, I assessed the functional contributions of individual extracellular domains and key residues. These analyses revealed that the Ig1–Ig2 tandem structure is indispensable for Agrin-dependent AChR clustering and clarified the distinct roles of each domain in MuSK activation.

2.3.2. The activated MuSK dimer adopts a highly constrained orientation

Our domain analyses demonstrate that both Ig1 and Ig2 are essential for Agrin-dependent induction of AChR clustering and that the rigid architecture formed by these two domains is functionally critical. Notably, the Ig1-deletion mutant MuSK(Ig23Fz) underwent robust Agrin-dependent autophosphorylation but completely failed to induce AChR clustering. This dissociation indicates that MuSK autophosphorylation alone is insufficient for downstream signaling and that productive activation requires not only receptor proximity but also a precisely defined dimer orientation, consistent with general principles of receptor tyrosine kinase activation⁶.

Because MuSK(Ig23Fz) retains autophosphorylation activity, the intracellular kinase domains must still approach one another following Agrin stimulation, similar to MuSK(WT). However, lack of AChR clustering implies that the extracellular dimer formed by this mutant is structurally misaligned. Consistent with this interpretation, MuSK(Ig23Fz) likely participates in AML-like assemblies but fails to adopt the activation-competent MuSK-MuSK geometry required for signal propagation.

Section 2.2.7 revealed that MuSK has two LRP4-binding interfaces: a primary interaction mediated by Ig1 domain and Ig3 domain. Accordingly, MuSK(Ig23Fz), which lacks Ig1, may still engage LRP4 through Ig3, forming an alternative AML-like complex distinct from the canonical Ig1/ β 3-mediated assembly^{15,16}. Such a complex could bring two MuSK molecules into partial proximity sufficient to trigger autophosphorylation yet fail to orient them appropriately for downstream signaling.

In contrast, mutants retaining both Ig1 and Ig2 domains supported robust AChR clustering. Moreover, insertion of a flexible GS linker between Ig1 and Ig2 abolished AChR clustering while preserving autophosphorylation. These results demonstrate that

it is the rigid geometry of the Ig1-Ig2 tandem—rather than the mere presence of these domains—that is essential for productive signaling. The Ig1-Ig2 unit therefore functions as a structurally constrained scaffold that enforces the correct extracellular alignment of MuSK molecules.

Collectively, the results of this chapter suggest that MuSK dimers fall into two mechanistic classes: those that support autophosphorylation alone and those that support both autophosphorylation and downstream AChR clustering. The transition between these states is governed by dimer geometry rather than phosphorylation *per se*.

A critical determinant of this transition is formation of the MuSK–DOK7 2:2 complex²⁵ (Fig. 16a-d). Phosphorylation of Tyr553 in the juxtamembrane region creates a binding site for DOK7, and simultaneous binding of two DOK7 molecules promotes dimerization and subsequent phosphorylation of additional tyrosine residues within the TKD. Structural analysis of the DOK7 dimer bound to MuSK-derived peptides (PDB: ABCD) shows that the C α –C α distance between two pTyr553 residues is approximately 60 Å²⁵ (Fig. 16e). By comparison, the MuSK Ig1-Ig2 dimer structure places the Ig2 C-terminal residues (E209) ~80 Å apart (Fig. 16f), whereas the Ig1 C-terminal residues (K116) are separated by only ~30 Å (Fig. 16f). The latter distance is incompatible with stable DOK7 dimer engagement.

This geometric constraint explains why constructs such as MuSK(Ig1) and MuSK(Ig23Fz) undergo autophosphorylation yet fail to induce AChR clustering: the extracellular dimer geometry they adopt cannot be propagated into an intracellular configuration that supports productive DOK7 dimerization and full kinase activation.

Finally, although these mutants undergo autophosphorylation, the specific pattern of tyrosine phosphorylation within the intracellular region remains unresolved. Elucidation of these patterns will require site-specific phospho-antibodies and will be important for fully understanding the spectrum of MuSK activation states.

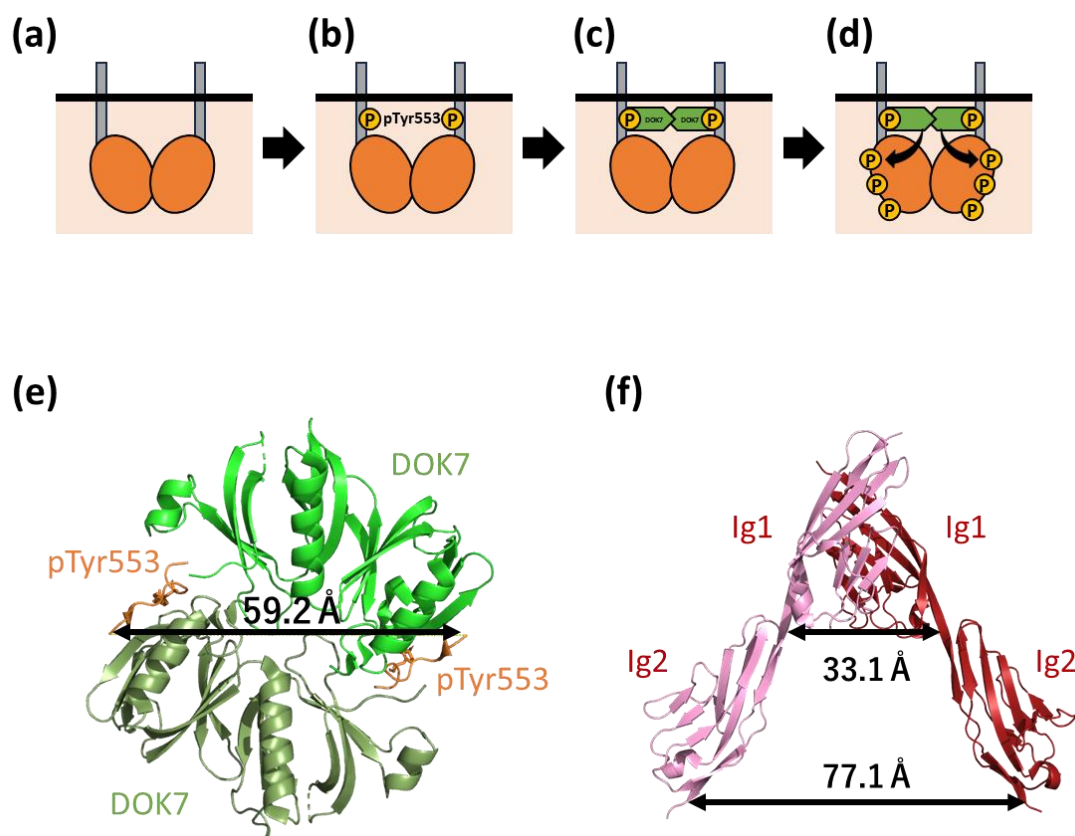


Fig.16 : Distances between key residues in MuSK and DOK7 structures

- (a-d) Schematic representation of intracellular events following MuSK dimerization at the cell surface. Upon formation of the AML complex and dimerization of MuSK (a), Tyr553 in the juxtamembrane region is autophosphorylated (b). The resulting pTyr553 is recognized by DOK7, which binds and dimerizes on the cytoplasmic side (c). Formation of this 2:2 MuSK-DOK7 complex promotes phosphorylation of the MuSK TKD, leading to full activation of MuSK (d).
- (e) Distance between the C-termini of Ig1 and Ig2 domains in the MuSK Ig1-Ig2 dimer (PDB ID: 2IEP). The C α -C α distance between K116 residues in Ig1 and between E209 residues in Ig2 is measured to estimate the spacing imposed by the extracellular dimer interface.
- (f) Distance between pTyr553-equivalent residues in the DOK7 dimer complexed with MuSK JM peptides (PDB ID: ABCD). The C α -C α distance provides a structural reference for the separation required for DOK7 to simultaneously engage two MuSK molecules.

2.3.3. Critical Ig1 residues required for MuSK activation

Building upon previous findings that the Ig1 domain is essential for Agrin-dependent AChR clustering⁴⁴, I performed a detailed structure-guided mutational analysis to identify residues critical for MuSK activation. I focused on two sets of residues: M48 and L83, which are thought to contribute to MuSK–MuSK dimerization⁴⁵, and E35, E110, Q116, and K118, which form side-chain hydrogen bonds with LRP4 in the reported ternary complex structure¹⁸. Our functional analysis showed that mutation of all residues except E35 nearly completely abolished AChR clustering.

For M48 and L83, substitution with arginine—previously shown to reduce (M48R) or eliminate (L83R) MuSK autophosphorylation⁴⁵—also completely lost AChR clustering activity. Even M48R, which retains weak autophosphorylation capability, failed to induce downstream signaling. This indicates that these residues are essential for forming a correctly oriented MuSK–MuSK dimer structure.

For E110, Q116, and K118, structural data show that these residues possess on the same β -strand of Ig1 and participate in hydrogen bonding with the LRP4 β 3 domain¹⁸. This β -strand continues uninterrupted into the Ig2 domain, suggesting that interactions mediated by these residues stabilize not only the MuSK–LRP4 interface but also the overall orientation of the Ig1–Ig2 tandem. Our results support this model: mutation of any of these three residues disrupts the LRP4–Ig1 interface, destabilizes the relative alignment of Ig1 and Ig2, and ultimately prevents formation of the activation-competent MuSK dimer within the AML complex.

Consistent with this interpretation, the regions involved in Ig1–Ig1 dimerization (M48/L83) and Ig1–LRP4 binding (E110/Q116/K118) do not physically overlap (Fig.17a). Therefore, when MuSK engages LRP4 through these residues, the Ig1–Ig2 tandem is positioned in a defined orientation that facilitates proper MuSK–MuSK dimerization and enables downstream DOK7 engagement (Fig. 17b–d). Conversely, disruption of any of these interactions leads to misalignment within the AML complex and consequently abolishes AChR clustering.

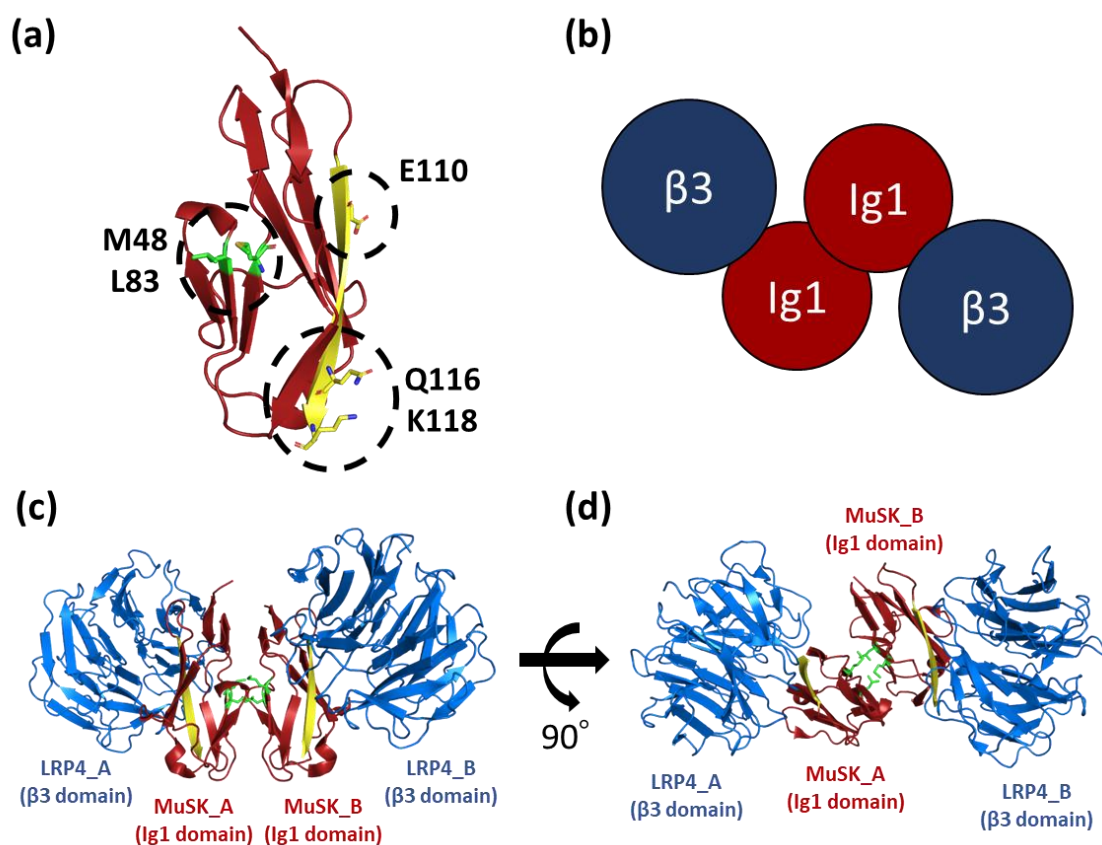


Fig.17 : Predicted orientation of MuSK Ig1 and LRP4 β 3 within the AML complex

- (a) Location of the MuSK Ig1 dimer interface and LRP4 β 3-contacting residues within the Ig1 domain.
- (b) Schematic of the predicted relative orientation of the MuSK Ig1 domain and LRP4 β 3 domain within the AML complex.
- (c) Structural model of the MuSK(Ig1)–LRP4(β 3) complex. The β -strand in Ig1 that contains LRP4-contacting residues (E110, Q116, K118) is colored yellow, and the dimer interface is colored green.
- (d) Top view of the modeled MuSK(Ig1)–LRP4(β 3) complex, illustrating the spatial arrangement of the MuSK dimer within the AML complex.

2.3.4. The extracellular region ensures correct intracellular geometry

Taken together, these results indicate that the rigid Ig1–Ig2 geometry pre-sets the relative positioning of intracellular Tyr553 residues, thereby determining whether productive DOK7 dimer assembly can occur downstream signaling.

Although the Ig1–Ig2 tandem structure is indispensable for Agrin-dependent AChR clustering, results from Section 2.2.11 demonstrate that it is not required for Agrin-independent activation by exogenous agonists. The Fz-directed agonist antibody X17 can induce AChR clustering even when only the Ig3-Fz region of MuSK is present. This observation highlights a crucial conceptual point: the extracellular domains of MuSK do not directly execute signaling but rather serve as a geometric scaffold that aligns two MuSK intracellular regions in a configuration compatible with DOK7-mediated activation.

In physiological activation, this alignment is achieved through Agrin-LRP4-MuSK complex, which enforces a precise extracellular dimer orientation and thereby ensures correct intracellular spacing. However, non-physiological agonists such as X17 antibody can bypass the Ig1–Ig2 requirement by forcing two MuSK molecules into a geometry that happens to satisfy the intracellular constraints. Thus, the essential requirement is not the identity of the extracellular domain used for crosslinking, but rather the geometry it imposes.

Why, then, is extracellular dimerization so strictly regulated in vivo? I speculate that the physiological Agrin–LRP4–MuSK system ensures that MuSK activation occurs only at appropriate times and locations—specifically at developing or remodeling neuromuscular junctions—and prevents aberrant or ectopic AChR clustering. This tight regulation safeguards NMJ homeostasis and prevents pathological signaling.

2.3.5. Structural differences among the three Ig-like domains

Finally, I examined why Ig1 and Ig3 bind LRP4, whereas Ig2 does not, despite all three being Ig-like domains. Structural comparison of human Ig1, human Ig3, and rat Ig2 revealed that overall folds are similar but local structural features differ (Fig. 18). The most notable difference is that a β -strand that forms a single continuous strand in Ig1 and Ig3 is split into two shorter strands in Ig2. This β -strand corresponds to the region in Ig1 where LRP4-binding residues cluster. Thus, Ig3 resembles Ig1 in this structural motif, enabling weak LRP4 binding, whereas Ig2 lacks the necessary β -strand architecture and therefore does not interact with LRP4.

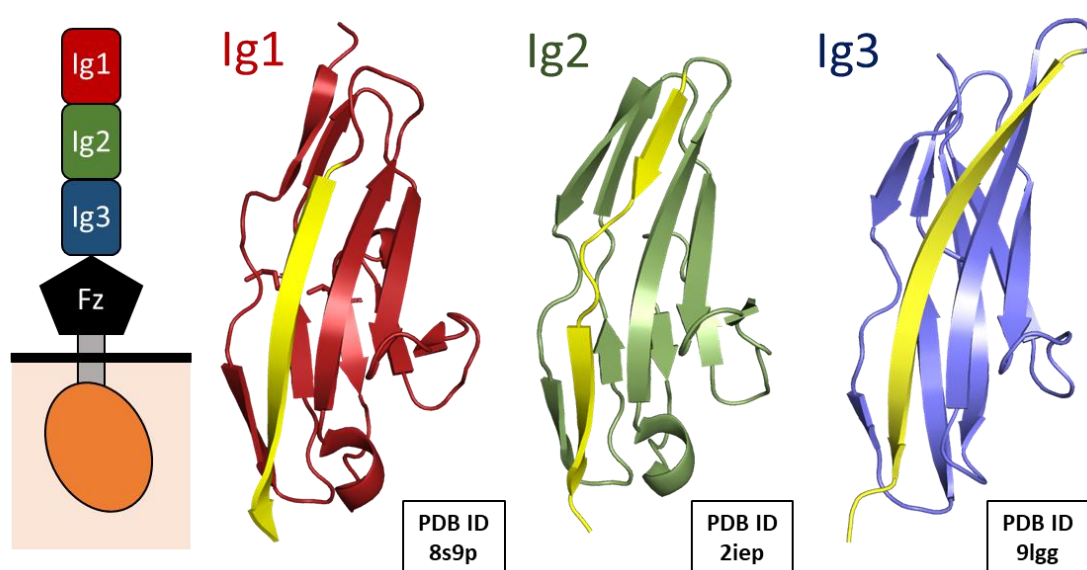


Fig.18 : Structural comparison of the three Ig-like domains of MuSK

Structural comparison of MuSK Ig1-Ig3 domains. The PDB ID used for each domain is indicated at the lower right of each structure. In Ig1, the β -strand containing residues E110, Q116, and K118, which are important for LRP4 β 3 binding, is colored orange. For Ig2 and Ig3, the β -strand corresponding to this region (when structurally aligned with Ig1) is also highlighted in orange, illustrating similarities and differences among the three Ig-like domains.

2.4. Material & Method

2.4.1. DNA constructs

All MuSK, LRP4, and Agrin constructs were made by PCR or gene synthesis using sequences (MuSK: UniProt O15146/LRP4: UniProt O75096/Agrin: UniProt O00468), and were generated by inserting the desired coding sequences into pcDNA3.1-based expression vectors. For constructs used in MuSK phosphorylation assays, full-length human MuSK was fused at the N-terminus to a MAP tag, yielding MAP–MuSK(WT). Domain-deletion mutants were designed based on the MuSK amino acid sequence, defining the domains as follows: Ig1 (Ser23–Lys118), Ig2 (Met119–Lys205), Ig3 (Val210–Ala304), and Fz (Lys308–Phe463). Each mutant lacked the specified domain(s). Point mutants and GS-linker insertion mutants were generated by PCR-based mutagenesis using the full-length MAP–MuSK construct as a template. For soluble MuSK ectodomain fragments, constructs encoded the MuSK extracellular region followed by a TEV protease cleavage site and a PA tag. The MuSK segments used were: Ig12 (Ser23–Phe211), Ig1 only (Ser23–Lys118), and Ig2 only (Met119–Lys205). LRP4 ectodomain constructs encoded the LRP4 extracellular region followed by human IgG Fc and a C-terminal 6×His tag. The LRP4 fragments used were: full-length ectodomain (Ser21–Ser1722), 1st β -propeller (Ser21–Ser739), 2nd β -propeller (Cys702–Gly1046), 3rd β -propeller (Cys1006–Ser1351), and 4th β -propeller (Cys1313–Gly1650). The Agrin fragment construct encoded, from the N-terminus, an 8×His tag, a Myc tag, and the C-terminal region of human Agrin (Lys1589–Pro2068) containing the Z8-loop, which is essential for MuSK activation. For generation of the MuSK KO C2C12 cell line, the Cas9 construct was based on lentiCRISPR v2 (Addgene #52961), into which the gRNA sequence 5′ -CATTCTCCCGGATGCTGTAG-3′ was inserted.

2.4.2. Cell culture

Expi293F cells (Thermo Fisher Scientific) were cultured in Expi293 Expression medium (Thermo Fisher Scientific) at 37° C in a humidified atmosphere of 8% CO₂, with rotation at 125 rpm. C2C12 myoblasts (CRL-1772, ATCC) were maintained in an adherent culture at 37° C in a 5% CO₂ environment using Dulbecco's Modified Eagle's Medium (WAKO) supplemented with 10% (v/v) FBS, 1% (v/v) NEAA, and 0.5% (v/v) Penicillin-Streptomycin.

2.4.3. Protein expression

All proteins except for that used in the crystallization were produced in Expi293F cells using the Expi293F Expression system and the supernatant was harvested four days post-transfection. For the purification of His-tagged hAgrinC50, culture supernatants were mixed with Ni-NTA agarose (QIAGEN) and rotated for 2 h at room temperature. The agarose was transferred to an Econo-Column (Bio-Rad) and washed three times with 10 column volumes of TBS (20 mM Tris, 150 mM NaCl, pH 7.5). Proteins were eluted with TBS containing 250 mM imidazole. The fractions containing target molecules were combined and dialyzed against PBS (10 mM Na-phosphate, 140 mM NaCl, 2.7 mM KCl, pH 7.4) at 4° C. Purification of Fc-containing proteins were performed by affinity chromatography using rProtein A Fast Flow Sepharose (Cytiva). Culture supernatants were mixed with rProtein A resin and rotated for 2 h at room temperature. The beads were transferred to an Econo-Column and washed three times with 10 column volumes of TBS. Fc-containing proteins were eluted with the IgG elution buffer (Thermo Scientific) and immediately neutralized with 1/10 volume of 1 M Tris-HCl, pH 9.0. The protein fractions were combined and dialyzed against PBS. For L1-fused Fc proteins, a second purification step of size-exclusion chromatography on a Superdex 200 Increase 10/300 GL column (Cytiva), equilibrated in PBS, was included to remove oligomeric species. All purified proteins were concentrated using an Amicon Ultra-4 Centrifugal Filters Ultracel-30K and stored at –80° C until use.

2.4.4. AChR clustering assay using wild-type C2C12 cells

C2C12 myoblasts were seeded onto a 24-well plate at 2.5×10^4 cells/well. After three days in growth medium, the cells were switched to differentiation medium (DMEM with 2% horse serum, 1% NEAA, 0.5% Penicillin-Streptomycin) to induce differentiation into myotube and cultured for two days. Stimulation was started by replacement of medium with that containing Agrin C50 or X17 antibody⁴⁰, and the cells were cultured for additional 18 h. Myotubes were incubated with Alexa Fluor 594-conjugated α -bungarotoxin (Life Technologies) for 1 h at 37° C, washed three times with PBS, and fixed in 3% paraformaldehyde for 10 min. After three additional washes with PBS, images were captured from 33 fields using a BZ-X800 microscope (KEYENCE), and data were analyzed using BZ-X800 analysis software (KEYENCE).

2.4.5. AChR clustering assay using MuSK KO C2C12 cells

MuSK KO C2C12 myoblasts were seeded onto a 24-well plate at 2.5×10^4 cells/well. On the following day, the medium was refreshed and DNA transfection was performed

using Xtreme (ATCC) according to the manufacturer's instructions. After two days in growth medium, the cells were switched to differentiation medium (DMEM with 2% horse serum, 1% NEAA, 0.5% Penicillin-Streptomycin) to induce differentiation into myotube and cultured for two days. Stimulation was started by replacement of medium with that containing Agrin C50 or X17 antibody, and the cells were cultured for additional 18 h. Myotubes were incubated with Alexa Fluor 594-conjugated α -bungarotoxin (Life Technologies) for 1 h at 37° C, washed three times with PBS, and fixed in 3% paraformaldehyde for 10 min. After three additional washes with PBS, images were captured from more than 30 fields using a BZ-X800 microscope (KEYENCE), and data were analyzed using BZ-X800 analysis software (KEYENCE).

2.4.6. Establishment of MuSK KO C2C12 cell line

C2C12 myoblasts were transfected with the Cas9/gRNA construct using Xtreme, and 1 day after transfection, the medium was replaced with C2C12 culture medium containing 10 μ g/ml puromycin. After 3 days of selection, surviving cells were subjected to limiting dilution to obtain single-cell-derived clones. Clones with detectable growth were analyzed for MuSK expression in the myoblast state by flow cytometry and after differentiation into myotubes by IP-Western, to identify MuSK-deficient clones.

2.4.7. Flow cytometric analysis of MuSK mutant expression

Expi293F cells transfected with the plasmids containing hMuSK(WT), hMuSK mutants, or mMuSK that had been modified to contain N-terminal MAP-tag were harvested two days post-transfection, transferred into a V-bottom 96-well plate at 2.0×10^5 cells/well and incubated with 5.0 μ g/ml purified PMab-1 (anti-MAP tag antibody, rat IgG2a)⁵⁰ or 1 μ M L1-fused Fc in DMEM containing 10% FBS, 1% NEAA, and 0.5% Penicillin-Streptomycin for 1 hour on ice. After washing twice with PBS(-), the cells were further incubated with Alexa Fluor 488-labeled goat anti-rat IgG (Thermo Fisher Scientific) or Alexa Fluor 488-labeled goat anti-human IgG (Thermo Fisher Scientific) diluted 1:10,000 in DMEM containing 10% FBS, 1% NEAA, and 0.5% Penicillin-Streptomycin for 30 minutes on ice. After three washes with PBS(-), the cells were analyzed on EC3800 (Sony). Data were analyzed using FlowJo software (TOMY Digital Biology).

2.4.8. Flow cytometric validation of MuSK KO C2C12 cells

Wild-type and MuSK KO C2C12 myoblasts were detached by trypsin-EDTA. Cells were transferred into a V-bottom 96-well plate at 2.0×10^5 cells/well and incubated with

5.0 µg/ml anti-MuSK polyclonal antibody (R&D System, goat IgG) or 1.0 µg/ml X17 antibody in DMEM containing 10% FBS, 1% NEAA, and 0.5% Penicillin-Streptomycin for 1 hour on ice. After washing twice with PBS(-), the cells were further incubated with Alexa Fluor 488-labeled donkey anti-goat IgG (Thermo Fisher Scientific) or Alexa Fluor 488-labeled goat anti-human IgG (Thermo Fisher Scientific) diluted 1:10,000 in DMEM containing 10% FBS, 1% NEAA, and 0.5% Penicillin-Streptomycin for 30 minutes on ice. After three washes with PBS(-), the cells were analyzed on EC3800 (Sony). Data were analyzed using FlowJo software (TOMY Digital Biology).

2.4.9. IP–Western analysis of MuSK expression

Wild-type and MuSK KO C2C12 myoblasts were differentiated into myotubes and lysed in lysis buffer. Lysates were incubated with X17-conjugated CNBr-activated Sepharose to immunoprecipitate MuSK. After washing the resin with TBS (pH 7.5), bound proteins were eluted with SDS sample buffer and separated by SDS–PAGE on polyacrylamide gels (ATTO). Proteins were transferred to PVDF membranes (Bio-Rad) using a Trans-Blot Turbo system (Mixed MW program). Membranes were dried, blocked with Intercept TBS blocking buffer (LI-COR), and then incubated with primary antibodies overnight at 4 °C in presence of Signal Enhancer Hikari (Nacalai Tesque). After three washes with TBS-T, membranes were incubated with secondary antibodies for 1 h at room temperature, washed again, rinsed with Milli-Q water, dried, and scanned using an Odyssey XF imaging system (LI-COR). For MuSK detection, AF562 was used as the primary antibody, followed by HRP-conjugated anti-goat IgG (Sigma) as the secondary antibody.

2.4.10. MuSK phosphorylation assay

The culture and differentiation of C2C12 myoblasts (Wild-type or MuSK KO cell line) into myotubes were carried out in a 24-well plate as described above. To prepare the activation medium, 50 µL of PBS containing either Agrin C50 or L1-fused Fc proteins was mixed with 450 µL of differentiation medium. For MuSK activation, the differentiation medium was replaced with the activation medium, and C2C12 myotubes were incubated at 37° C in 5% CO₂ for 30 min. After incubation, activation medium was removed, and cells were lysed in 150 µL of lysis buffer (50 mM HEPES, 150 mM NaCl, 1 µM pepstatin A, 10 µM leupeptin, 1 mM PMSF, 1 mM NaF, 10 mM Na₃VO₄, 0.5% (v/v) NP-40). The lysates were stored on ice for 30 min. The soluble fraction was collected by centrifugation and incubated with anti-MuSK antibody X17-immobilized Sepharose for 1.5 h at 4 °C. Sepharose was washed three times with TBS and

resuspended in SDS-PAGE sample buffer. Proteins were separated by SDS-PAGE and transferred onto a low-fluorescence PVDF membrane (Bio-Rad) using a Trans-Blot Turbo system (Bio-Rad, Program: Mixed MW). The membrane was blocked with Intercept (TBS) blocking buffer (LI-COR) at room temperature for 2 h. For detection of MuSK and phosphorylated tyrosine, the membrane was incubated overnight at 4° C with 0.5 μ g/mL anti-MuSK polyclonal antibody (R&D System, goat IgG), or 1 μ g/mL anti-phosphotyrosine antibody 4G10 (Merk Millipore, mouse IgG) in Intercept (TBS) blocking buffer containing 0.01% (v/v) Tween 20. After washing with TBS containing 0.01% (v/v) Tween 20 (TBS-T), the membrane was incubated with 0.1 μ g/mL IRDye 680RD donkey anti-goat IgG (LI-COR), or 0.1 μ g/mL IRDye 800CW donkey anti-mouse IgG (LI-COR) in Intercept (TBS) blocking buffer containing 0.01% (v/v) Tween 20, and 0.001% (w/v) SDS. After washing by TBS-T, detection was performed using an Odyssey XF imaging system (LI-COR). Band intensities were quantified using Empire Studio v2.2 software (LI-COR).

2.4.11. MuSK-LRP4 binding assay

Expi293F culture supernatants containing secreted MuSK ectodomain fragments were collected by centrifugation and used directly for binding assays. For LRP4 ectodomain fragments, cells were pelleted by centrifugation, lysed in lysis buffer, and clarified by centrifugation; the resulting supernatants were used. For pull-down assays, LRP4-containing lysates were incubated with rProtein A Sepharose (Cytiva) for 1 h at room temperature to capture Fc-fused LRP4 fragments. After washing to remove unbound material, MuSK-containing supernatants were added and incubated for an additional 2 h with rotation. To examine the interaction between membrane-expressed MuSK and LRP4, Expi293F cells were co-transfected with plasmids encoding MuSK and LRP4. Two days after transfection, cells were harvested and lysed in lysis buffer. After clarification by centrifugation, the lysates were incubated with PMab-1-conjugated CNBr-activated Sepharose to immunoprecipitate LRP4. The mixture was rotated for 2 h at 4 °C prior to washing and subsequent analysis. The resin was then washed three times with TBS (pH 7.5) and eluted with SDS sample buffer. Western blotting was performed as described for MuSK expression analysis, except that low-background PVDF membranes were used. For MuSK detection, NZ-1 (anti-PA tag antibody, rat IgG2a)⁵¹ was used as the primary antibody and IRDye 800CW goat anti-rat IgG (LI-COR) as the secondary antibody. For LRP4 detection, anti-His tag antibody (Sigma) and IRDye 680RD goat anti-mouse IgG (LI-COR) were used as primary and secondary antibodies, respectively.

3. Engineering Fc-based MuSK agonists

3.1. Introduction

As described in Chapter 1, MuSK activation is initiated by dimerization driven by its physiological ligands, Agrin and LRP4. In Chapter 2, I demonstrated that among the four extracellular domains of MuSK, the Ig1-Ig2 tandem structure plays an essential role in Agrin-dependent AChR clustering, primarily by imposing a specific and rigid geometry on the MuSK dimer. In contrast, the Ig3-Fz region of MuSK, which is located closer to the plasma membrane, has not been shown to be required for Agrin-dependent signaling, and its functional contribution to MuSK dimerization remains unclear. In contrast, the observation that addition of the X17 antibody to MuSK(Fz) is sufficient to induce AChR clustering indicates that domains other than Ig1-Ig2 can also support downstream signaling. These findings suggest that, even in the absence of Agrin, downstream signaling can be induced if two MuSK molecules are brought together in an appropriate orientation by alternative ligands.

A powerful approach to probe such structural requirements is to artificially dimerize MuSK using molecules that bind defined epitopes on its extracellular region. If MuSK signaling is induced only when the Ig3-Fz domains are crosslinked in a particular orientation, this would directly inform how MuSK must be arranged within the physiological Agrin-MuSK-LRP4 (AML) complex. In this regard, Fc-based artificial dimerizers—such as Mirabodies developed in our laboratory⁵²—provide an attractive platform for constructing receptor tyrosine kinase (RTK) agonists with well-defined geometrical constraints (Fig. 19a–b). Mirabodies are generated by grafting macrocyclic peptide pharmacophores into surface-exposed loops of human IgG1 Fc, yielding bivalent binders whose rigidity and limited conformational freedom enforce specific receptor dimer geometries. Such molecules have been successfully used to create Fc-based agonists for several RTKs, and in some cases have enabled modeling of activation-competent dimer structure^{53 54 55}.

Even when peptide hits are linear rather than macrocyclic, they can be converted into Fc-based bivalent dimerizers by fusing the peptide sequence directly to either terminus of the Fc polypeptide⁵⁶ (Fig. 19c). This strategy allows peptide binders lacking intrinsic structural constraints to be displayed in a bivalent and geometrically restricted format, making them suitable tools for interrogating RTK activation mechanisms.

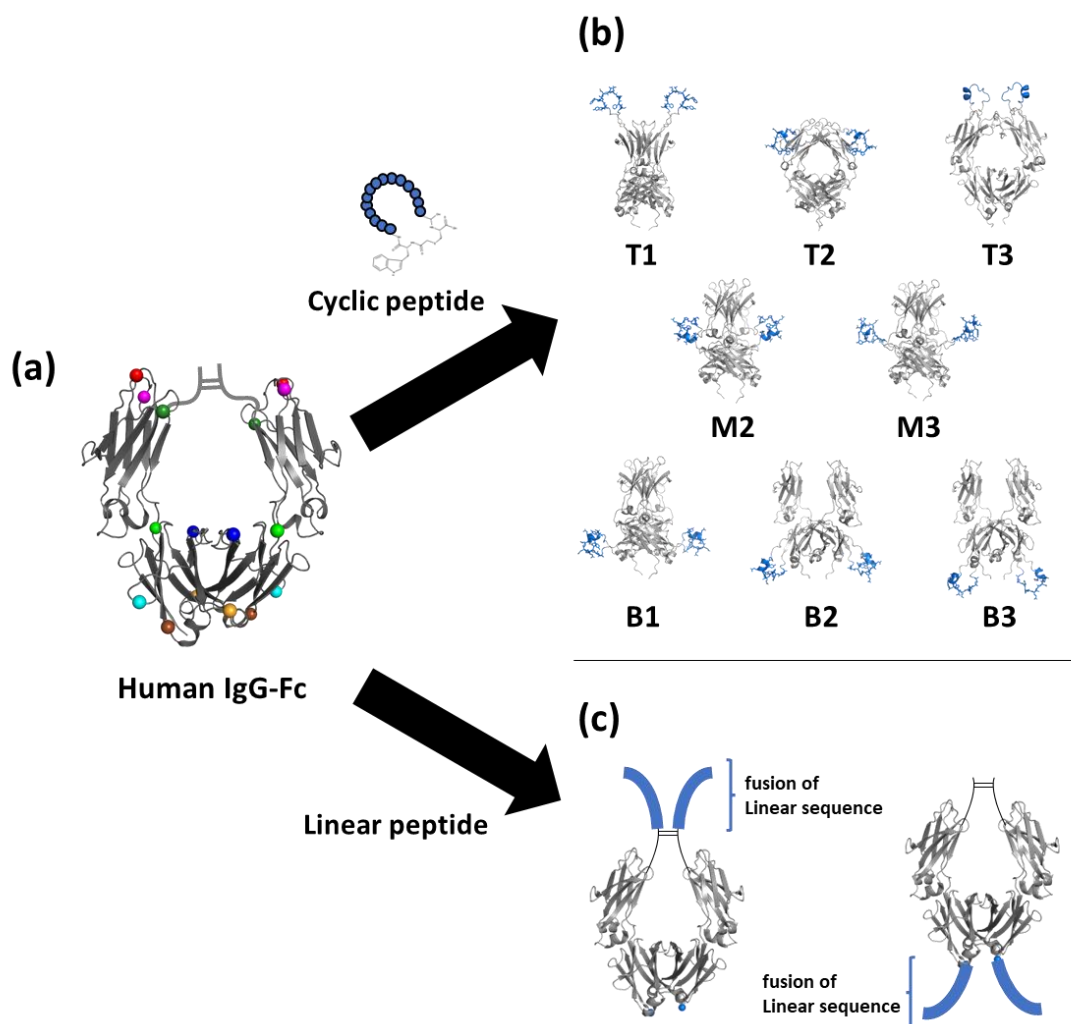


Fig.19 : Design of dimerizers using human IgG Fc

- (a) Crystal structure of human IgG Fc (PDB ID: 1FC1). Dots indicate loop regions where cyclic peptides can be grafted.
- (b) Representative models of Mirabodies, in which macrocyclic peptides (blue) are inserted into eight permissive loop sites on Fc. The insertion site determines the orientation with which the peptide is displayed.
- (c) Conceptual design of Fc-based dimerizers bearing linear peptides. In this format, the linear peptide is fused to the N- or C-terminus of Fc rather than inserted into a loop.

Prior to this study, using the RaPID (Random non-standard Peptides Integrated Discovery) system ⁵⁷ (Fig. 20a), I performed a library screening against the human MuSK extracellular region and identified a 27-residue linear peptide, termed L1, which binds to human MuSK with high affinity ($K_D \approx 1.6$ nM) (Fig. 20b). L1 arose from a one-nucleotide frameshift during translation, which prevented cyclization and resulted in a linear peptide rather than the macrocycle originally intended in the RaPID library design (Fig. 20c). Surface plasmon resonance measurements confirmed that L1 binds MuSK with a dissociation constant of approximately 1.6 nM, demonstrating exceptionally high affinity.

Subsequent biochemical and crystallographic analyses revealed that L1 binds at the interface between the Ig3 and Fz domains of MuSK. The co-crystal structure showed that L1 engages a composite binding pocket formed jointly by these two domains (Fig. 21a–b). This finding opened the possibility of constructing MuSK agonists by fusing L1 to Fc, thereby enforcing defined orientations of the Ig3–Fz region and enabling structural inference regarding the activation-competent MuSK dimer—an otherwise poorly characterized portion of the receptor lacking direct structural information in its dimeric state.

In addition to providing mechanistic insight, artificial MuSK agonists hold considerable therapeutic potential for neuromuscular disorders. In patients who produce autoantibodies against Agrin, LRP4, or MuSK, formation of the AML-complex is impaired, leading to reduced MuSK activity and destabilization of the neuromuscular junction (NMJ) ²⁹. In such cases, exogenous MuSK agonists capable of inducing AChR clustering could, in principle, restore NMJ structure independently of the native ligands. While several agonistic anti-MuSK antibodies have entered clinical development ^{35 36 37 38 37 39 40 41}, non-antibody agonist formats have not yet been reported. Fc-based agonists derived from peptide binders therefore represent a promising alternative modality.

In this chapter, I generated two Fc-based MuSK dimerizers by fusing the L1 sequence to either the N-terminus or the C-terminus of human IgG1 Fc. I show that although both constructs bind MuSK and are capable of bridging two MuSK molecules in solution, only one of them exhibits agonistic activity in muscle cells. By combining functional analyses with structural modeling based on the MuSK–L1 co-crystal structure, I propose a model describing how the activation-competent MuSK dimer is achieved and how differences in Ig3–Fz orientation determine whether downstream AChR clustering signaling is induced.

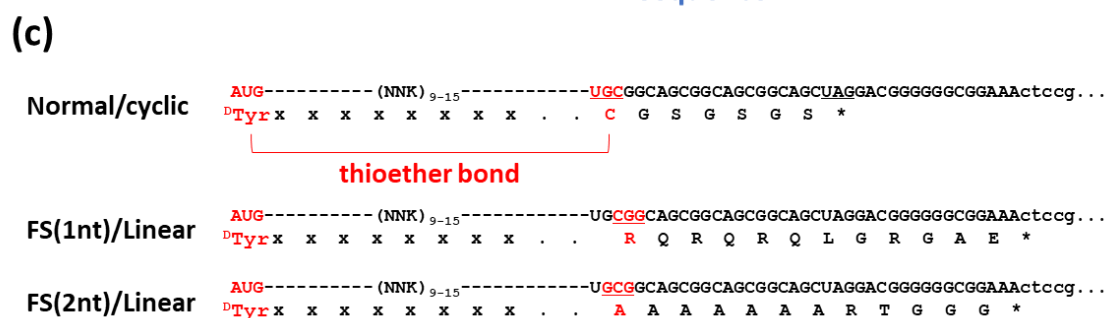
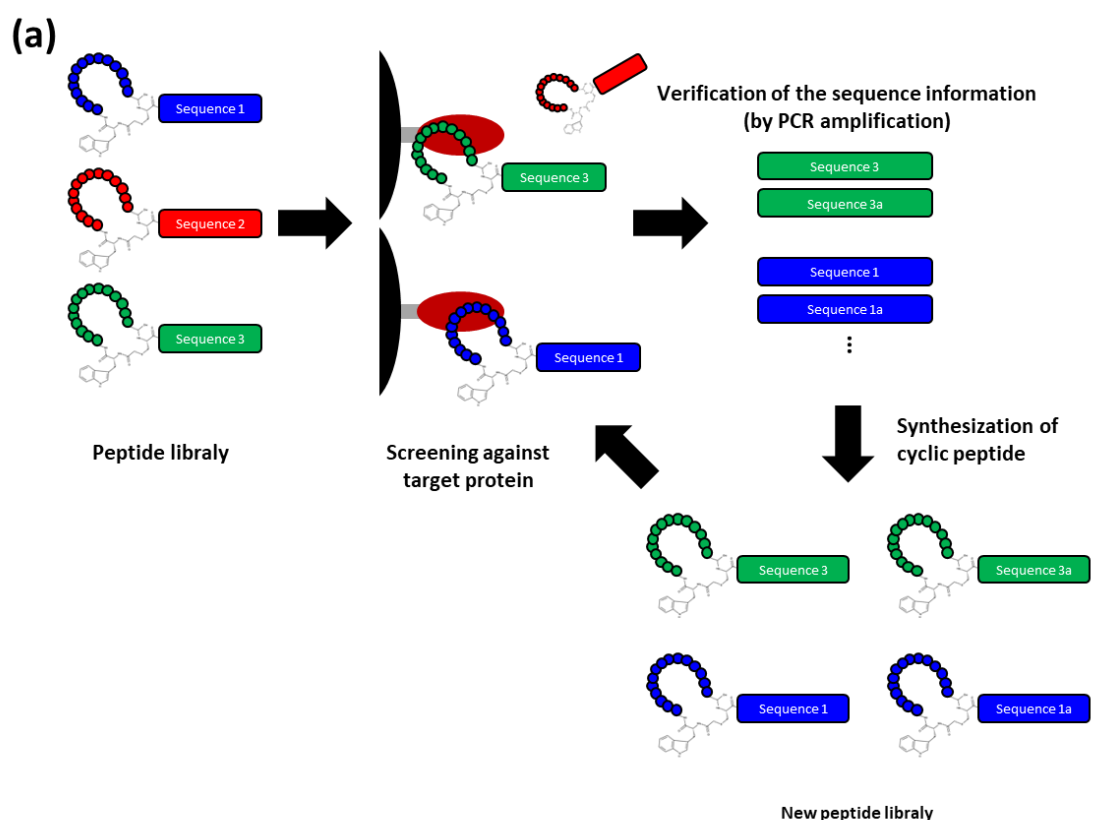
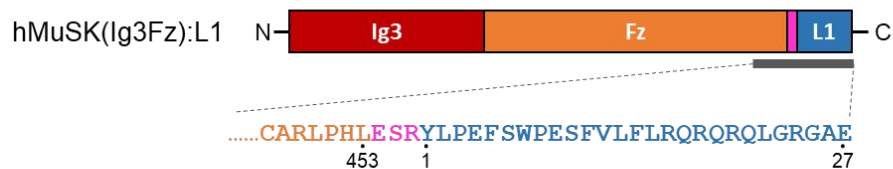


Fig.20 : Isolation of MuSK-binding peptides by RaPID screening

- (a) Schematic overview of the RaPID screening process. A macrocyclic peptide library is translated in vitro, and peptide-mRNA fusions are selected for binding to the immobilized target protein. After reverse transcription and sequencing of enriched cDNAs, new libraries are constructed based on enriched sequences and subjected to additional rounds of selection to enrich for higher-affinity binders.
- (b) Sequence and design of the L1 peptide, the highest-affinity binder obtained from RaPID screening against the human MuSK ectodomain. The sequence indicated by the blue underline corresponds to the selected peptide, and additional N- and C-terminal modifications are introduced to improve stability.
- (c) Schematic of peptide sequences obtained from the RaPID library. The variable region is represented by "X". Under normal translation, the N-terminal D-tyrosine and the downstream cysteine form a thioether bond, generating a macrocyclic peptide. Frameshift events during reverse transcription can disrupt the cysteine codon, resulting in linear peptides such as L1.

(a)



(b)

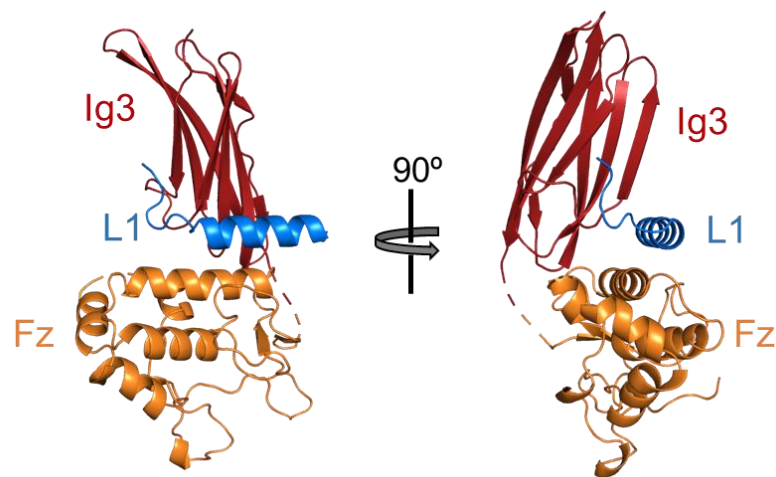


Fig.21 : Binding mode of the L1 sequence to human MuSK

- (a) Design of the fusion protein used for crystallization. The L1 sequence is fused to the C-terminus of the hMuSK Ig3-Fz fragment via a three-residue linker. The amino acid sequence from the C-terminus of hMuSK(Ig3Fz) to the N-terminus of L1 is shown below.
- (b) Overall structure of hMuSK(Ig3Fz) bound to L1 (PDB ID: 9LGG). L1 binds at the junction between the Ig3 and Fz domains, recognizing a composite interface formed by both domains.

3.2. Result

3.2.1. Design of MuSK binder peptide fused Fc

To generate bivalent MuSK-binding proteins, I designed two Fc fusion constructs in which the 27-residue L1 peptide was fused directly to either the N-terminus or the C-terminus of human IgG1 Fc, yielding L1-Fc and Fc-L1, respectively (Fig. 22a). The macrocyclic peptides used in Mirabody, L1 is a linear peptide and therefore cannot be grafted into surface-exposed loop regions of Fc. Instead, terminal fusion was selected to endow the Fc scaffold with two spatially defined MuSK-binding sites.

Because Fc forms a rigid homodimer, each fusion construct is expected to present two copies of the L1 peptide at fixed relative positions. Importantly, depending on whether L1 is fused to the N-terminus or the C-terminus of Fc, the relative orientation of the two L1 peptides—and consequently the geometry with which two MuSK molecules are brought together—is expected to differ.

I first examined whether these fusion proteins could be expressed as soluble, secreted proteins. Transient expression in Expi293F cells yielded both L1-Fc and Fc-L1 at sufficient levels. SDS-PAGE analysis revealed an upward shift in apparent molecular weight relative to plain Fc, consistent with the addition of the L1 peptide sequence (Fig. 22b). I next assessed whether L1-Fc and Fc-L1 retained MuSK-binding capability. As expected, both constructs bound to the MuSK extracellular fragment, whereas plain Fc showed no detectable binding (Fig. 22c). These results demonstrate that the L1 peptides appended to Fc remain functionally competent for MuSK recognition in this fusion format.

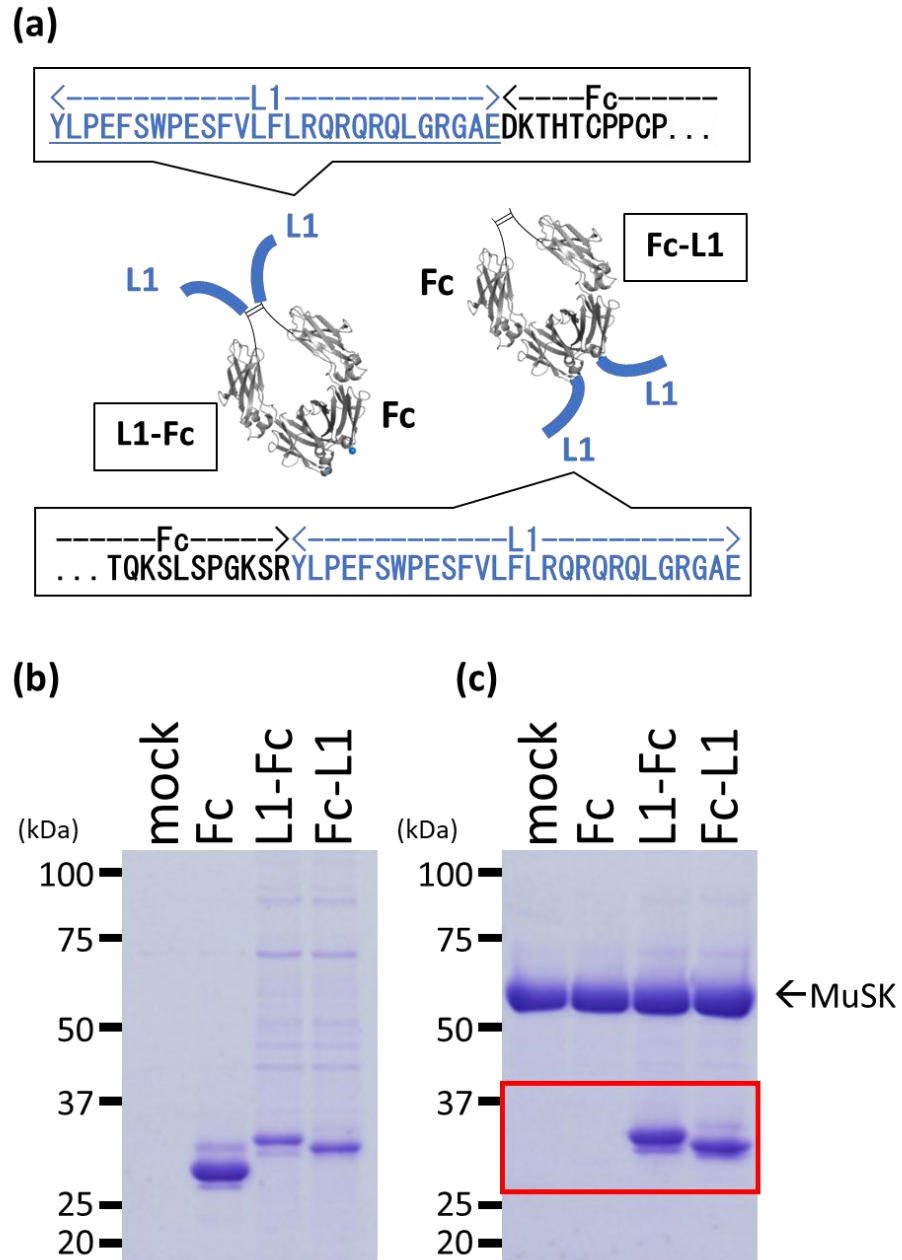


Fig.22 : Design of MuSK dimerizers based on the L1 sequence

- (a) Schematic design of L1-based MuSK dimerizers. The L1 sequence is fused either to the N-terminus (L1-Fc) or C-terminus (Fc-L1) of human IgG1 Fc to generate bivalent MuSK binders.
- (b) Pull-down assay confirming expression of L1-Fc and Fc-L1.
- (c) Binding ability of L1-fused Fc to hMuSK. Both fusion proteins bind hMuSK.

3.2.2. Purification of L1-fused Fc

To quantitatively compare the binding affinities of the two L1-fused Fc constructs and to reliably assess their abilities to induce AChR clustering, it was essential to prepare monodisperse protein samples for each construct. SDS-PAGE analysis of culture supernatants indicated that both L1-Fc and Fc-L1 contained substantial amounts of high-molecular-weight species, suggesting that affinity purification alone was insufficient and that aggregated forms were prevalent. These observations indicated that additional purification steps would be required following ProteinA affinity chromatography.

Indeed, when L1-Fc and Fc-L1 purified using rProtein A Sepharose were analyzed by SDS-PAGE under both reducing and non-reducing conditions, multiple bands corresponding to contaminating or heterogeneous species were detected (Fig. 23a). I therefore subjected both samples to size-exclusion chromatography (SEC) for further separation.

Analysis of L1-Fc by SEC revealed that the majority of the protein eluted as a large aggregate peak, while an additional, smaller peak was observed around fraction 30 (Fig. 23b). The absence of a single sharp monodisperse peak suggested heterogeneity within this fraction. Consistent with this interpretation, SDS-PAGE analysis of individual fractions revealed two bands under reducing conditions around fraction 33, with the lower band likely corresponding to species in which one of the two appended L1 peptides had been proteolytically cleaved (Fig. 23c).

SEC analysis of Fc-L1 showed an even higher proportion of aggregated species than observed for L1-Fc, with only a small monodisperse peak detected around fraction 33 (Fig. 23d). In contrast to L1-Fc, however, SDS-PAGE analysis indicated that proteolytic cleavage of the appended L1 sequence was substantially less pronounced in Fc-L1 (Fig. 23e).

Although the majority of both preparations consisted of aggregated species, fractions corresponding to the presumed monodisperse populations (fractions 25–44 for L1-Fc and fractions 18–44 for Fc-L1) were pooled and concentrated to generate final samples for subsequent analyses. The overall recovery of Fc-L1 after SEC was low, amounting to approximately 5% of the material prior to gel filtration. SDS-PAGE analysis of the final concentrated samples is shown in Fig. 23f. Although minor contaminating bands remained detectable, the majority of each preparation consisted of monodisperse L1-fused Fc proteins suitable for downstream biochemical and cell-based assays.

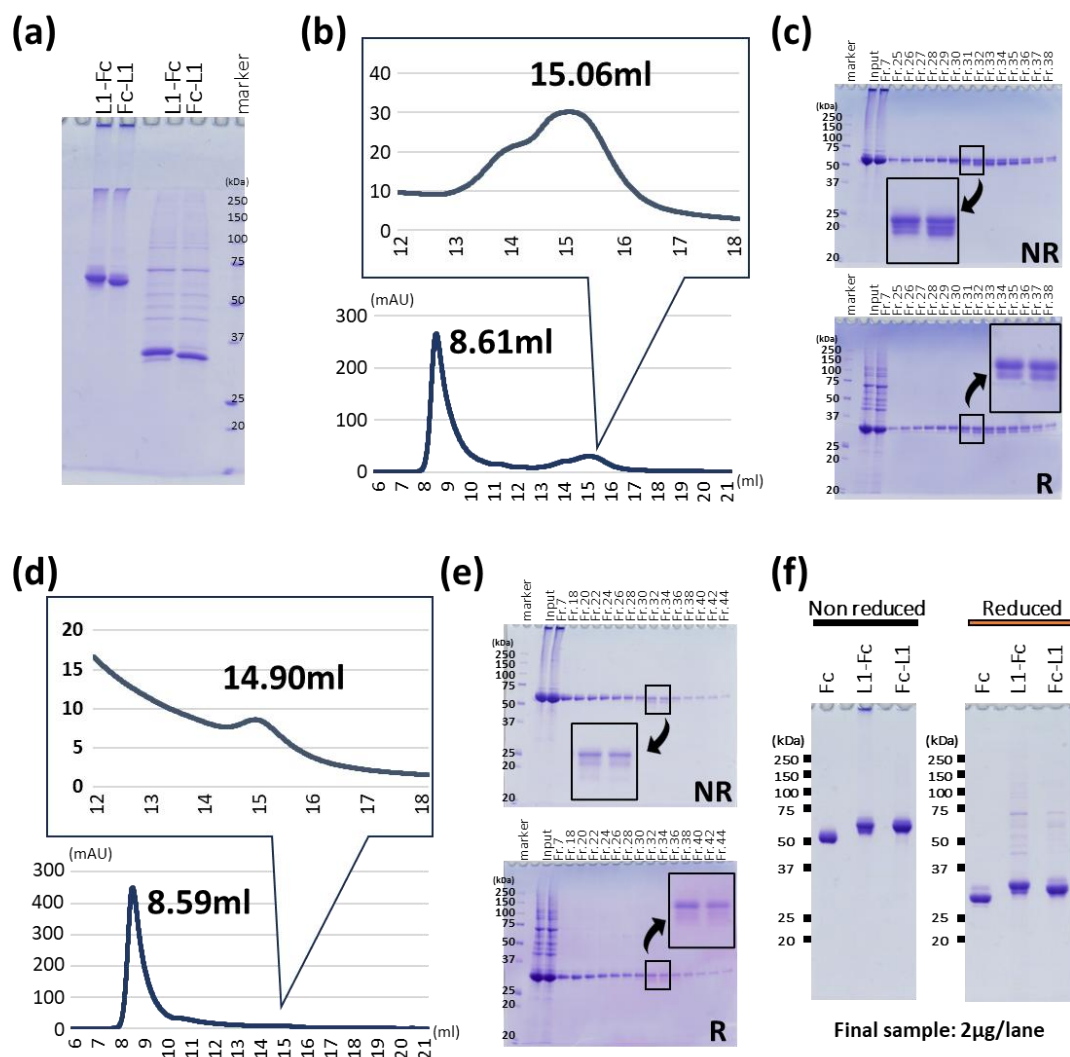


Fig.23 : Preparation of monodisperse L1-Fc and Fc-L1 samples

- SDS-PAGE analysis of L1-Fc and Fc-L1 after ProteinA affinity purification, showing substantial high-molecular-weight species and degradation products.
- SEC profile of L1-Fc (Y-axis: A280(mAU)/X-axis: Elution volume (mL)). The overall profile (bottom) and comparison with molecular weight standards, an enlarged view of the elution range corresponding to approximately 12–18 mL is shown (top). The peak observed at 8.61 mL corresponds to aggregated proteins.
- SDS-PAGE of fractions from the L1-Fc SEC run. Fractions 25–38 (Elution volume: 13–16.5 mL) were analyzed under reducing and nonreducing conditions; fractions 31–32 are shown at larger magnification. The upper band corresponds to intact L1-Fc, whereas the lower band likely represents species in which one L1 sequence has been proteolytically removed. Selected fractions (Fr. 25–38) were pooled for subsequent assays.
- SEC profile of Fc-L1, annotated as in (b).
- SDS-PAGE of fractions from the Fc-L1 SEC run. Fractions 18–44 (Elution volume: 11.25–18 mL) were analyzed under reducing and nonreducing conditions; fractions 32 and 34 are shown at larger magnification. As for L1-Fc, intact and cleaved Fc-L1 species are observed. Selected fractions (Fr. 18–44) were pooled for subsequent assays.

3.2.3. Affinity of L1-Fc and Fc-L1 for human MuSK

I next quantitatively assessed the binding affinities of L1-Fc and Fc-L1 for the recombinant human MuSK ectodomain using the Meso Scale Discovery (MSD) electrochemiluminescence platform. A biotinylated human MuSK ectodomain construct (hMuSKec-BAS-His; Fig. 24a) was purified using Ni-NTA resin, and successful biotin incorporation was verified by fluorescence-based streptavidin blotting (Fig. 24b–c).

Prior to comparing the binding activities of the L1-fused Fc constructs, I first optimized the coating conditions for hMuSKec on the MSD plates. Wells were coated with 0.1, 0.03, or 0.01 pmol of hMuSKec, and binding was detected using the agonistic anti-MuSK antibody X17 as a positive control. Although coating with 0.1 pmol hMuSKec yielded the highest signal intensity, I selected 0.03 pmol per well for subsequent experiments to ensure that measurements remained within the linear detection range of the instrument (Fig. 24d).

Dose–response analyses were then performed using L1-Fc, Fc-L1, and plain Fc. As expected, plain Fc showed no detectable binding to hMuSKec. In contrast, both L1-Fc and Fc-L1 exhibited clear concentration-dependent increases in signal, reaching saturation at approximately 10 nM (Fig. 24e–f). Nonlinear regression analysis yielded apparent dissociation constants (K_{Dapp}) of 0.45 nM for L1-Fc and 1.99 nM for Fc-L1. These results indicate that both L1-fused Fc constructs retain high-affinity binding to MuSK, with only modest differences in apparent affinity.

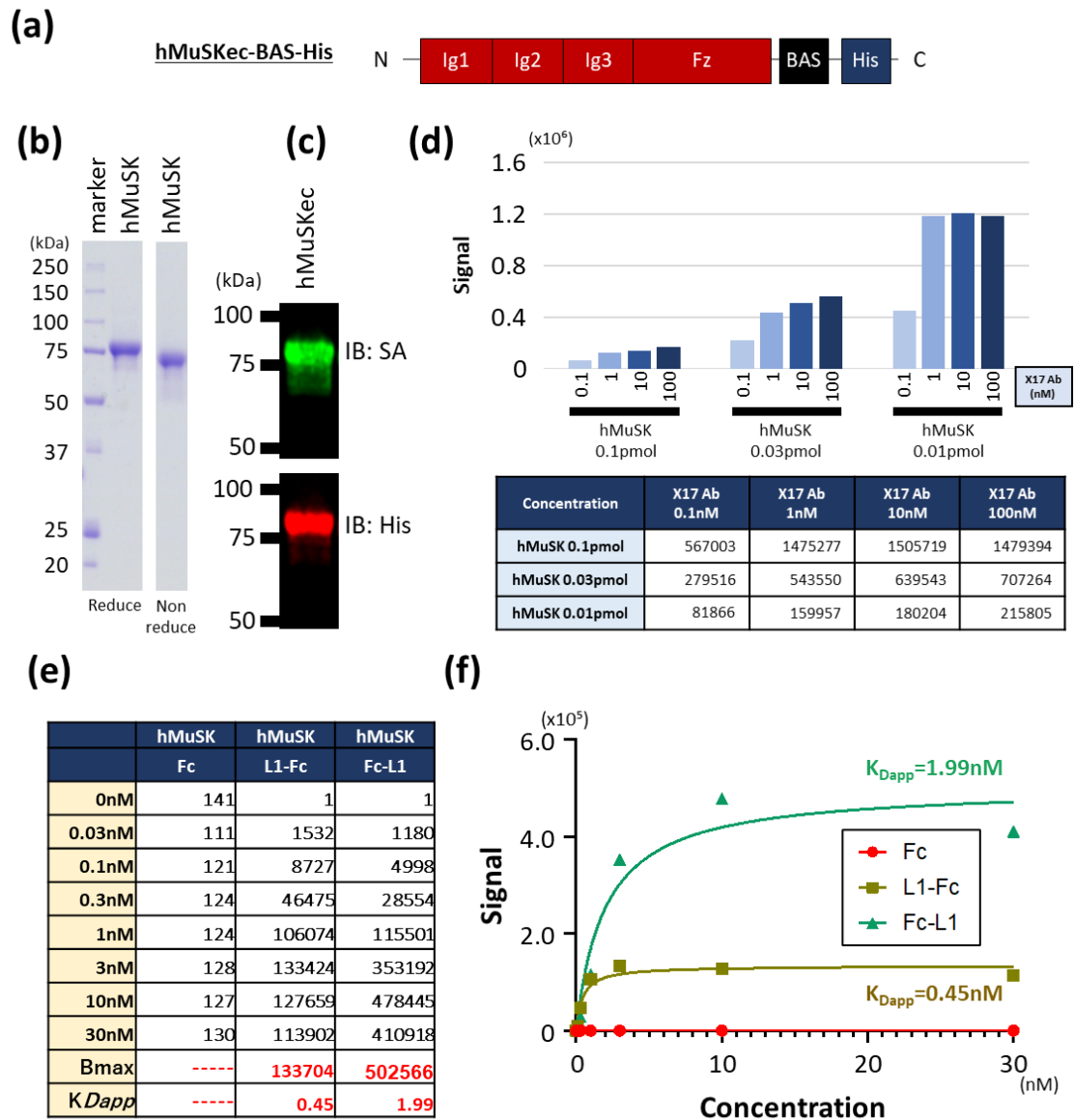


Fig.24 : Comparison of hMuSK binding by L1-Fc and Fc-L1 in MSD assays

- (a) Schematic of the hMuSK ectodomain construct used for MSD assays (hMuSKec-BAS-His).
 (b) SDS-PAGE analysis of purified hMuSKec-BAS-His.
 (c) Confirmation of biotinylation of hMuSKec-BAS-His by streptavidin-based detection.
 (d) Optimization of hMuSKec-BAS-His coating amount for MSD.
 (e) Concentration-dependent binding of L1-Fc and Fc-L1 to hMuSKec-BAS-His coated at 0.03 pmol/well. Apparent KD values (KDapp) were estimated using GraphPad Prism and are indicated in the panel.
 (f) Plot of MSD signal as a function of L1-Fc or Fc-L1 concentration, based on data from (e).

3.2.4. Ability of L1-Fc and Fc-L1 to bridge two MuSK molecules

Because MuSK agonists must crosslink two receptor molecules to induce activation, I next tested whether L1-Fc and Fc-L1 can physically bridge two MuSK molecules. To this end, I used the NanoBiT assay, which reports dimerization of soluble receptors by reconstituting split luciferase fragments (SmBiT and LgBiT) appended to MuSK ectodomain fragments (Fig. 25a).

Purified hMuSKec-SmBiT and hMuSKec-LgBiT constructs (Fig. 25b) were mixed at 10 nM each and increasing concentrations of L1-Fc or Fc-L1 were added. Both fusion proteins induced a bell-shaped increase in luminescence, with maximal signals at ~30 nM (Fig. 25c). This indicates that both constructs efficiently bridge two MuSK molecules in solution with comparable potency. However, the maximal luminescence induced by Fc-L1 was substantially higher than that induced by L1-Fc, suggesting that the two constructs impose different MuSK dimer geometries, which may have functional consequences for downstream signaling.

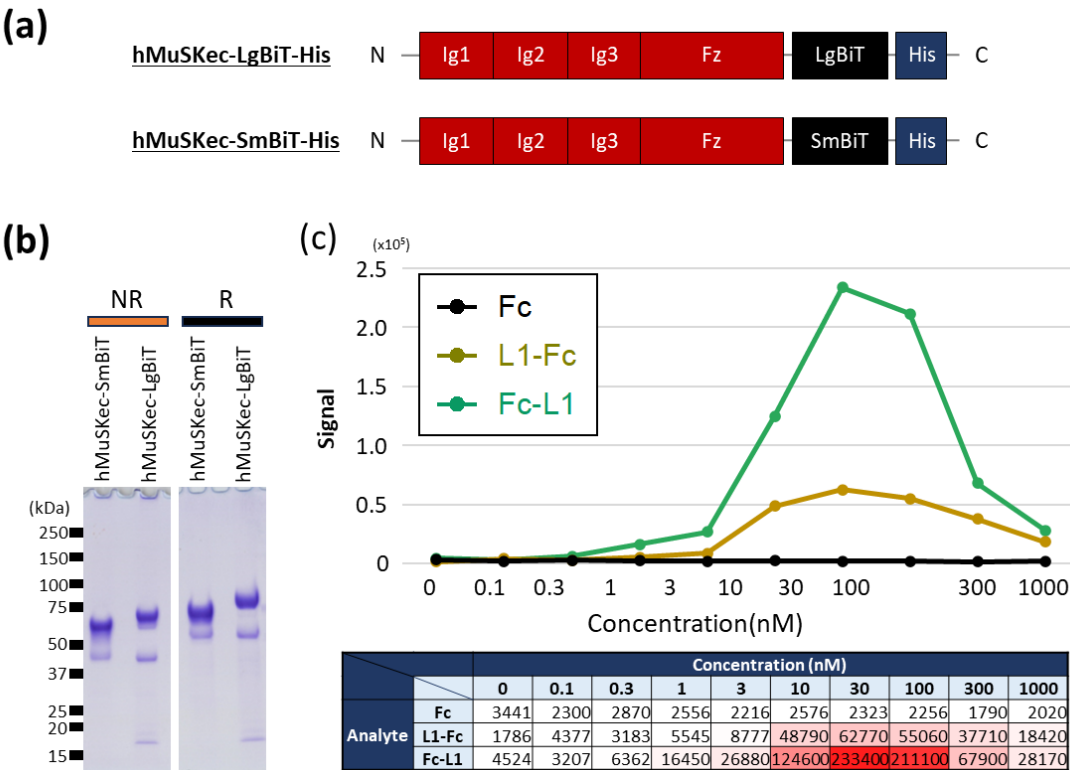


Fig.25 : Evaluation of MuSK dimerization efficiency by L1-Fc and Fc-L1

- (a) Schematic of NanoBiT constructs used in the dimerization assay (hMuSKec-LgBiT-His and hMuSKec-SmBiT-His).
- (b) SDS-PAGE analysis of purified hMuSKec-LgBiT and hMuSKec-SmBiT.
- (c) NanoBiT assay measuring luminescence signals upon addition of L1-Fc or Fc-L1 to 10 nM each of hMuSKec-LgBiT and hMuSKec-SmBiT. Both constructs induce MuSK dimerization in a concentration-dependent manner, with Fc-L1 producing higher maximal signals.

3.2.5. Binding of L1-fused Fc constructs to membrane-anchored MuSK

Before evaluating the agonist activities of L1-Fc and Fc-L1 in muscle cells, I examined whether these constructs could recognize MuSK expressed on the plasma membrane. Because differentiated C2C12 myotubes express endogenous mouse MuSK (mMuSK) rather than human MuSK (hMuSK), it was essential to determine whether the L1-fused Fc constructs are capable of binding membrane-anchored MuSK from both species.

MAP-tagged hMuSK and mMuSK were transiently expressed on the surface of Expi293F cells. Flow cytometric analysis using an anti-MAP tag antibody⁵⁰ confirmed robust surface expression of both constructs, although hMuSK exhibited slightly higher expression levels than mMuSK. Upon incubation with 1000 nM L1-Fc or Fc-L1, both constructs induced clear fluorescence shifts relative to unstained controls, demonstrating that they bind to membrane-anchored MuSK (Fig. 26).

Notably, binding to mMuSK was consistently weaker than to hMuSK. Whereas more than 80% of hMuSK-expressing cells displayed strong positive staining, only slightly above 50% of mMuSK-expressing cells fell within the same fluorescence range. These results indicate that L1-fused Fc constructs can recognize both human and mouse MuSK on the cell surface, but with reduced apparent affinity toward mMuSK. This observation is consistent with the biochemical binding analyses described in Chapter 4.

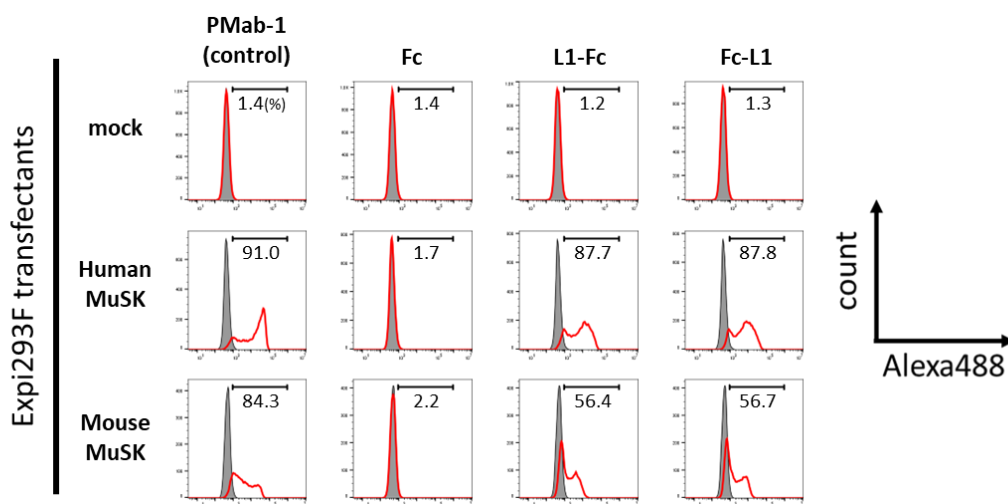


Fig.26 : Binding of L1-Fc and Fc-L1 to full-length human and mouse MuSK

Flow cytometric analysis of L1-Fc and Fc-L1 binding to membrane-anchored hMuSK and mMuSK. Unstained cells are shown in gray, and cells stained with PMab-1 (expression control) or L1-Fc / Fc-L1 (test) are shown in red. The percentages in the upper right of each plot indicate the fraction of cells with fluorescence within the indicated gate.

3.2.6. Agonist activity of L1-Fc and Fc-L1

Using the established C2C12 myotube assay system, I next evaluated whether the L1-fused Fc constructs function as MuSK agonists by assessing both MuSK phosphorylation and induction of AChR clustering (Fig. 27a). Differentiated C2C12 myotubes were treated overnight with plain Fc, L1-Fc, or Fc-L1 across a range of concentrations, followed by fluorescent labeling of AChRs.

Neither plain Fc nor L1-Fc induced detectable AChR clustering at any concentration tested. In contrast, Fc-L1 robustly induced AChR clustering in a dose-dependent manner, generating punctate clusters similar to those induced by Agrin. Quantitative analysis of 32 independent microscopic fields per condition confirmed these observations (Fig. 27b). While plain Fc and L1-Fc showed no concentration-dependent effects, Fc-L1 exhibited a characteristic bell-shaped response, with maximal clustering observed at approximately 3 nM. These data demonstrate that Fc-L1, but not L1-Fc, functions as a MuSK agonist.

Although Fc-L1 clearly induced AChR clustering, its maximal activity was lower than that achieved by stimulation with Agrin, indicating partial agonistic activity.

Analysis of MuSK autophosphorylation under identical conditions revealed a closely correlated pattern. L1-Fc failed to induce detectable MuSK phosphorylation, whereas Fc-L1 promoted MuSK phosphorylation in a concentration-dependent manner (Fig. 27c). As with AChR clustering, the maximal phosphorylation induced by Fc-L1 was lower than that observed following Agrin stimulation. Together, these results demonstrate that Fc-L1 uniquely possesses agonistic activity toward MuSK, whereas L1-Fc is inactive despite comparable MuSK-binding ability.

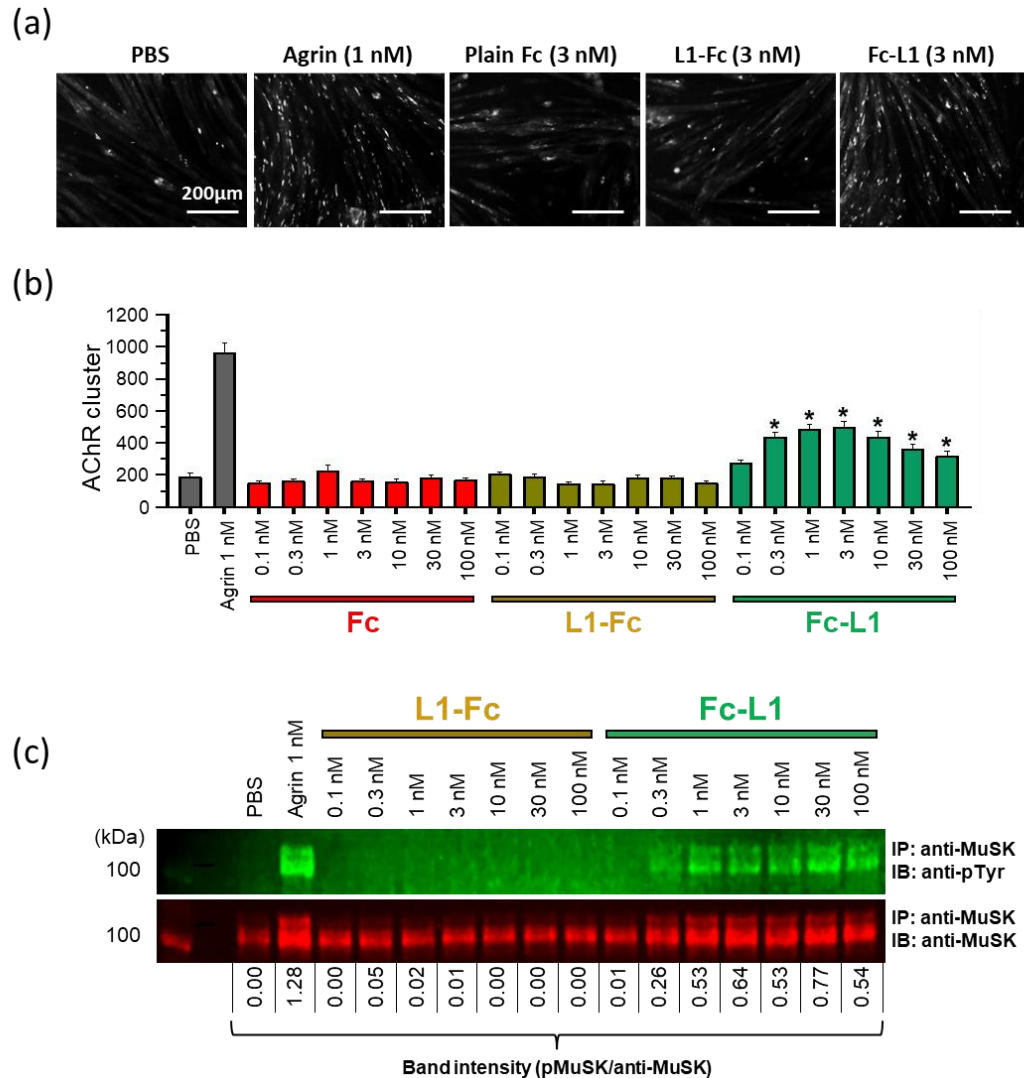


Fig.27 : Agonist activity of L1-Fc and Fc-L1 toward MuSK

- (a) Representative images of AChR clustering in C2C12 myotubes treated with L1-Fc or Fc-L1 at various concentrations. Lower panels are magnified views of the boxed regions in the upper panels. Robust AChR clustering is observed with 1 nM AgrinC50 and 3 nM Fc-L1, but not with L1-Fc.
- (b) Quantification of AChR clusters induced by L1-Fc or Fc-L1 at different concentrations. For each condition, 35 fields were analyzed. Bars represent mean cluster numbers per field; error bars indicate SEM. An independent t-test shows no significant difference between PBS and 3 nM L1-Fc, whereas 3 nM Fc-L1 induces a significant increase.
- (c) MuSK phosphorylation in response to L1-Fc or Fc-L1. Consistent with AChR clustering, only Fc-L1 induces detectable MuSK phosphorylation.

3.2.7. Structural modeling of MuSK dimer geometries induced by L1-Fc and Fc-L1

Both L1-Fc and Fc-L1 bind MuSK with comparable affinity and are capable of bridging two MuSK molecules in solution. However, only Fc-L1 activates MuSK in C2C12 myotubes. This observation indicates that agonist activity is determined not simply by receptor crosslinking per se, but by the specific orientation in which two MuSK molecules are juxtaposed.

To investigate the structural basis underlying this difference, I constructed models of MuSK dimers induced by L1-Fc and Fc-L1, using the crystal structure of the MuSK Ig3-Fz fragment bound to the L1 peptide as a template (Fig. 28a). In the L1-Fc construct, the Fc moiety is fused to the C-terminus of the L1 peptide, whereas in Fc-L1 it is fused to the N-terminus. Because L1 binds asymmetrically at the Ig3-Fz interface, these two fusion topologies impose fundamentally different spatial relationships between MuSK and the Fc scaffold.

In the L1-Fc configuration, the Fc domain is positioned on the C-terminal side of the L1 peptide, adjacent to the flexible linker connecting the Ig3 and Fz domains (Fig. 28b). In contrast, in the Fc-L1 configuration, the Fc domain is located on the opposite side of the bound peptide, oriented away from the Ig3-Fz linker (Fig. 28c). As a result, the two constructs orient the L1 peptides—and consequently the attached MuSK molecules—in nearly opposite directions.

Dimer models generated by arranging two MuSK-L1 complexes around the Fc homodimer revealed pronounced geometric differences. In L1-Fc-induced dimers, the C-terminal ends of the L1 peptides face inward (Fig. 28d), whereas in Fc-L1-induced dimers, the N-terminal ends face inward (Fig. 28e). This inversion leads to substantial differences in the lateral spacing and relative tilt of the Ig3-Fz domains, providing a structural explanation for the distinct agonistic activities of the two constructs.

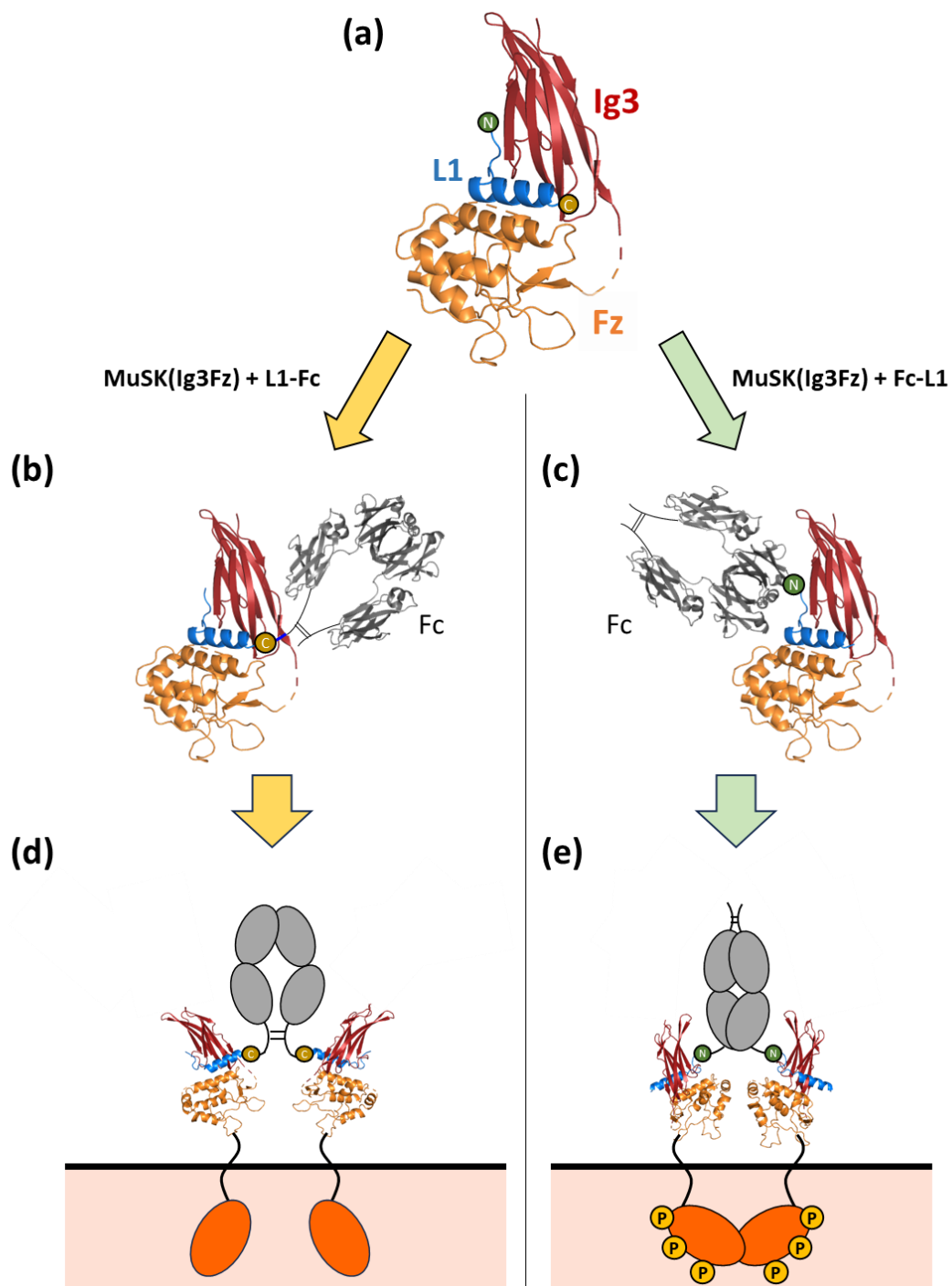


Fig.28 : Structural models of MuSK dimers induced by L1-Fc and Fc-L1

- (a) Binding mode of L1 to hMuSK(Ig3Fz). The N-terminus of L1 is colored green and the C-terminus is colored yellow.
- (b) Model of L1-Fc bound to hMuSK(Ig3Fz), illustrating the relative positioning of Fc and the MuSK ectodomain.
- (c) Model of Fc-L1 bound to hMuSK(Ig3Fz).
- (d) Predicted arrangement of two MuSK(Ig3Fz) molecules crosslinked by L1-Fc. Two MuSK ectodomains are brought together such that the C-terminal ends of the L1 peptides face inward.
- (e) Predicted arrangement of two MuSK(Ig3Fz) molecules crosslinked by Fc-L1, in which the N-terminal ends of the L1 peptides face inward.

3.3. Discussion

3.3.1. Summary

In this chapter, I sought to construct Fc-based MuSK agonists using the high-affinity linear peptide L1 obtained from RaPID screening, and to determine how the orientation of two MuSK molecules affects downstream activation. To this end, I generated two bivalent L1-fused Fc constructs, L1-Fc and Fc-L1. Although both constructs bound MuSK with high affinity and were capable of bridging two MuSK ectodomains in solution, only Fc-L1 exhibited agonist activity in C2C12 myotubes. These results indicate that MuSK activation is critically dependent on the orientation in which two receptor molecules are crosslinked, rather than on affinity or dimerization efficiency alone. Using the crystal structure of the MuSK-L1 complex, I constructed structural models that explain how L1-Fc and Fc-L1 enforce distinct extracellular geometries, and why only one of these geometries is compatible with productive MuSK activation.

3.3.2. Structural requirements for an AChR-clustering-competent MuSK dimer

Although both Fc-fused constructs induced dimerization of soluble MuSK in the NanoBiT assay, only Fc-L1 activated MuSK on the surface of myotubes. This indicates that simple crosslinking of two MuSK molecules is not sufficient; rather, MuSK activation requires a dimeric configuration that allows both DOK7 engagement and proper downstream phosphorylation.

MuSK activation is initiated by autophosphorylation of Tyr553 in the juxtamembrane region, followed by DOK7 binding and formation of the MuSK–DOK7 complex²⁵ (Fig. 16a-d), leading to phosphorylation of the activation loop in the tyrosine kinase domain. To achieve this activation mechanism, the two MuSK intracellular regions must be positioned at an appropriate distance and angle to allow simultaneous recognition by dimeric DOK7. Thus, even if autophosphorylation is detected, downstream AChR clustering will not occur unless the extracellular architecture enables stable formation of the MuSK–DOK7 complex.

Structural modeling revealed that the mutual orientation of the two L1 peptides differs drastically depending on whether Fc is fused to the N-terminus or C-terminus of L1. When Fc-L1 binds MuSK, the Fc domain resides on the N-terminal side of the L1 peptide, generating a dimer in which the Ig3-Fc domains are drawn together in a configuration that positions the Ig1–Ig2 region at a lateral spacing compatible with the physiological AML complex (Fig. 29a). In contrast, L1-Fc positions Fc on the opposite

side of the MuSK–L1 interface, resulting in a broadly “splayed” dimer with excessive Ig1 separation (Fig. 29b). Although this arrangement is sufficient to support partial autophosphorylation, it is predicted to hinder proper DOK7 dimer engagement, explaining why L1-Fc lacks agonist activity.

Importantly, Ig1-domain separation predicted for L1–Fc-induced dimers (~ 220 Å) is far larger than that predicted for Fc–L1 (~ 70 Å), the latter being much closer to the inter-Ig1 distance expected for the physiological AML complex (Fig. 29c). Such excessive lateral separation imposed by L1–Fc is therefore likely incompatible with formation of the downstream DOK7-mediated MuSK–MuSK 2:2 complex, consistent with its inability to activate MuSK despite successful dimerization in solution. These results indicate that activation-competent MuSK dimers must satisfy strict geometric requirements—particularly in the spatial arrangement of the Ig1–Ig2 and Ig3–Fz regions—and that Fc–L1, unlike L1–Fc, approximates this active-state geometry.

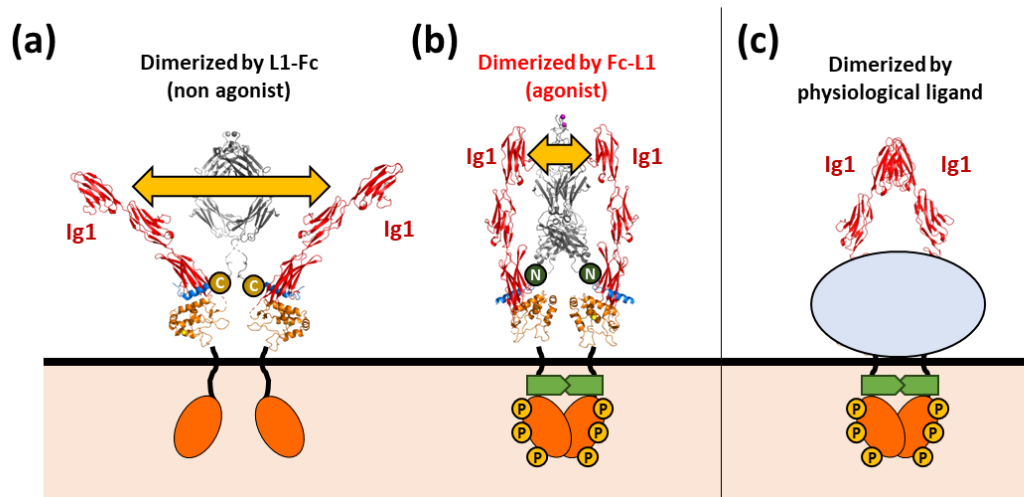


Fig.29 : Impact of MuSK dimer orientation on downstream signaling

Comparison of full-length MuSK dimer models formed by L1–Fc or Fc–L1 with the expected Agrin-induced MuSK dimer. Dimer models for L1–Fc (a) and Fc–L1 (b) were constructed by superimposing AlphaFold2-predicted Ig1–Ig3 structures onto the Ig3–Fz–L1 complex (PDB ID: 9LGG). The distance between Ig1 domains differs markedly between the two models (L1–Fc: ~ 200 Å; Fc–L1: ~ 70 Å). For the Agrin-induced dimer, only the experimentally determined Ig1–Ig2 dimer structure is shown (c).

3.3.3. Limitations and potential for therapeutic application of Fc-L1

Although Fc-L1 functions as a MuSK agonist in cell-based assays, several limitations must be addressed before it could be considered as a therapeutic candidate. First, the L1 sequence is highly susceptible to proteolysis when displayed at the terminus of Fc, as evidenced by multiple bands detected in SDS-PAGE following purification. While such proteolysis did not prevent Fc-L1 from activating MuSK in vitro, it would be unacceptable for a therapeutic molecule, as cleavage of either L1 copy would abolish agonist activity.

Second, Fc-L1 exhibited poor biophysical properties. Both SEC and SDS-PAGE analyses revealed substantial aggregation, likely due to the hydrophobic residues within the L1 peptide. Aggregation not only reduces yield but may also compromise pharmacokinetic behavior and safety in vivo. Thus, significant engineering of the L1 sequence—for example by replacing nonessential hydrophobic residues with polar ones—would be required to improve solubility while maintaining MuSK affinity.

Despite these limitations, Fc-based dimerizers remain an attractive modality for RTK agonist development. Compared with monoclonal antibodies, Fc-based proteins are smaller and offer highly tunable geometries, while retaining favorable pharmacological stability conferred by Fc scaffold. With further engineering to enhance stability and protease resistance, Fc-L1 derived molecules may serve as useful research tools or potential therapeutic candidates for neuromuscular disorders in which MuSK activation is impaired.

3.4. Material & Method

3.4.1. Plasmid constructs

All MuSK constructs were made by PCR or gene synthesis using the human or mouse MuSK sequences (UniProt O15146 or Q61006, respectively). To produce soluble hMuSK ectodomain proteins, the full-length mature sequence (residues 24-495) or its truncated fragments were appended to an artificial signal sequence and fused with various tags including human IgG1 Fc, biotin acceptor sequence (BAS)⁵⁸, 10×His tag, LgBiT/SmBiT (Promega), or PA tag⁵¹ at the C-terminus. The hMuSK segments used for the pull-down assay are as follows: Ig123Fz (Leu24-Leu453), Ig23Fz (Lys110-Leu453), Ig3Fz (Val210-Leu453), Fz (Gln310-Leu453), Ig123 (Leu24-Ser307), and Ig12 (Leu24-Phe221). For soluble mMuSK ectodomain proteins, the full-length mature sequence (residues 24-495) was appended to an artificial signal sequence and fused with BAS and a 10×His tag. For the construction of L1-Fc and Fc-L1, the L1 sequence was fused to the N- and C-termini, respectively, of human IgG1 Fc (residues Asp221-Lys478). The Agrin C50 fragment was constructed from the C-terminal region of human Agrin (Lys1589-Pro2068) containing the Z8 loop and an N-terminal 8×His tag. All constructs were cloned into the pcDNA3.1 vector (Thermo Fisher Scientific), except for the hMuSKec-LgBiT and SmBiT plasmids, which were cloned into the NanoBiT® PPI MCS Starter System Vectors (Promega).

3.4.2. Cell culture

Expi293F cells (Thermo Fisher Scientific) were cultured in Expi293 Expression medium (Thermo Fisher Scientific) at 37° C in a humidified atmosphere of 8% CO₂, with rotation at 125 rpm. C2C12 myoblasts (CRL-1772, ATCC) were maintained in an adherent culture at 37° C in a 5% CO₂ environment using Dulbecco's Modified Eagle's Medium (WAKO) supplemented with 10% (v/v) FBS, 1% (v/v) NEAA, and 0.5% (v/v) Penicillin-Streptomycin.

3.4.3. Protein expression and purification

All proteins were produced in Expi293F cells using the Expi293F Expression system (Thermo Fisher Scientific) and the supernatant was harvested four days post-transfection. For expressing of biotinylated hMuSKec and mMuSKec, cells were co-transfected with human/mouse MuSKec-BAS-His and BirA-ΔKDEL (an ER-resident mutant of the biotin ligase BirA)⁵⁸, followed by addition of 100 μM D-biotin (Nacalai Tesque) to the culture medium one day post-transfection. For the purification of His-

tagged proteins, culture supernatants were mixed with Ni-NTA agarose (QIAGEN) and rotated for 2 h at room temperature. The agarose was transferred to an Econo-Column (Bio-Rad) and washed three times with 10 column volumes of Tris-buffered saline (TBS, 20 mM Tris, 150 mM NaCl, pH 7.5). Proteins were eluted with TBS containing 250 mM imidazole. The fractions containing target molecules were combined and dialyzed against phosphate-buffered saline (PBS, 10 mM Na-phosphate, 140 mM NaCl, 2.7 mM KCl, pH 7.4) at 4° C. Purification of Fc-containing proteins were performed by affinity chromatography using rProtein A Fast Flow Sepharose (Cytiva). Culture supernatants were mixed with rProtein A resin and rotated for 2 h at room temperature. The beads were transferred to an Econo-Column and washed three times with 10 column volumes of TBS. Fc-containing proteins were eluted with the IgG elution buffer (Thermo Scientific) and immediately neutralized with 1/10 volume of 1 M Tris-HCl, pH 9.0. The protein fractions were combined and dialyzed against PBS. For L1-fused Fc proteins, a second purification step of size-exclusion chromatography on a Superdex 200 Increase 10/300 GL column (Cytiva), equilibrated in PBS, was included to remove oligomeric species. All purified proteins were concentrated using an Amicon Ultra-4 Centrifugal Filters Ultracel-30K and stored at –80° C until use.

3.4.4. Pull-down assay

Proteins expressed in the Expi293F culture medium were collected via centrifugation and used for pull-down binding assays. Briefly, anti-PA tag NZ-1 immobilized Sepharose beads were mixed with the culture supernatant containing PA-tagged hMuSK ectodomain fragments and rotated for 1.5 h. After removing the supernatant, the culture supernatant containing L1-fused Fc was added, and the mixture was rotated for an additional 1.5 h. The NZ-1 resin was washed three times with TBS, and elution was performed using 0.1 μ g/mL PA14 peptide in TBS. The eluted sample was mixed with SDS-PAGE sample buffer and subjected to SDS-PAGE using a 5–20 % gradient gel (ATTO). After electrophoresis, the gel was stained with Coomassie Brilliant Blue (CBB) and then destained.

3.4.5. Meso Scale Discovery (MSD) binding assay

For binding analysis of the L1-fused Fc proteins against hMuSKec, each well of the MSD GOLD 96-Well Streptavidin Plate (MSD) was coated with 50 μ L of biotinylated hMuSKec in PBS and incubated overnight at 4 °C. MuSK-coated wells were blocked with 150 μ L of 3 % (w/v) BSA in PBS containing 0.05% (v/v) Tween-20 (PBS-T) for 30 min, followed by washing with 150 μ L of PBS-T. Next, 50 μ L of various concentrations

of L1-fused Fc in PBS-T containing 1 % (w/v) BSA were added. The plate was shaken at 400 rpm at room temperature for 2 h in the dark, followed by three washes with PBS-T. Subsequently, 50 μ L/well of 1 μ g/mL SULFO-TAG Goat Anti-human Antibody (MSD) was added, and the plate was shaken again at 400 rpm at room temperature for 1 h. After three more washes with 150 μ L of PBS-T, 150 μ L of 2 $\times$ Read Buffer was added, and plates were read using the MESO QuickPlex SQ 120. The half-maximal binding concentration (KDapp) was calculated using GraphPad Prism10 software.

3.4.6. NanoBiT assay

Twenty-five μ L each of hMuSKec-SmBiT (10 nM) and hMuSKec-LgBiT (10 nM) in PBS were added to a 96-well F-bottom black plate (Greiner). Subsequently, 25 μ L of L1-fused Fc (0 nM–2,000 nM) in PBS was added to each well and mixed well. After incubation at 37° C for 15 min, 12.5 μ L of Nano-Glo Live Cell Reagent (Promega) was added, and the plate was shaken for 15 s at 400 rpm. Luminescence was detected using the GloMax Navigator (Promega).

3.4.7. Flow cytometry

Expi293F cells transfected with the plasmids containing full length hMuSK or mMuSK that had been modified to contain N-terminal MAP-tag were harvested two days post-transfection, transferred into a V-bottom 96-well plate at 2.0×10^5 cells/well and incubated with 5.0 μ g/ml purified PMab-1 (anti-MAP tag antibody, rat IgG2a) or 1 μ M L1-fused Fc in DMEM containing 10% FBS, 1% NEAA, and 0.5% Penicillin-Streptomycin for 1 hour on ice. After washing twice with PBS(-), the cells were further incubated with Alexa Fluor 488-labeled goat anti-rat IgG (Thermo Fisher Scientific) or Alexa Fluor 488-labeled goat anti-human IgG (Thermo Fisher Scientific) diluted 1:10,000 in DMEM containing 10% FBS, 1% NEAA, and 0.5% Penicillin-Streptomycin for 30 minutes on ice. After three washes with PBS(-), the cells were analyzed on EC3800 (Sony). Data were analyzed using FlowJo software (TOMY Digital Biology).

3.4.8. MuSK phosphorylation assay

The culture and differentiation of C2C12 myoblasts into myotubes were carried out in a 24-well plate as described above. To prepare the activation medium, 50 μ L of PBS containing either Agrin C50 or L1-fused Fc proteins was mixed with 450 μ L of differentiation medium. For MuSK activation, the differentiation medium was replaced with the activation medium, and C2C12 myotubes were incubated at 37 °C in 5% CO₂ for 30 min. After incubation, activation medium was removed, and cells were lysed in 150

μ L of lysis buffer (50 mM HEPES, 150 mM NaCl, 1 μ M pepstatin A, 10 μ M leupeptin, 1 mM PMSF, 1 mM NaF, 10 mM Na₃VO₄, 0.5 % (v/v) NP-40). The lysates were stored on ice for 30 min. The soluble fraction was collected by centrifugation and incubated with anti-MuSK antibody X17-immobilized Sepharose for 1.5 h at 4 °C. Sepharose was washed three times with TBS and resuspended in SDS-PAGE sample buffer. Proteins were separated by SDS-PAGE and transferred onto a low-fluorescence PVDF membrane (Bio-Rad) using a Trans-Blot Turbo system (Bio-Rad, Program: Mixed MW). The membrane was blocked with Intercept (TBS) blocking buffer (LI-COR) at room temperature for 2 h. For detection of MuSK and phosphorylated tyrosine, the membrane was incubated overnight at 4 °C with 0.5 μ g/mL anti-MuSK polyclonal antibody (R&D System, goat IgG), or 1 μ g/mL anti-phosphotyrosine antibody 4G10 (Merk Millipore, mouse IgG) in Intercept (TBS) blocking buffer containing 0.01% (v/v) Tween-20. After washing with TBS containing 0.01 % (v/v) Tween-20 (TBS-T), the membrane was incubated with 0.1 μ g/mL IRDye 680RD donkey anti-goat IgG (LI-COR), or 0.1 μ g/mL IRDye 800CW donkey anti-mouse IgG (LI-COR) in Intercept (TBS) blocking buffer containing 0.01 % (v/v) Tween-20, and 0.001 % (w/v) SDS. After washing by TBS-T, detection was performed using an Odyssey XF imaging system (LI-COR). Band intensities were quantified using Empire Studio v2.2 software (LI-COR).

3.4.9. AChR clustering assay

C2C12 myoblasts were seeded onto a 24-well plate at 2.5×10^4 cells/well. After three days in growth medium, the cells were switched to differentiation medium (DMEM with 2 % horse serum, 1 % NEAA, 0.5 % Penicillin-Streptomycin) to induce differentiation into myotube, and cultured for two days. Stimulation was started by replacement of medium with that containing Agrin C50, human IgG Fc, or L1-fused Fc, and the cells were cultured for additional 18 h. Myotubes were incubated with Alexa Fluor 594-conjugated α -bungarotoxin (Life Technologies) for 1 h at 37 °C, washed three times with PBS, and fixed in 3 % paraformaldehyde for 10 min. After three additional washes with PBS, images were captured from 33 fields using a BZ-X800 microscope (KEYENCE), and data were analyzed using BZ-X800 analysis software (KEYENCE).

4. Functional Analysis of the MuSK-Binder Peptide L1

4.1. Introduction

In Chapter 3, I demonstrated that the L1 peptide can serve as a basis for constructing Fc-fused MuSK agonists, and that the orientation in which L1 crosslinks two MuSK molecules critically determines whether downstream AChR clustering is induced. A notable characteristic of L1, however, is its reduced affinity for mouse MuSK (mMuSK) compared with human MuSK (hMuSK). Because RaPID screening identifies peptides against the specific protein used as the target⁵⁹—in this case, the human MuSK ectodomain—it is expected that the selected peptide would preferentially recognize hMuSK. Nevertheless, high affinity for human MuSK does not necessarily guarantee comparable affinity toward MuSK from other species, as even a single amino acid difference within the binding epitope can substantially alter peptide recognition.

The co-crystal structure of the MuSK Ig3–Fz region in complex with L1 (Fig. 30a) revealed that the L1 peptide can be divided into three segments: (i) Tyr1–Trp7, (ii) Pro8–Leu22, and (iii) Gly23–Glu27. The first segment does not adopt a defined secondary structure but interacts primarily through backbone-mediated contacts with a loop region of the Ig3 domain (Fig. 30b). Within this segment, Phe5 and Trp7 insert into a hydrophobic pocket formed by the Ig3 and Fz domains; in particular, the aromatic ring of Trp7 penetrates deeply into this pocket and forms a side-chain hydrogen bond with Ser254 of Ig3.

The second segment adopts an α -helical conformation, in which hydrophobic residues—Phe11, Val12, Phe14, and Leu15—constitute the core of the MuSK–L1 interface, forming extensive hydrophobic interactions with both the Ig3 and Fz domains (Fig. 30c). In addition, Arg18 engages in electrostatic interactions with residues such as Arg321, Glu345, and Glu346 within the Fz domain (Fig. 30d). By contrast, the third segment, Gly23–Glu27, is disordered in the crystal structure and appears to contribute minimally to MuSK binding.

Flow cytometry experiments described in Chapter 3 further indicated that L1–Fc binds mMuSK less efficiently than hMuSK, suggesting a species-dependent difference in the L1–MuSK interaction. To explore the structural basis for this difference, I compared the amino acid sequences of the Fz domains from hMuSK, mMuSK, and rat MuSK. This comparison revealed several amino acid substitutions within the L1-binding region (Fig. 30e). Notably, the residue corresponding to Arg321 in hMuSK is replaced by Glu in mMuSK. Moreover, structural comparison between the previously reported rat MuSK Fz domain⁶⁰ and the hMuSK Fz domain revealed additional conformational differences in loop regions that are

relevant to peptide recognition (Fig. 30f–h). Together, these sequence and structural differences are likely to contribute to the reduced affinity of L1 for mMuSK.

However, these considerations are based primarily on structural inference, and functional evaluation requires direct biochemical validation. In particular, two key questions must be addressed: (i) which regions or residues within the L1 sequence are essential for MuSK binding, and (ii) which specific differences between hMuSK and mMuSK are responsible for the observed species-dependent affinity.

In addition, Fc–L1 exhibits poor biophysical properties, including aggregation and susceptibility to proteolysis, largely due to the hydrophobic nature of the L1 sequence. If Fc–L1 or its derivatives are to be developed as therapeutic candidates or robust research tools, substantial optimization of the peptide sequence will be required. A detailed functional analysis of individual L1 residues is therefore essential not only for understanding binding specificity but also for guiding rational improvements in peptide stability and solubility.

In this chapter, I investigate the functional contributions of individual residues within the L1 sequence and dissect the molecular origin of species-dependent differences in MuSK binding. Using a series of truncated and alanine-substituted L1-Fc variants, together with mMuSK mutants engineered to mimic hMuSK at key positions, I identify residues within both L1 and MuSK that govern binding strength and specificity. These results provide a molecular framework for understanding the L1-MuSK interaction and establish a foundation for the future optimization of L1-derived MuSK agonists.

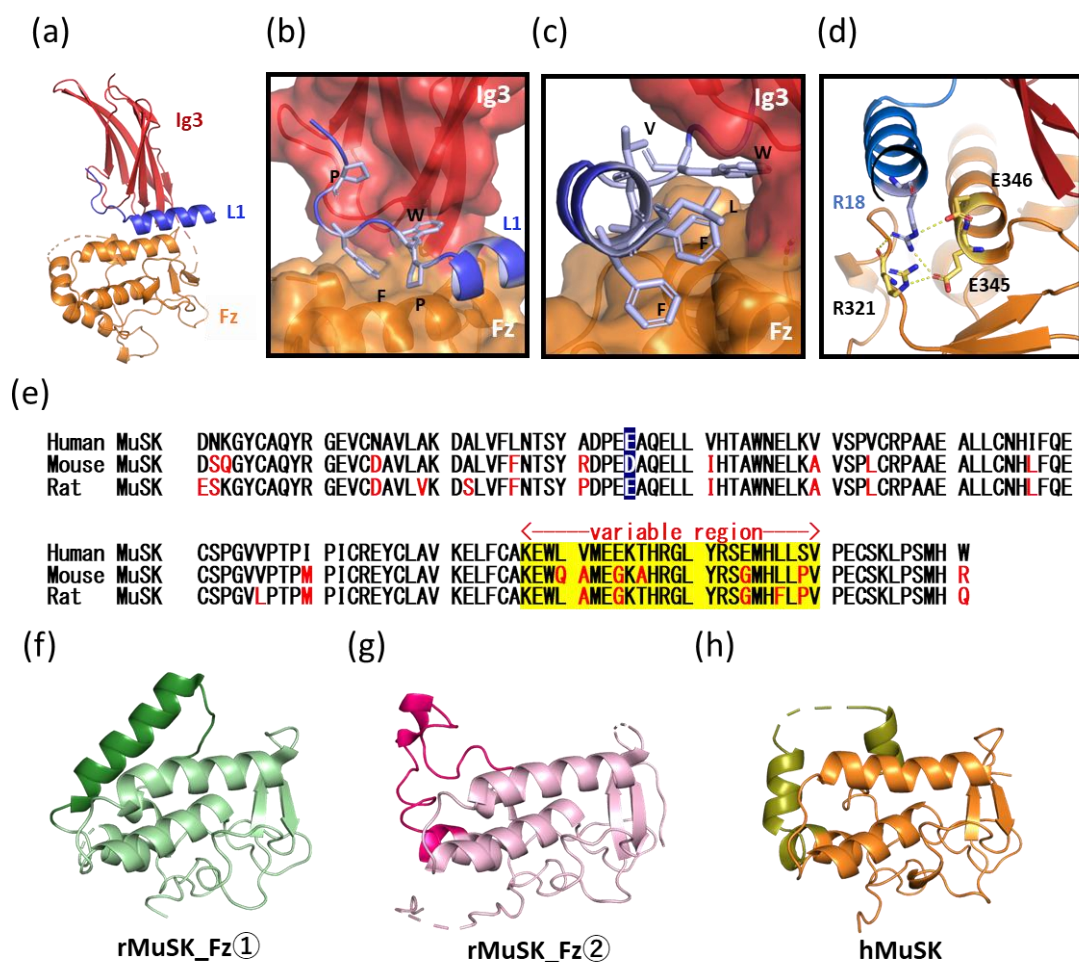


Fig.30 : Binding mode of L1 to hMuSK and comparison of Fz domains across species

- (a) Overall structure of the hMuSK(Ig3Fz)-L1 complex. Electron density for L1 is observed for residues Tyr1-Gln19.
- (b) Interactions between MuSK and the Tyr1-Pro8 segment of L1. Pro3, Phe5, and Trp7 contact MuSK via their side chains.
- (c) Interactions between MuSK and the Glu9-Leu15 segment of L1. Phe11, Val12, Phe14, and Leu15 form hydrophobic contacts with MuSK.
- (d) Interaction of L1 Arg18 with MuSK Fz residues Arg321, Glu345, and Glu346; these form salt bridges and electrostatic contacts.
- (e) Sequence alignment of the Fz domain from human, rat, and mouse MuSK. Residues that differ from the human sequence are shown in red. The residue equivalent to hMuSK Glu345 (which contacts Arg18 of L1) is highlighted in blue. The variable region that lacks a defined structure in rat MuSK is highlighted in yellow.
- (f-h) Structures of rat and human MuSK Fz domains, with the variable region highlighted in bold color.

4.2. Result

4.2.1. Functional analysis of L1 sequence

I first sought to identify residues within the L1 peptide that are essential for MuSK binding. Guided by the MuSK–L1 co-crystal structure, the peptide was divided into three segments—Tyr1-Trp7, Pro8-Leu22, and Gly23-Glu27. Because the central segment adopts an α -helical conformation, Pro8-Leu22 was further subdivided into two blocks (Pro8-Leu15 and Arg16-Leu22) for systematic deletion analysis (Fig. 31a), and a series of corresponding L1-Fc truncation variants was generated.

Upon expression in Expi293F cells, all C-terminal truncation variants were produced at detectable levels, whereas N-terminal deletions—including removal of only Tyr1-Trp7—failed to yield stable secreted protein (Fig. 31b). Owing to this expression defect, only C-terminal truncation variants were subjected to biochemical evaluation of MuSK binding using pull-down assays.

Deletion of residues Arg16-Glu27 (L1(1-15)-Fc) retained robust MuSK-binding activity, whereas further truncation to Pro8 (L1(1-7)-Fc) completely abolished binding (Fig. 31c). These results define residues 1-15 of L1 as the minimal region required for MuSK recognition.

To delineate the contributions of individual residues within this minimal region, alanine-scanning mutagenesis was performed across residues 1-15. All alanine-substituted variants were expressed at comparable levels (Fig. 31d). Pull-down analyses revealed that eight residues—Pro3, Phe5, Trp7, Pro8, Phe11, Val12, Phe14, and Leu15—are indispensable for MuSK binding, as substitution at any of these positions markedly reduced MuSK association (Fig. 31e).

These biochemical findings are in excellent agreement with structural observations from the MuSK-L1 complex. Phe5 and Trp7, together with the hydrophobic cluster formed by Phe11-Leu15, occupy the hydrophobic cleft at the Ig3-Fc interface, accounting for their essential contribution. Pro3 supports backbone-mediated interactions along the Ig3 loop surface, while Pro8 appears to stabilize the α -helical conformation of residues 8-15, thereby indirectly promoting formation of a MuSK-binding-competent structure. Collectively, these results demonstrate that MuSK recognition by L1 is governed predominantly by hydrophobic interactions and conformational constraints within the N-terminal half of the peptide.

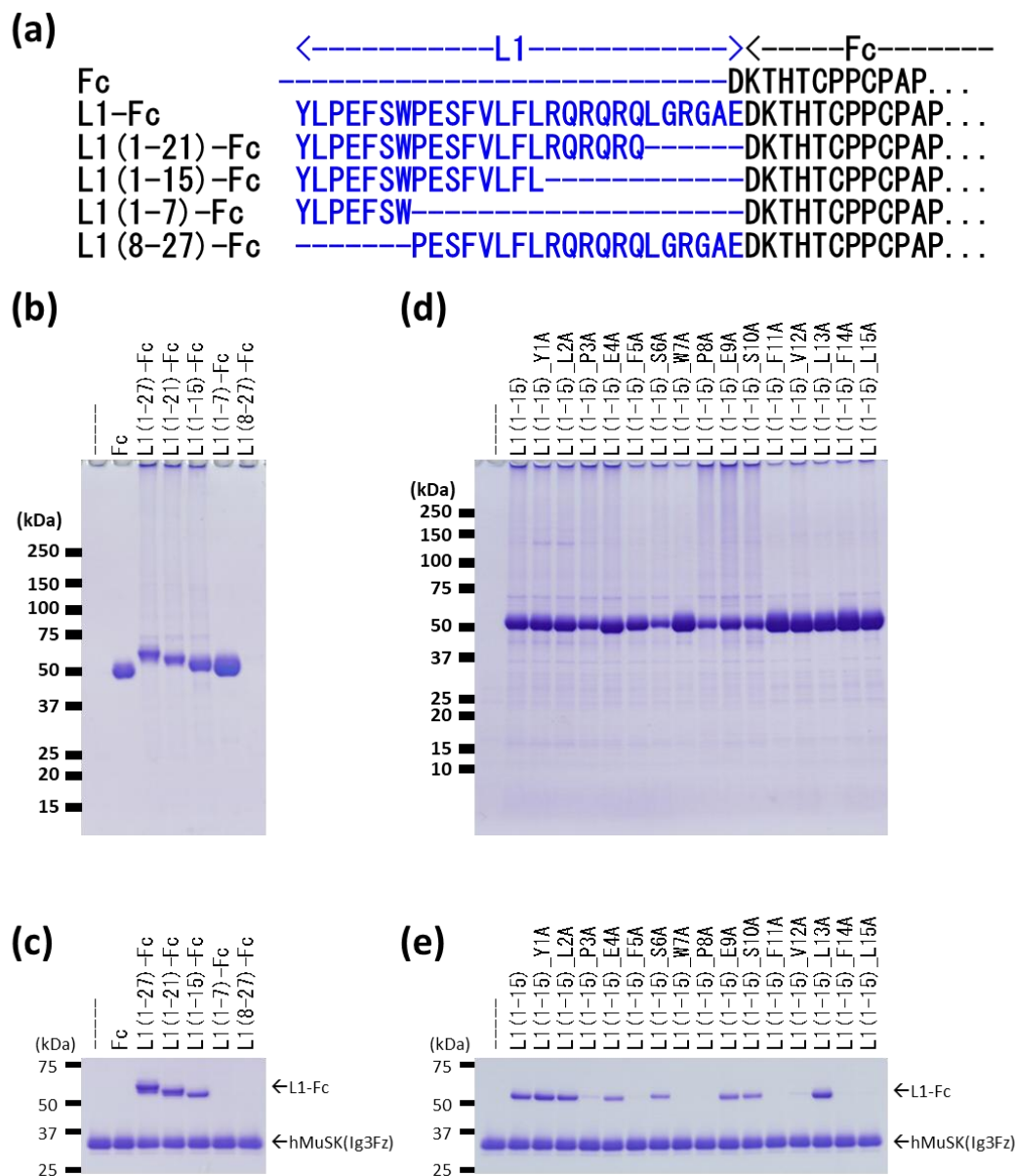


Fig.31 : Binding of L1-Fc variants to hMuSK

- (a) Design of L1-Fc truncation variants in which portions of the L1 sequence were deleted.
- (b) Expression of truncation variants in Expi293F cells, assessed by SDS-PAGE or flow cytometry.
- (c) Pull-down assay evaluating hMuSK binding by L1-Fc truncation variants.
- (d) Expression of each alanine-substituted variant.
- (e) Binding to hMuSK assessed by pull-down assays.

4.2.2. Species-dependent differences in L1 affinity for human and mouse MuSK

I next quantitatively compared the affinities of L1-Fc for human and mouse MuSK. Our previous flow cytometric analyses suggested that L1-Fc binds mouse MuSK (mMuSK) less efficiently than human MuSK (hMuSK). To rigorously quantify this species-dependent difference, I generated a biotinylated mMuSK ectodomain construct analogous to the hMuSKec-BAS-His protein used in earlier experiments. SDS-PAGE and streptavidin blotting confirmed efficient expression and biotinylation of mMuSK at levels comparable to those of hMuSK (Fig. 32a-c), and comparable coating conditions produced robust signals in the MSD assay.

Dose-response analysis revealed a pronounced species-dependent disparity in binding. L1-Fc binding to hMuSK reached saturation at approximately 10 nM, in agreement with previously determined affinity values. In contrast, binding to mMuSK did not reach saturation even at 1000 nM, yielding only ~40% of the maximal signal observed for hMuSK (Fig. 32d-e). These results clearly demonstrate that L1 exhibits a strong preference for hMuSK over mMuSK, establishing a marked species specificity in the L1-MuSK interaction.

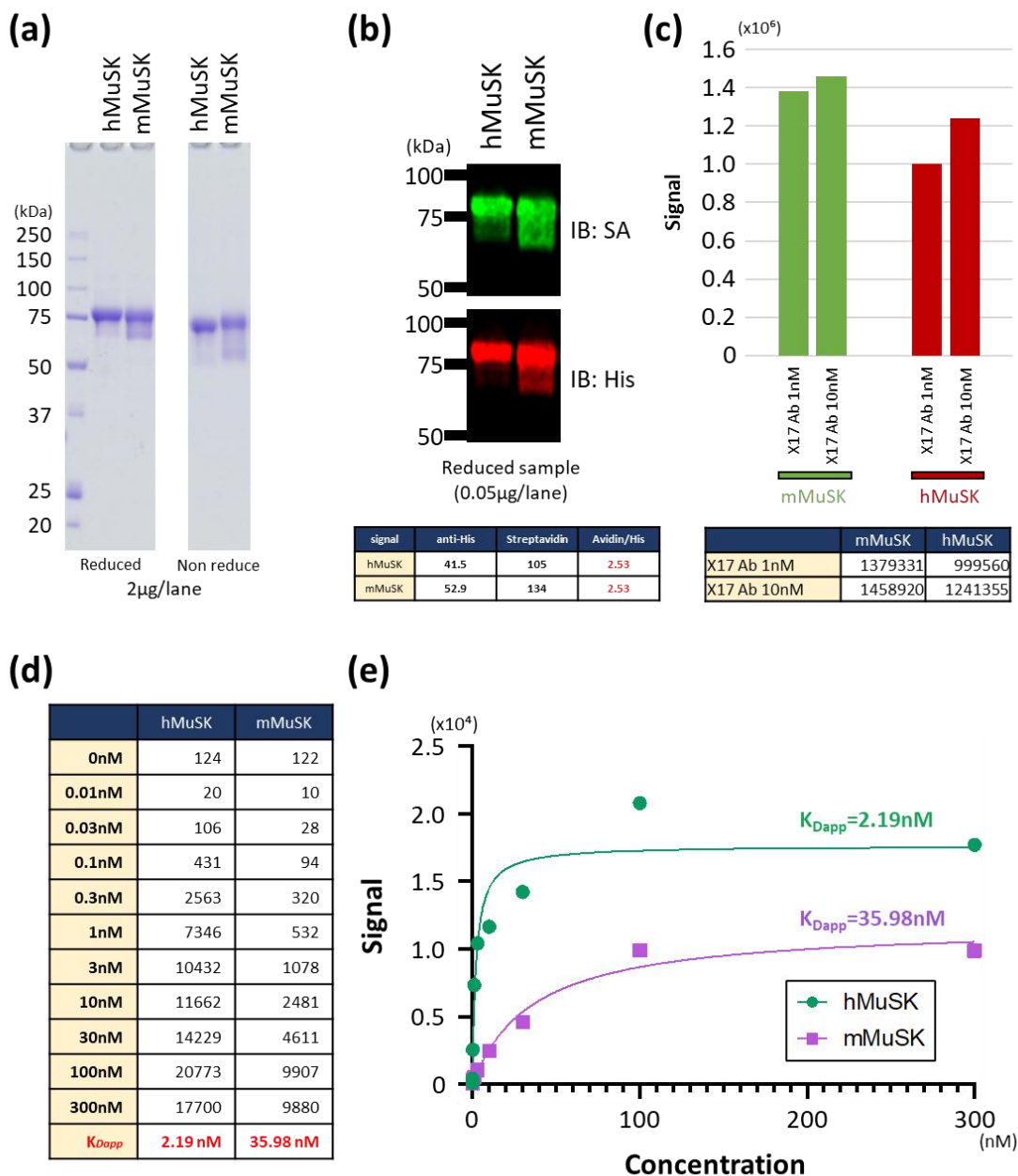


Fig.32 : Binding of L1-Fc to mouse MuSK in MSD assays

- (a) SDS-PAGE analysis of purified mMuSKec-BAS-His used in MSD.
- (b) Confirmation of biotinylation of mMuSKec-BAS-His.
- (c) Optimization of mMuSKec-BAS-His coating conditions, using X17 Ab as a control ligand that binds both hMuSK and mMuSK with similar affinity.
- (d-e) Comparison of L1-Fc binding to hMuSKec and mMuSKec. (d) Raw MSD signals at each L1-Fc concentration. (e) Plots of signal versus concentration for hMuSKec and mMuSKec.

4.2.3. Dissecting the structural basis of species-specific L1 binding in mMuSK mutants

To elucidate the molecular basis underlying the reduced affinity of L1-Fc for mouse MuSK, I generated mMuSK-based mutants and examined how specific sequence differences affect L1-Fc binding. Sequence comparison of the L1-binding region revealed two major divergences between hMuSK and mMuSK. First, the residue corresponding to Glu345 in hMuSK is replaced by Asp346 in mMuSK. Second, the region spanning Lys402-Val431 in hMuSK exhibits substantial sequence variability in mMuSK.

To assess the contribution of these differences, I generated two mMuSK mutants: a point mutant in which Asp346 was replaced with Glu [mMuSK(D346E)], and a chimeric mutant in which the entire variable region corresponding to residues 402-431 was replaced with the human MuSK sequence (mMuSK(K402-V431_human chimera)). L1-Fc binding to these mutants was evaluated by flow cytometry (Fig. 33).

Flow cytometric analysis confirmed that both mutants were expressed at levels comparable to mMuSK(WT), albeit lower than hMuSK. Importantly, both mutants exhibited increased L1-Fc binding relative to mMuSK(WT). The mMuSK(D346E) mutant showed enhanced binding in both MSD and flow cytometry assays, indicating a direct contribution of this residue to L1 recognition. In contrast, the mMuSK(K402-V431_human chimera) mutant displayed improved binding only in flow cytometry, but not in the MSD assay. These results demonstrate that species-dependent differences in L1 binding arise from multiple sequence features within the Ig3-Fz region, with distinct contributions depending on the experimental context.

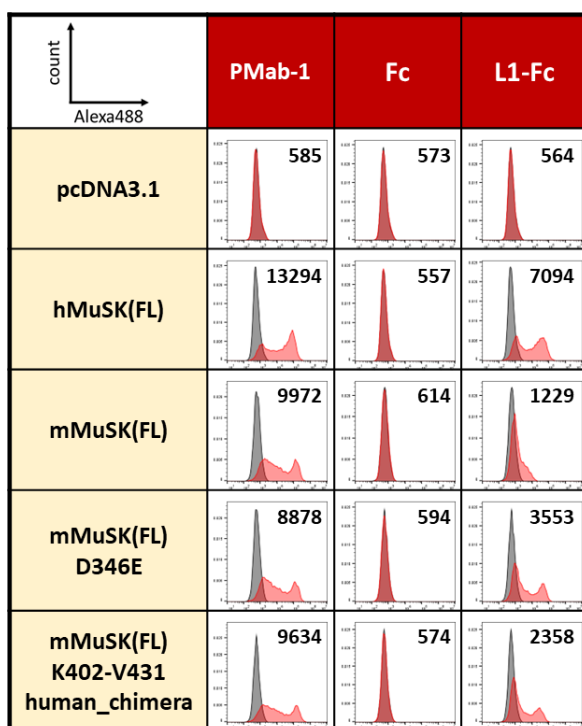


Fig.33 : Binding of L1-Fc to mMuSK variants

Flow cytometric analysis of L1-Fc binding to wild-type and mutant MuSK constructs. Histograms show unstained cells (gray) and cells stained with PMAb-1 (expression control) or L1-Fc (test). All constructs carry an N-terminal MAP tag and were expressed in Expi293F cells. The geometric mean fluorescence intensity for each condition is indicated in the upper right of each panel.

4.3. Discussion

4.3.1. Summary of findings from L1 functional analysis

In this chapter, I carried out a detailed functional analysis of the L1 peptide, which was originally isolated as a high-affinity binder for human MuSK through RaPID screening. Our results provide insight into the molecular determinants of the L1-MuSK interaction and clarify why L1 exhibits pronounced species specificity. The combined data also suggest strategies for improving the physicochemical properties of L1-derived agonists.

Our truncation analysis demonstrated that residues 1-15 of L1 constitute the minimal MuSK-binding region. This portion of the peptide corresponds precisely to the segment that occupies the composite hydrophobic pocket formed by the Ig3 and Fz domains in the co-crystal structure, and it includes nearly all residues whose side chains participate directly in the interface. Alanine scanning further revealed that eight residues—Pro3, Phe5, Trp7, Pro8, Phe11, Val12, Phe14, and Leu15—are indispensable for MuSK binding. The structural basis for these effects is readily interpretable: the hydrophobic cluster of Phe11, Val12, Phe14, and Leu15 anchors the α -helical segment of L1 deep within the Ig3-Fz cleft, whereas Phe5 and Trp7 contribute to the N-terminal docking surface. Pro3 enhances main-chain interactions along the Ig3 loop, and Pro8 plays an important role in maintaining the α -helical conformation required for MuSK docking.

Arg18, by contrast, contributes stabilizing electrostatic interactions with residues in the Fz domain but was not required to maintain minimal binding when the C-terminal half of L1 was deleted. Although the Arg18–Glu345 salt bridge observed in the crystal structure likely contributes to tightening of the interface, the truncation series suggests that this interaction is not absolutely essential for MuSK association. It remains possible, however, that Arg18 modulates affinity or on/off rates in ways not captured by pull-down assays. A more quantitative determination—such as measuring binding of an R18A mutant using SPR or MSD—would help clarify the extent of its contribution.

4.3.2. Structural basis for species-dependent differences in L1 binding

The functional differences between human and mouse MuSK revealed an additional layer of complexity. Both MSD and flow cytometry analyses demonstrated that L1 binds mMuSK with much lower affinity than hMuSK. This species specificity can be rationalized by differences in the L1-contacting interface. Our mutational analysis showed that replacing Asp346 of mMuSK with Glu—the residue present in hMuSK—enhanced L1 binding in both soluble and membrane-bound contexts, consistent with a model in which the Asp $\rightarrow$ Glu substitution restores favorable electrostatics or alleviates

steric constraints that impede L1 docking.

The second region of divergence, spanning residues Lys402–Val431, influenced L1 recognition in a more subtle manner. Although substituting this entire segment with the human sequence did not markedly improve binding to soluble MuSK in MSD assays, it increased binding to membrane-anchored MuSK in flow cytometry. This observation suggests that the K402-V431 region may contribute indirectly to recognition by modulating the local conformational dynamics of the Fz domain or by stabilizing the relative arrangement of the Ig3-Fz domains in the membrane-bound state. The fact that this effect is assay-dependent further indicates that MuSK ectodomains may adopt different conformational ensembles in solution and on the cell surface, and that such conformational differences can influence the apparent contribution of individual residues to peptide binding.

Altogether, these results provide a coherent structural explanation for why L1 binds human MuSK far more strongly than mouse MuSK: species-dependent variations within the Ig3-Fz binding pocket alter the shape and electrostatic environment of the L1-docking surface. Importantly, restoring specific hMuSK-like features in mMuSK was sufficient to partially rescue L1 recognition, demonstrating that L1-MuSK specificity can be rationally modulated through targeted mutagenesis.

4.3.3. Implications for improving L1-derived MuSK agonists

Finally, these findings have practical implications for engineering improved MuSK agonists. Several residues identified as non-essential for MuSK binding—particularly hydrophobic residues within the C-terminal half of L1—likely contribute to the poor solubility and aggregation propensity of Fc-L1. Substituting such residues with more polar amino acids may improve the physicochemical properties of L1-based agonists without compromising affinity. Conversely, residues that proved essential in the alanine scan define the minimal pharmacophore that must be preserved in any optimized ligand. Thus, the functional map established in this chapter provides a foundation for designing more stable and more broadly reactive MuSK-binding peptides.

4.4. Materials and Methods

4.4.1. Plasmid construction

L1-Fc variants were generated on plain L1-Fc construct using PCR-based site-directed mutagenesis unless otherwise noted. Truncation variants such as L1(1-15)-Fc and L1(1-7)-Fc were constructed by inverse PCR followed by self-ligation. Alanine-substituted variants were generated by introducing single-residue substitutions within residues 1-15 of L1 using standard PCR mutagenesis.

For species-dependent MuSK binding analysis, mutations were introduced into full-length mouse MuSK (mMuSK) using analogous strategies. The D346E substitution was generated by site-directed mutagenesis, whereas the K402-V431 human-chimera construct was prepared by inverse PCR to replace the corresponding region with the human MuSK sequence. All constructs were cloned into pcDNA3.1 unless otherwise stated.

4.4.2. Protein expression and purification

All proteins except for that used in the crystallization were produced in Expi293F cells using the Expi293F Expression system (Thermo Fisher Scientific) and the supernatant was harvested four days post-transfection. For expressing of biotinylated hMuSK(WT)_{ec}, MuSK(mutant)_{ec}, and mMuSK_{ec}, cells were co-transfected with human/mouse MuSK_{ec}-BAS-His and BirA-ΔKDEL (an ER-resident mutant of the biotin ligase BirA)⁵⁸, followed by addition of 100 μM D-biotin (Nacalai Tesque) to the culture medium one day post-transfection. For the purification of His-tagged proteins, culture supernatants were mixed with Ni-NTA agarose (QIAGEN) and rotated for 2 h at room temperature. The agarose was transferred to an Econo-Column (Bio-Rad) and washed three times with 10 column volumes of Tris-buffered saline (TBS, 20 mM Tris, 150 mM NaCl, pH 7.5). Proteins were eluted with TBS containing 250 mM imidazole. The fractions containing target molecules were combined and dialyzed against phosphate-buffered saline (PBS, 10 mM Na-phosphate, 140 mM NaCl, 2.7 mM KCl, pH 7.4) at 4 °C. All purified proteins were concentrated using an Amicon Ultra-4 Centrifugal Filters Ultracel-30K and stored at −80° C until use.

4.4.3. Pulldown assay

Proteins expressed in the Expi293F culture medium were collected via centrifugation and used for pull-down binding assays. Briefly, anti-PA tag NZ-1⁵¹ immobilized Sepharose beads were mixed with the culture supernatant containing PA-tagged hMuSK

ectodomain fragments and rotated for 1.5 h. After removing the supernatant, the culture supernatant containing L1-fused Fc was added, and the mixture was rotated for an additional 1.5 h. The NZ-1 resin was washed three times with TBS, and elution was performed using 0.1 μ g/mL PA14 peptide in TBS. The eluted sample was mixed with SDS-PAGE sample buffer and subjected to SDS-PAGE using 5–20 % gradient gel (ATTO). After electrophoresis, the gel was stained with Coomassie Brilliant Blue (CBB) and then destained.

4.4.4. Flow cytometry for assessing L1–Fc binding to hMuSK and mMuSK

Expi293F cells transfected with the plasmids containing full length hMuSK(WT), hMuSK(mutant), or mMuSK that had been modified to contain N-terminal MAP-tag42 were harvested two days post-transfection, transferred into a V-bottom 96-well plate at 2.0×10^5 cells/well and incubated with 5.0 μ g/ml purified PMAb-1 (anti-MAP tag antibody, rat IgG2a) or 1 μ M L1-Fc in DMEM containing 10 % FBS, 1% NEAA, and 0.5% Penicillin-Streptomycin for 1 hour on ice. After washing twice with PBS(-), the cells were further incubated with Alexa Fluor 488-labeled goat anti-rat IgG (Thermo Fisher Scientific) or Alexa Fluor 488-labeled goat anti-human IgG (Thermo Fisher Scientific) diluted 1:10,000 in DMEM containing 10 % FBS, 1 % NEAA, and 0.5 % Penicillin-Streptomycin for 30 minutes on ice. After three washes with PBS(-), the cells were analyzed on EC3800 (Sony). Data were analyzed using FlowJo software (TOMY Digital Biology).

4.4.5. Mesoscale Discovery (MSD) binding assay

or binding analysis of the L1-fused Fc proteins against hMuSKec or mMuSKec, each well of the MSD GOLD 96-Well Streptavidin Plate (MSD) was coated with 50 μ L of 0.6 nM biotinylated hMuSKec or mMuSKec in PBS and incubated overnight at 4° C. MuSK-coated wells were blocked with 150 μ L of 3 % (w/v) BSA in PBS containing 0.05 % (v/v) Tween-20 (PBS-T) for 30 min, followed by washing with 150 μ L of PBS-T. Next, 50 μ L of various concentrations of X17 Ab or Fc-L1 in PBS-T containing 1% (w/v) BSA were added. The plate was shaken at 400 rpm at room temperature for 2 h in the dark, followed by three washes with PBS-T. Subsequently, 50 μ L/well of 1 μ g/mL SULFO-TAG Goat Anti-human Antibody (MSD) was added, and the plate was shaken again at 400 rpm at room temperature for 1 h. After three more washes with 150 μ L of PBS-T, 150 μ L of $2 \times$ Read Buffer was added, and plates were read using the MESO QuickPlex SQ 120. The half-maximal binding concentration (K_{Dapp}) was calculated using GraphPad Prism10 software.

5. Conclusion

5.1.1. Integrative Summary of Chapters 2–4

Through a combination of precise functional analyses in MuSK-knockout C2C12 cells, structure-guided mutagenesis, and peptide-based agonist engineering, this study delineates the spatial constraints that dictate activation of muscle-specific kinase (MuSK).

Systematic dissection of extracellular domains revealed that the Ig1–Ig2 tandem is indispensable for Agrin-dependent induction of AChR clustering. Although mutants lacking Ig1 or bearing a flexible linker between Ig1 and Ig2 retained autophosphorylation at Tyr553, they failed to support AChR clustering. These results indicate that MuSK activation requires not merely receptor dimer proximity, but a rigidly defined extracellular orientation enforced by the Ig1-Ig2 unit (Fig. 15).

Structural considerations further suggest that Ig1-Ig2 architecture pre-sets the spacing between intracellular Tyr553 residues to match the geometry required for formation of MuSK-DOK7 2:2 complex, which initiates the full activation cascade. Thus, extracellular geometry is consistent with not merely permissive, but deterministic for correct intracellular molecular alignment.

In contrast, analysis of the Ig3–Fz region demonstrated that although this domain is not essential for Agrin-dependent activation, it can also support signaling when two MuSK molecules are brought together in an appropriate orientation by artificial agonists. Engineering of L1-based Fc dimerizers showed that distinct Ig3–Fz dimer geometries result in markedly different signaling outcomes. These findings indicate that the Ig3–Fz domain also exhibits orientation-dependent constraints and can modulate dimer architecture in a manner that either permits or prevents productive downstream signaling.

In parallel, functional analysis of the MuSK-binding peptide L1 identified the minimal pharmacophore (residues 1-15) required for MuSK recognition and clarified the structural basis for pronounced species specificity (human > mouse). These findings provide concrete guidelines for improving the biophysical properties of L1 and for designing next-generation MuSK agonists.

Together, these results establish that MuSK activation is a domain-coordinated, geometry-restricted process in which Ig1-Ig2 and Ig3-Fz work can also modulate to enforce a productive dimer configuration. This model aligns with emerging principles of allosteric dimer control observed in receptor tyrosine kinases such as EGFR and MET, suggesting that MuSK exemplifies a broader activation paradigm among RTKs.

5.1.2. MuSK adopts a specific dimer orientation that is optimal for activation

A decisive demonstration of orientation-dependent MuSK activation was provided by engineered Fc-based artificial agonists. Both L1-Fc and Fc-L1 exhibited high affinity for MuSK and comparable abilities to promote MuSK dimerization in solution; however, only Fc-L1 functioned as a MuSK agonist, robustly inducing AChR clustering in C2C12 myotubes.

Structural modeling revealed the mechanistic basis for this stark functional divergence (Fig. 29). Because the L1 peptide binds asymmetrically at the Ig3-Fz interface, the direction in which Fc is fused dictates the resulting dimer geometry. Fc-L1 aligns two MuSK molecules in a compact configuration in which the spacing between Ig1 domains approximates that expected in the physiological Agrin-LRP4-MuSK complex. In contrast, L1-Fc produces an excessively splayed dimer that cannot induce MuSK activation

These results provide direct structural–functional evidence that MuSK activation is governed by orientation, rather than by affinity or bivalency alone. The Fc-L1 data therefore serve as a synthetic reconstruction of the physiological requirement: MuSK activation requires a precise extracellular angle and inter-molecular distance, without which downstream signaling cannot proceed.

Furthermore, comparison of the MuSK dimer architecture induced by Fc-L1 with that formed under physiological Agrin-dependent conditions reveals a notable structural convergence: in both cases, the Ig1 domains of the two MuSK molecules are positioned in close proximity (Fig. XX and Fig. XX). This similarity suggests that productive MuSK dimers capable of inducing downstream signaling share a common feature, namely a compact extracellular arrangement in which the Ig1-containing regions are tightly apposed. Taken together, these observations support the notion that a compact packing of the MuSK extracellular domains represents a signaling-competent orientation that is particularly well suited for triggering AChR clustering.

5.1.3. Proposed model of Agrin–MuSK–LRP4 complex and active MuSK dimer

Based on previous studies and the findings presented in this work, I propose a stepwise model for assembly of the Agrin-MuSK-LRP4 (AML) complex and subsequent activation of MuSK.

In the absence of Agrin, MuSK exhibits weak but detectable binding to LRP4⁶¹. Even under basal conditions, MuSK is therefore likely to undergo transient association and dissociation with LRP4 on the muscle cell membrane, mediated primarily through the Ig1 domain and, to a lesser extent, the Ig3 domain (Fig. 34a–b). In this state, these interactions

are insufficient to induce MuSK activation or downstream signaling.

Upon secretion of Agrin from motor neuron terminals, Agrin first binds to LRP4 on the muscle cell surface (Fig. 34c). This interaction promotes formation of a ternary complex containing one molecule each of Agrin, MuSK, and LRP4. Whether MuSK directly contacts Agrin within this 1:1:1 complex remains unclear. However, given that MuSK activation requires dimerization of two receptor molecules, this intermediate complex is unlikely to be sufficient to trigger downstream signaling.

Subsequently, two of these ternary complexes are proposed to associate with each other via Agrin- and/or MuSK-mediated interactions. This association results in formation of a higher-order AML complex containing two molecules each of Agrin, MuSK, and LRP4 (Fig. 34d). Within this assembly, two MuSK molecules are brought together in a defined orientation, enabling dimerization of the extracellular regions. This dimerization event initiates autophosphorylation of the intracellular region, recruitment and dimerization of DOK7, and ultimately induction of AChR clustering signaling (Fig. 34e).

A critical determinant of productive MuSK activation is the Ig1 domain, which mediates two distinct but complementary interactions. First, Ig1 provides a binding interface for LRP4. Second, Ig1 contributes to homotypic MuSK-MuSK interactions that stabilize the receptor dimer (Fig. 34f-g). Together, these interactions fix the relative orientation of Ig1 domains within the AML complex, thereby constraining the overall geometry of the MuSK dimer. Consistent with this model, disruption of either the Ig1-LRP4 interaction or the Ig1-mediated dimer interface abolishes Agrin-dependent AChR clustering.

The Ig2 domain does not directly participate in LRP4 binding. However, crystallographic analysis demonstrates that Ig1 and Ig2 form a rigid tandem unit without an intervening flexible linker. Our functional analyses reveal that this rigid Ig1-Ig2 architecture is essential for MuSK activation, indicating that Ig1-Ig2 is incorporated into the AML complex in a conformation closely resembling that observed in the crystal structure (Fig. 34h). Thus, the primary role of Ig2 appears to be structural, maintaining the precise orientation and spacing of Ig1 domains required for formation of an activation-competent MuSK dimer.

By contrast, the Ig3-Fz region is not required for Agrin-dependent signaling. Nevertheless, analysis using the MuSK-binding peptide L1 and Fc-based agonists demonstrates that the relative orientation of the Ig3-Fz domains can critically influence MuSK activation when dimerization is enforced artificially. Structural modeling suggests that productive signaling requires the linker region connecting Ig3 and Fz to be oriented outward, whereas inward-facing configurations are incompatible with activation (Fig. 34h). These observations suggest that, while the Ig3-Fz domains are not essential for physiological activation, their orientation within the dimeric structure is likely to play a

critical role in downstream signal induction.

The extracellular geometry established within the AML complex is transmitted to the intracellular region, where it determines whether productive DOK7 dimerization can occur. DOK7 recognizes phosphorylated Tyr553 in the juxtamembrane region of MuSK and must form a dimer to promote full activation of the tyrosine kinase domain. If the extracellular dimer geometry fails to position the intracellular regions appropriately, DOK7 dimerization and subsequent AChR clustering cannot proceed, even when autophosphorylation is observed.

Taken together, our results support a unified model in which MuSK activation is governed by orientation-dependent dimerization within the AML complex. The Ig1-Ig2 domains play a central and indispensable role in enforcing the geometry required for Agrin-dependent signaling, while other extracellular domains function as auxiliary structural elements that preserve or modulate this geometry. This work provides a comprehensive structural and functional framework for understanding MuSK activation and offers a rational basis for the design of MuSK agonists targeting neuromuscular disorders.

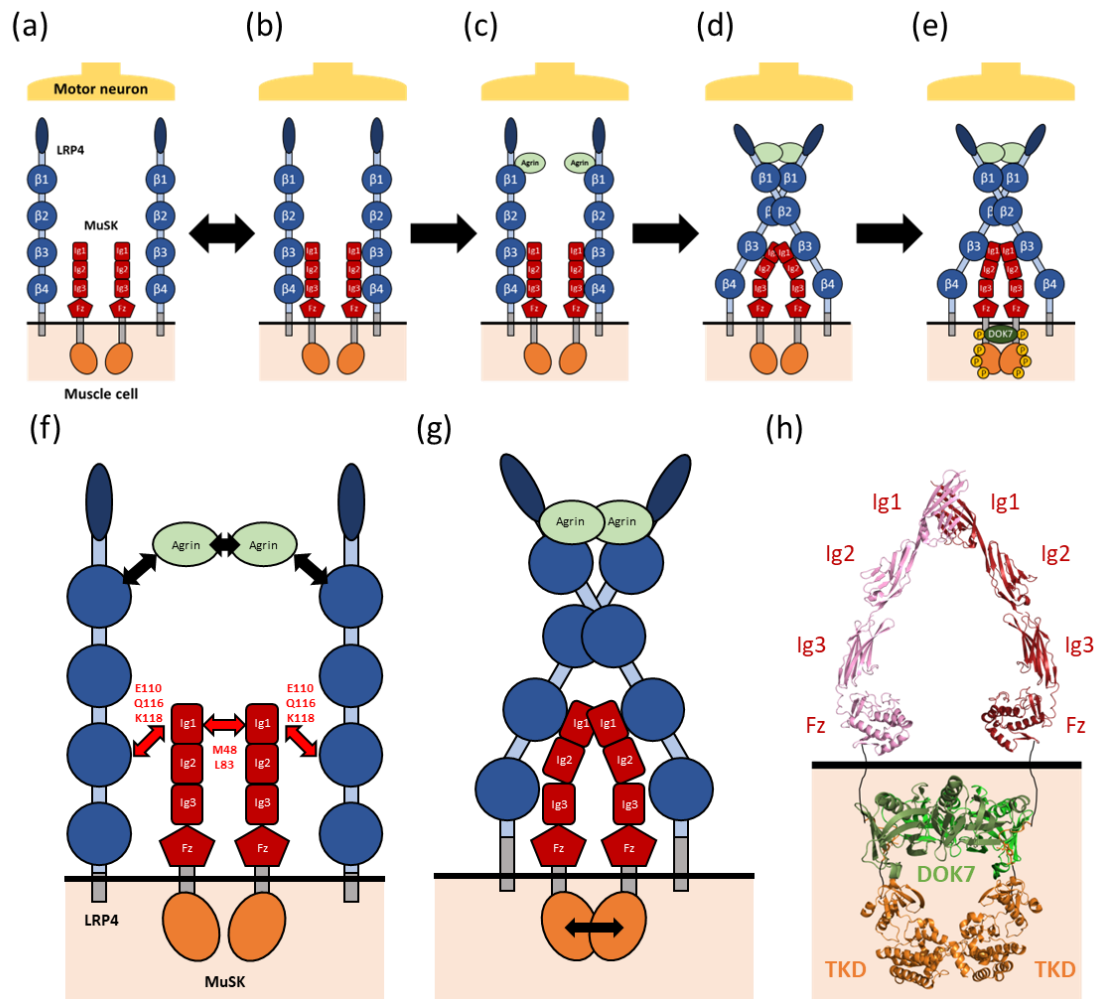


Fig.34 : Predicted MuSK dimer structure within the AML complex

- (a–e) Model of MuSK activation via AML-complex formation. In the absence of Agrin, MuSK interacts weakly and transiently with LRP4 (a,b). Upon secretion of Agrin from motor nerve terminals, Agrin binds LRP4 (c). Agrin dimerization and additional interactions then bring together two LRP4–MuSK complexes, forming an AML complex containing two molecules each of Agrin, MuSK, and LRP4 (2:2:2) (d). Subsequent formation of a 2:2 MuSK–DOK7 complex leads to activation of the intracellular TKDs and induction of AChR clustering (e).
- (f–g) Schematic of protein–protein interactions within the AML complex. Agrin and MuSK each interact with LRP4 and also form homotypic dimers (f). MuSK engages both Agrin–LRP4 and MuSK–MuSK interactions through its Ig1 domain, which fixes the position of Ig1 within the complex. The rigid Ig1–Ig2 tandem then maintains an appropriate distance between intracellular TKDs.
- (h) Predicted MuSK dimer structure within the AML complex. Two MuSK molecules form a dimer via Ig1–Ig1 interactions, with Fz domains oriented toward the inside of the dimer. This arrangement is proposed to facilitate formation of the DOK7–MuSK 2:2 complex in the cytoplasm and to promote TKD activation. Structures used for model building are as follows: Ig1/Ig2 domain, PDB ID: 2IEP; Ig3/Fz domain, PDB ID: 9LGG; DOK7, PDB ID: 3ML4; MuSK kinase domain, PDB ID: 1LUF.

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7. Publication

1. Mizutani F, Matoba K, Peacock H, Yamada M, Mihara E, Higuchi O, Suga H, Arimori T, Takagi J. 「Muscle-specific tyrosine kinase activation by a peptide-based dimerizer is orientation dependent」 Structure, Volume 34, Issue 2, 2026, Pages 284-295.e5, ISSN 0969-2126, <https://doi.org/10.1016/j.str.2025.10.018>

8. International meeting abstract

1. Fumiya Muzutani, Hayden Peacock, Hiroaki Suga, Junichi Takagi 「Elucidation of the recognition mechanism of LRP4 by Muscle-specific kinase (MuSK) critical for the establishment of the neuromuscular junction」 EMBO Workshop & Yamada Conference Wnt 2022

9. Domestic meeting abstract

1. Fumiya Muzutani, Hayden Peacock, Kyoko Matoba, Osamu Higuchi, Hiroaki Suga, Junichi Takagi 「Isolation and characterization of a linear peptide that binds to human MuSK extracellular domain」 第 20 回日本蛋白質科学会年会
2. Fumiya Muzutani, Hayden Peacock, Hiroaki Suga, Junichi Takagi 「神経筋接合部形成に関わる筋特異的キナーゼ(MuSK)－LRP4 相互作用様式に関する研究」 第 23 回日本蛋白質科学会年会
3. Fumiya Muzutani, Junichi Takagi 「MuSK KO マウス筋芽細胞(C2C12)を用いた MuSK の機能ドメイン解析」 第 98 回日本生化学会大会
4. Fumiya Muzutani, Junichi Takagi 「筋特異的キナーゼ(MuSK)シグナリングには Ig1-2 ドメインが必須である」 第 24 回日本蛋白質科学会年会

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