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Developing tools to study MuSK and Lrp4 during neuromuscular disorders

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2. Zusammenfassung

Die neuromuskuläre Endplatte (NMJ) ist die hochspezialisierte Verbindung zwischen einem peripheren Motoneuron und einer Skelettmuskelzelle. Diese chemische Synapse wandelt vom Gehirn ausgelöste Stimulation in Muskelkontraktion um und ermöglicht Bewegung und Atmung. Ihre Entwicklung wird durch die Muskel-spezifische Kinase (MuSK), eine Rezeptor-Tyrosinkinase (RTK), sowie das lipoproteinrezeptor-verwandte Protein 4 (Lrp4) und das vom Motoneuron abgeleitete Proteoglykan Agrin gesteuert. Gemeinsam kontrollieren sie die Assemblierung der Acetylcholin-Neurotransmitterrezeptoren (AChRs) zu funktionellen Einheiten. Erkrankungen der NMJ sind oft schwerwiegend und teils lebensbedrohlich. Ihre Vielfalt und unterschiedliche Symptomatik machen Diagnose und personalisierte Therapie entscheidend.

Myasthenia gravis (MG) ist eine Autoimmunerkrankung, die durch Autoantikörper gegen Schlüsselproteine der NMJ wie Lrp4, MuSK oder AChR verursacht wird. Im ersten Teil dieser Arbeit wurde ein zellbasierter Assay mit HeLa-Zellen entwickelt, um Lrp4-positive MG-Patientenseren zu identifizieren. Hierfür wurde das PiggyBac-System genutzt, um stabile HeLa-Zelllinien zu erzeugen, die mCherry-markiertes Lrp4 überexprimieren. Obwohl die Färbung mit einem kommerziellen Antikörper erfolgreich war, zeigte keines der getesteten Patientenseren eine positive Diagnose. Das System benötigt daher weitere Optimierung, um höhere Sensitivität und Zuverlässigkeit zu gewährleisten, bleibt jedoch ein vielversprechender Ansatz.

Im zweiten Teil wurden die Auswirkungen der seltenen MuSK-Mutationen N103S und L545P auf AChR-Clustering untersucht. Die Experimente erfolgten mit zwei Modellen: MuSK-Knockout-Myotuben mit ektopter Expression von Wildtyp- oder mutiertem MuSK und Wildtyp-Myotuben mit induzierbarer MuSK-Überexpression. Die MuSK-/- Zellen zeigten erhebliche Probleme in ihrer Differenzierung und im AChR-Clustering, was sie als Modell derzeit unzuverlässig macht. Theoretisch sind sie jedoch besser geeignet, wenn technische Herausforderungen überwunden werden.

In den Wildtyp-Myotuben mit MuSK-Überexpression zeigte sich nach Agrin-Stimulation eine vergleichbare AChR-Clusterbildung bei mutierten und Wildtyp-MuSK-Zelllinien. Dies deutet darauf hin, dass in Anwesenheit von endogenem Wildtyp-MuSK die Mutationen die MuSK-Aktivierung nicht beeinflussen. Weitere Untersuchungen zu MuSK-Expressionsniveau und -Aktivierung sind notwendig, um belastbare Daten zu erhalten.

Diese Arbeit beleuchtet die Herausforderungen und das Potenzial der Systeme, um MG und CMS besser zu verstehen. Trotz technischer Einschränkungen liefern die Ansätze wichtige Einblicke für zukünftige diagnostische und therapeutische Entwicklungen.

3. Abstract

The neuromuscular junction (NMJ) is the highly specialized connection between a peripheral motor neuron and a skeletal muscle cell. This chemical synapse transforms brain-elicited stimulation into muscle contraction and enables us to move and to breathe. Development of NMJ is governed by Muscle Specific Kinase (MuSK), a receptor tyrosine kinase (RTK) together with lipoprotein receptor-related protein 4 (Lrp4) and the motor-neuron-derived proteoglycan Agrin, which control the assembly of the acetylcholine neurotransmitter receptors (AChRs) into functional units. Diseases affecting the NMJ are severe, sometimes even life-threatening. These disorders are diverse and present distinct symptoms. Their heterogeneity makes diagnosis and personalized therapy the key factors in treatment of patients.

Myasthenia gravis (MG) is an autoimmune disease caused by autoantibodies against key molecules of the neuromuscular junction such as Lrp4, MuSK or AChR. In the first part of this thesis, a cell-based assay using HeLa cells was developed to identify Lrp4-positive MG patient sera. For this purpose, the PiggyBac system was used to generate stable HeLa cell lines able to overexpress mCherry-tagged Lrp4. Although staining with a commercial antibody was successful, none of the available patient sera tested showed a positive diagnosis. This suggests that the system needs further optimization to ensure higher sensitivity and reliability. However, the concept remains promising, especially if existing challenges are addressed.

In the second part of this thesis, the effects of two rare MuSK mutations, N103S and L545P, on AChR clustering were investigated. The experiments were performed using two models: MuSK knockout myotubes with ectopic expression of wildtype or mutant MuSK and wildtype myotubes with inducible MuSK overexpression. The MuSK^{-/-} cells showed significant problems in their differentiation and AChR clustering, making them currently unreliable as a model. However, theoretically, they are the better model to study MuSK mutations if these technical challenges are overcome.

In the wildtype myotubes with MuSK overexpression, the results indicated that upon agrin stimulation, the ability of myotubes to form AChR clusters was comparable for both of the mutant MuSK cell lines compared to the wildtype MuSK cell line. This suggests that in the presence of endogenous wildtype MuSK the mutations do not affect MuSK activation. Further investigations on MuSK expression level and activation are required to generate conclusive data from this model system.

In summary, this work highlights both the challenges and the potential of the systems used to better understand MG and CMS. While technical limitations influenced the results, the approaches and findings provide important insights into future diagnostic and therapeutic developments.

4. Introduction

4.1 The Neuromuscular Junction

The neuromuscular junction (NMJ) is the synapse between a motor neuron and a skeletal muscle fiber (Figure 1A). The NMJ enables voluntary movement by facilitating communication from the motor neuron to the muscle. This process begins when the action potential reaches the termini of the motor neuron, causing the release of the neurotransmitter acetylcholine (ACh) stored in vesicles into the synaptic cleft. Released ACh is then recognized by the nicotinic acetylcholine receptors (AChR) on the muscle fiber which function as ligand gated ion channels that allow the movement of sodium ions into and potassium ions out of the muscle fiber. This movement of ions creates a net positive potential inside the muscle fiber, creating what's called the end plate potential (EPP). If the EPP is large enough, it results in the formation of an action potential enabling muscle contraction.

The NMJ is made up of three distinct regions: the presynaptic terminal, the synaptic cleft, and the postsynaptic membrane (Figure 1B).

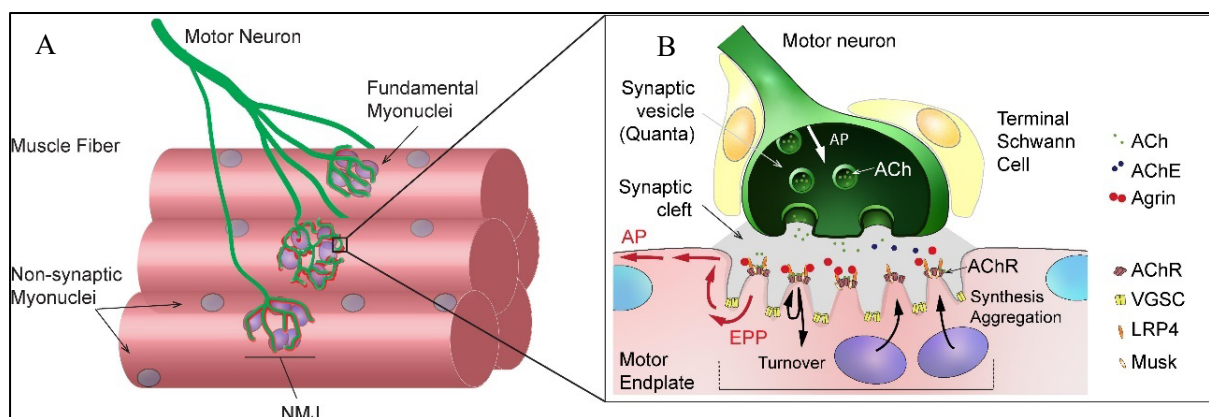


Figure 1: The structure and the participating components of the neuromuscular junction (NMJ).
A: A motor neuron innervating multiple muscle fibers. Every muscle fiber contains only one NMJ where it is innervated by a motor neuron. **B:** Detailed depiction of the NMJ with its participating components. The propagating action potential in the motor neuron results in the release of ACh, stored in synaptic vesicles, into the synaptic cleft. Released ACh binds to acetylcholine receptors on the crests of the muscle fiber, enabling the movement of sodium and potassium ions. This creates a local depolarization called the end plate potential (EPP), which if great enough results in the formation of an action potential and subsequent muscle contraction. (Castets et al., 2020)

The presynaptic terminal comes about from the growth cone of the axon and is the end of the motor neuron that interacts with the muscle fiber at the NMJ (Figure 1B). The presynaptic terminals are also covered with terminal Schwann cells, which are non-myelinated glial cells that play a role in the maintenance and regeneration, especially after nerve injury, of the NMJ (Alvarez-Suarez et al., 2020; Santosa et al., 2018).

The synaptic cleft is the narrow, 50-80 nm wide space between the motor neuron and the muscle fiber (Tintignac et al., 2015). It is the space through which ACh diffuses from the presynaptic terminal towards the postsynaptic membrane, which is the muscle fiber. Acetylcholine esterase

(AChE), an enzyme present in the basal lamina in the synaptic cleft rapidly degrades the released ACh into choline and acetic acid ensuring that prolonged exposure to ACh does not occur and it is degraded in a timely manner (Trang & Khandhar, 2024).

The postsynaptic membrane, the area of the muscle fiber directly beneath the nerve terminal, is made up of crests and troughs where on top of the crests, AChRs are found at a very high concentration, around $10.000/\mu\text{m}^2$ in the electric organ of *Torpedo californica* (Heuser & Salpeter, 1979), and in adult murine muscle they are found at a density of over $15.000/\mu\text{m}^2$ (Merlie & Sanes, 1985) and on the bottom of the troughs, voltage gated sodium channels are concentrated. The AChRs and the voltage gated sodium channels enable movement of ions across membranes, creating a potential across the membrane as the EPP.

4.2 Key regulators of NMJ development

4.2.1 Muscle-Specific Kinase (MuSK)

Muscle specific kinase (MuSK) is a receptor tyrosine kinase (RTK) that plays a central role in the formation of NMJs during development. Without proper activity of MuSK, AChR clusters cannot form on muscle fibers and thus, preventing signals coming from the motor neuron from being conveyed to the muscle fiber, inhibiting contraction. Total absence of MuSK results in death of mice embryos at birth due to lack of ability to breathe (DeChiara et al., 1996).

MuSK is a single pass RTK that is made up of 3 immunoglobulin-(Ig) like domains, followed by a Frizzled-like Cysteine-rich domain (Fz-CRD) on the extracellular side, with a short juxtamembrane region and a tyrosine kinase domain (TKD) (Figure 2).

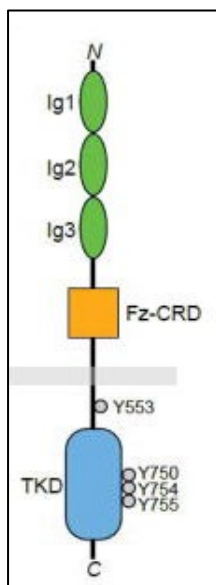


Figure 2: **Domains of MuSK.** The RTK MuSK contains 3 immunoglobulin-(Ig) like domains and a Frizzled-like Cysteine-rich domain (Fz-CRD) on the extracellular side, one transmembrane domain and the tyrosine kinase domain (TKD) on the intracellular side. The phosphorylated tyrosine residues are indicated in the figure, Tyr553 is in the juxtamembrane region and Tyr750, Tyr754, and Tyr755 are found in the activation loop in the kinase domain (Hubbard & Gnanasambandan, 2013a).

Important for this thesis are the extracellular Ig1 domain, which is crucial for the interaction between Lrp4 and MuSK (W. Zhang et al., 2011), and the juxtamembrane region where the Tyr553 residue forms a place for interaction with Dok7 (Hubbard & Gnanasambandan, 2013b).

4.2.2 Agrin

Agrin is a heparan sulfate proteoglycan indispensable for the formation and maintenance of NMJ. Agrin deficient mice are not able to survive birth because the formation of their NMJs are hindered by the absence of Agrin, they either die in utero or are stillborn (Gautam et al., 1996). Neuronal Agrin is secreted by motor neurons, and it binds to Lrp4 on the surface of the muscle fibers to activate MuSK. The activation of MuSK leads to AChR clustering on the postsynaptic membrane. Disruption in this signaling pathway leads to complications to the function of the NMJ and can result in diseases such as MG and CMS.

4.2.3 Low-density Lipoprotein Receptor Related Protein 4 (Lrp4)

During early research of the NMJ, it was discovered that the presence of both Agrin and MuSK was necessary for the proper formation of AChR clusters and survival. It was discovered that Agrin does not directly interact and form a complex with MuSK (DeChiara et al., 1996; Glass et al., 1996). Rather, it was suggested that there was another key element in the signaling cascade, named at the time as myotube associated specificity component (MASC) (Glass et al., 1996; Glass & Yancopoulos, 1997). MASC was thought to be the protein bridging the interaction between the neuronally released Agrin and MuSK on the surface of the myofiber. Later, it was discovered that the suggested MASC was a transmembrane protein called low-density lipoprotein receptor related protein 4 (Lrp4) (Kim et al., 2008; B. Zhang et al., 2008).

Lrp4 is a multidomain single pass transmembrane protein member of the large low-density lipoprotein (LDL) receptor family with its extracellular domain containing 8 LDLa repeats, 6 EGF-like and 4 β -propeller domains (Figure 3). As explained in the section “Activation of MuSK” neuronally released Agrin binds Lrp4. Crystal structure and truncational study investigations regarding the domains of interaction has revealed that the region of binding with Agrin is observed to be occurring on the LDLa 6-8, the first two EGF-like and the first of the four β -propeller domains, the β -1 (W. Zhang et al., 2011; Zong & Jin, 2012). The intracellular region of Lrp4 has proved to be nonessential for the signaling cascade to successfully cluster AChRs, through the use of a chimeric Lrp4-CD4 where the intracellular region of Lrp4 has been replaced with the intracellular region of CD4, a completely unrelated transmembrane protein. It was discovered that the chimeric protein was able to form proper AChR clusters and mice with the chimeric Lrp4 were able to survive postnatally (Gomez & Burden, 2011). Mice lacking Lrp4 die at birth because of a failure to form NMJs, preventing them from taking their

first breaths since no signals from the motor neuron can be conveyed to muscles (Weatherbee et al., 2006).



Figure 3: **Domains of Low-density lipoprotein receptor related protein 4 (Lrp4).** The extracellular region of Lrp4 is made up of 8 LDLa repeats, 6 EGF-like and 4 β -propeller domains. (Modified from Xie et al., 2023).

Lrp4 interacts with MuSK, resulting in its phosphorylation and activation, especially after binding with Agrin. This interaction, even in the absence of Agrin, enables prepatternning of AChRs before nerve innervation. The Agrin-independent AChR prepatternning sets the stage for when and where innervation occurs and the AChRs can be clustered at the endplate, and this is proven when Lrp4 mutants fail to show this early clustering ability that prevent proper NMJ formation *in vivo* (Weatherbee et al., 2006). Lrp4 mutant muscle cell lines are not able to achieve MuSK phosphorylation when stimulated with Agrin *in vitro* (Kim et al., 2008), however heterologous HEK293 cells are in fact able to achieve MuSK phosphorylation through co-expression of both Lrp4 and MuSK (B. Zhang et al., 2008), thus solidifying the fact that it is indeed Lrp4 binding to Agrin, and consequently activating MuSK for downstream signaling events.

4.3 Formation of AChR Clusters Through Activation of MuSK

The formation of AChR clusters and the NMJ begins with prepatternning and the activation of MuSK. The process called prepatternning is when Agrin-independent clusters form on the central region of skeletal muscle fibers, before innervation by a motor neuron. When the motor neuron migrates towards and innervates the fibers, the remaining AChRs are eliminated from the surface of the fiber (Figure 4). However, the role of prepatternning is not clear. It is known that without Lrp4 and MuSK, prepatternning does not occur (Lin et al., 2001; Remédio et al., 2016; Yang et al., 2001).

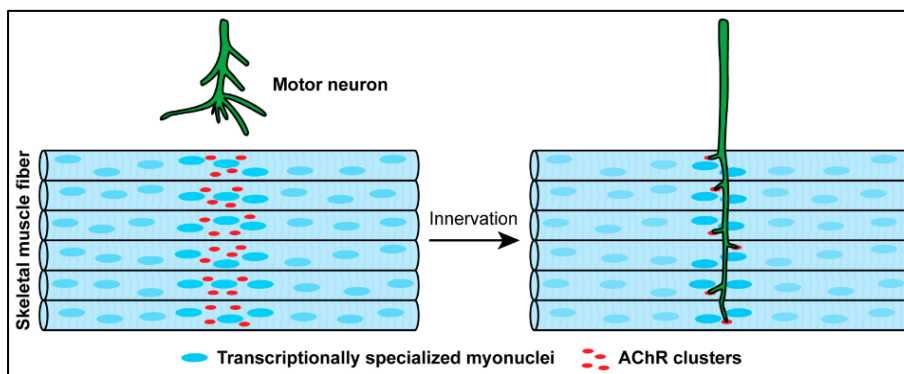


Figure 4: **Innervation of skeletal muscle fibers.** AChRs expressed in the central region of skeletal muscle fibers are innervated by the incoming motor neuron. After innervation, the clusters that formed but not innervated are eliminated from the muscle fiber (Burden et al., 2018).

Activation of MuSK is initiated by Agrin binding to Lrp4 on the surface of muscle fibers (Figure 5). This binding is proposed to cause a conformational change in Lrp4 that enables MuSK to get autophosphorylated on the Tyr553 residue (Hubbard & Gnanasambandan, 2013b; Kim et al., 2008), and this Lrp4-MuSK interaction occurs on the Ig1 domain on MuSK (Guarino et al., 2020). Phosphorylation of the Tyr553 residue enables an adaptor protein, Dok7, to bind to the juxtamembrane region of MuSK where the phosphorylated Tyr553 resides. This binding is possible through the phosphotyrosine binding domain (PTB) of Dok7 (Bergamin et al., 2010; Hubbard & Gnanasambandan, 2013b). Without phosphorylation of the Tyr553 residue, recruitment of Dok7 is not possible and further phosphorylation, downstream signaling and AChR cluster formation is prevented (Herbst & Burden, 2000). After binding of Dok7 to phosphorylated Tyr553, dimerization occurs through the pleckstrin homology (PH) domains of Dok7, further promoting MuSK phosphorylation on the tyrosine residues in the kinase domain, namely the Tyr750, Tyr754 and Tyr755 residues. This autophosphorylation event removes autoinhibition in the activation loop in the kinase domain, fully activating the RTK (Till et al., 2002). AChRs are clustered through the full activation of MuSK. Before its activation, through basal level interaction of Lrp4 and MuSK, AChR clusters are already present in the central area of the muscle fiber, called prepatternning. When MuSK is fully activated through these described signaling cascades, clustered AChRs become part of a fully functioning NMJ (Arber et al., 2002). Further, in the absence of rapsyn, which is the protein that binds to and anchors AChRs (Liao et al., 2023), the ability of muscle fibers to form AChR clusters is abolished and thus, mouse embryos born are non-viable (Gautam et al., 1995).

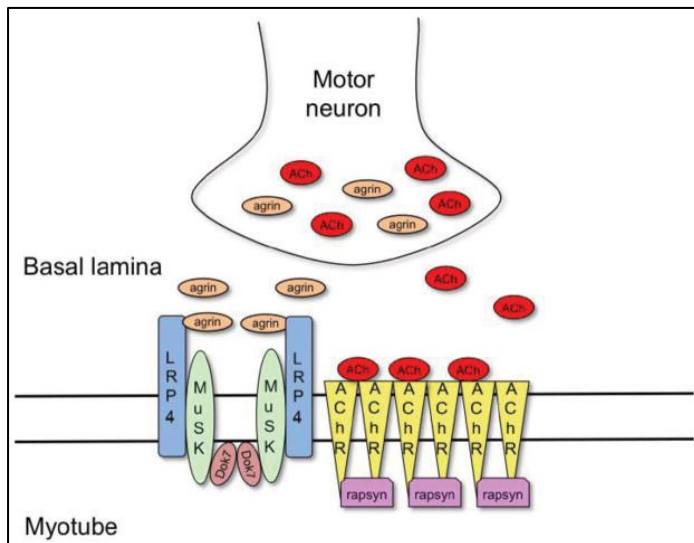


Figure 5: A closer look at the neuromuscular junction. Agrin, released by the motor neuron binds Lrp4, activating MuSK. After phosphorylation of MuSK, Dok7 interacts with it, further aiding in MuSK autophosphorylation and then it achieves full activation. Fully active MuSK enables AChR clusters to form through the help of the scaffolding protein, rapsyn (Hubbard & Gnanasambandan, 2013a).

4.4 Diseases of the NMJ

Due to the intricate nature of the signaling pathways and their importance during development, disruptions that occur in these critical pathways are the cause of a multitude of diseases. Various causes, such as autoimmune or genetic mutations, lead to the dysregulation of the signaling pathways eventually impacting proper function of the NMJ and cause debilitating diseases that range in severity. The dysregulation of the NMJ, the cornerstone of voluntary muscle contraction, results in fatigable muscles, especially after continuous exertion or towards the end of the day and can significantly impact the quality of life of people suffering from NMJ diseases, can even be life threatening if or when respiratory muscles are affected. Two examples of diseases that will be investigated in this thesis are myasthenia gravis (MG) and congenital myasthenic syndromes (CMS).

4.4.1 Myasthenia Gravis

4.4.1.1 Cause

Myasthenia gravis is a rare autoimmune disease characterized by the presence of antibodies against members of the NMJ. These antibodies are most commonly against the AChR, but also against MuSK, and more rarely against Lrp4. Around 80-85% of patients diagnosed with myasthenia gravis have antibodies against AChR (Hoch et al., 2001; Lindstrom et al., 1976), 70% of MG patients that are seronegative for AChR antibodies, meaning that they do not possess antibodies against AChR, but rather have antibodies against MuSK, which represents around 5% of all MG patients (Hoch et al., 2001). Data about Lrp4-MG is not completely clear. This is caused by the relatively new discovery of Lrp4, the infrequency of having antibodies against it and small sample sizes in patient groups. In literature, there is a lot of variation between available data, from 8% up to 50% but these are given as the percentage of double seronegative (dSN) that have Lrp4 antibodies and are not standardized in the way the data is presented and quantified. Higher number of tests and standardized presentation of data is needed to ascertain the overall percentage of Lrp4-MG laying within all MG patients (Cossins et al., 2012; Higuchi et al., 2011; Marino et al., 2015; Pevzner et al., 2012; Zisimopoulou et al., 2014).

Diagnosis depends on several factors including which NMJ protein the antibodies target, the age of onset and the symptoms the patient is presenting with. For AChR-positive MG, there are also multiple subtypes which are; (1) ocular, (2) generalized MG that can either be early onset MG (EOMG), late (LOMG) or very late onset, depending on the age of the patient when symptoms start to surface (<50, >50 and >60 years, respectively) and finally (3) thymoma associated MG (Berrih-Aknin & Le Panse, 2014; Dresser et al., 2021). When the autoantibodies are against MuSK or Lrp4, which are other important members of the NMJ, the type of MG is classified as such, different treatment options can be suggested, and the symptoms of patients are observed to be slightly different.

4.4.1.2 Clinical presentation of Myasthenia Gravis

Myasthenia gravis is, as mentioned before, characterized by fluctuating muscle weakness that is improved by rest. As the muscles are utilized, or as the day nears its end, continuous use of the affected muscles causes weakness in them, and this is the principal and main characteristic feature of the disease. Most patients are initially diagnosed with ocular myasthenia where the disease affects the ocular muscles, causing ptosis (drooping of the eyelid) and diplopia (double vision). Patients presenting with ocular MG often go on to develop generalized MG within 2 years of the onset of their symptoms. This is observed between 50-80% of patients (Bever Jr et al., 1983; Fang et al., 2022; Grob et al., 2008). However, 90% of patients who do not develop into generalized form within two years remain ocular (Dresser et al., 2021). On the other hand, the generalized form causes weakness in muscles other than the ocular muscles, such as limb muscles. Bulbar muscles are also affected that result in dysphagia (difficulty swallowing), and dysarthria (difficulty speaking). Muscles active in respiration can also be affected by weakness, sometimes causing respiratory crises that require assisted ventilation and weakness in neck muscles can cause a patient's head to droop. In mild cases of MG, muscle weakness can sometimes be revealed after intensive use of the effected muscle, such as a forced upwards gaze in ocular MG, or repetitive exertion in generalized MG (Dresser et al., 2021). In more severe cases of MG, typically MuSK-MG, patients are more likely to suffer from myasthenic crises requiring intense medical intervention caused by more involvement of bulbar muscles (Rodolico et al., 2020; He et al., 2024). Thymus involvement is usually associated with the presence of AChR antibodies. EOMG thymic hyperplasia and in thymoma associated MG thymic tumors are observed, whereas there is no thymic involvement in MuSK-MG, and not sufficient data is available regarding Lrp4-MG (Koneczny & Herbst, 2019).

4.4.1.3 Diagnosis

The diagnosis of the disease typically begins with the presentation of symptoms mentioned above, that indicate the possibility of MG. It is important to distinguish MG from CMS, since they are both diseases of the NMJ and might present with comparable symptoms. Suspicion of MG leads to administration of further tests, including serological, electrophysical and pharmacological tests. Since AChR-MG is the most common form of the disease (Lindstrom et al., 1976), the initially suggested serological test is to see whether the patient has antibodies towards AChR. This serological test to detect antibodies can be done using one of the many methods available, such as: radioimmunoprecipitation assay (RIPA) by radioactively labeling AChRs, by enzyme linked immunosorbent assay (ELISA) or by relatively recently developed cell-based assays (CBA) (Li et al., 2023; Zisimopoulou et al., 2013). Each test has its own positive and negative attributes, such as the high specificity and sensitivity of RIPA, as well as its availability as a commercial test but on the other hand, it cannot be performed by all testing facilities, based on the fact that it utilizes radioactivity and not all facilities can be expected to contain personnel and equipment that can handle this type of diagnostic test. ELISA, however, is safer in terms of not dealing with any radioactivity, but it slightly lacks in terms of specificity and sensitivity and the possibility of giving false positive results (Oger & Frykman, 2015). CBAs improve on the specificity and sensitivity, proved by the its ability to reveal positive diagnosis when low affinity antibodies are present in sera samples and the results were negative by other solution-based tests such as RIPA, especially for AChR- and MuSK-MG (Spagni et al., 2023). However it introduces new challenges in terms of adding more complexity to testing by incorporating cell culture, incubation, staining and interpretation of results (Spagni et al., 2023)

Even though serological testing is the method for a concrete diagnosis of MG, it can come later in line when compared to other tests. Other tests such as electrophysiological tests administered can reveal a MG diagnosis as well. One example is the repetitive nerve stimulation (RNS) test. This is done by placing a stimulating and a measuring electrode to a nerve and the muscle respectively to record compound muscle action potential (CMAP). The stimulating signal, typically 2-5 Hz, is repeated multiple times and the change in CMAP is observed. If there is a decrement equal or greater than 10% between the initial and the fourth CMAP, caused by fatigability, this result indicates a positive diagnosis (AAEM Quality Assurance Committee, 2001; Abraham et al., 2017). However, the test is less sensitive when the patient is presenting with either ocular, or mild generalized MG.

Another electrodiagnostic test that is also crucial in terms of diagnosis is single-fiber electromyography (SF-EMG). SF-EMG can detect abnormal muscle signals even if the results of the RNS test were within normal limits (Vahabi et al., 2021). The test is administered by inserting a needle electrode into a muscle in a way that the fibers of the muscle belong to the same motor unit, meaning that the signal for contraction is delivered to all of them by the same motor neuron. Then, either through voluntary contraction or through the use of a needle stimulator, muscle action potentials are triggered. The time difference between the occurrence of muscle action potentials is measured. The difference in time between the muscle action potentials are called as the “jitter”, and if the jitter values, which differ from muscle to muscle, are greater than the threshold value with 10% of the muscle fibers, or if all the measured values

of jitter exceeds the threshold of that muscle group, then the result is considered abnormal (AAEM Quality Assurance Committee, 2001; Sanders & Howard JR, 1986; Soriano, 2021).

Other than serological and electrophysiological tests, pharmacological tests can be done in MG diagnoses, these include AChE inhibitors, which aim to increase the amount of ACh that is present in the synapse and thus aiding in NMJ transmission. Edrophonium chloride is an example of one of many AChE inhibitors and is preferred for its fast and short-lived action duration, however its potential life threatening side-effects such as bradycardia brings about the need to administer this test in an emergency room setting with electrocardiographic monitoring. (Evoli et al., 1996; Jayam Trouth et al., 2012)

Another test that can be done in cases of ocular MG that presents with the symptom of ptosis is the ice pack test. This is a non-pharmacological test done by placing an ice pack over the eye with ptosis and holding the pack on the skin for up to 5 minutes. If an improvement in ptosis is observed, then this is indicative of ocular MG (Jayam Trouth et al., 2012; Kearsey et al., 2010). Finally, CT scans should be performed to confirm or rule out thymus involvement.

4.4.1.4 Treatment Options

Combating myasthenia gravis is a multifront effort. These efforts range from managing symptoms to surgery. The first line of treatment is the use of AChE inhibitors, mainly pyridostigmine (Evoli et al., 1996). AChE inhibitors function by inhibiting the enzyme AChE that serves the function of increasing the availability of ACh in the NMJ, ensuring that the present ACh can lead to successful transmission of signals from the motor neuron to the muscle fiber. AChE inhibitors are typically used for managing the muscle weakness of patients, rather than solving the underlying autoimmune problem. However, AChE inhibitors are generally not as effective in patients with MuSK-MG compared to patients with AChR- or Lrp4-MG, and can even lead to worsening of muscle weakness so different approaches are needed (Evoli et al., 2008).

Immunosuppressive drugs are another treatment method frequently used in cases where satisfactory improvements are not observed with the administration of AChE inhibitors, or especially in cases of MuSK-MG where AChE inhibitors can even cause detrimental effects (Maggi & Mantegazza, 2011). There are many different immunosuppressive drugs, whose intricacies will not be discussed in detail due to this being out of the scope of the thesis.

Steroidal immunosuppressive drugs, namely glucocorticoids, such as prednisone or prednisolone, as well as non-steroidal immunosuppressives such as azathioprine are used as first-line treatment options (Beloor Suresh & Asuncion, 2024; Keesey, 2004; Pascuzzi et al., 1984). They are used for modifying the underlying autoimmunity problem by aiming to reduce antibody production that leads to having the disease. Prednisone acts faster compared to azathioprine, 2 weeks with high dose prednisone compared to as much as 15 months in case of azathioprine (Jayam Trouth et al., 2012), however azathioprine can be utilized in cases where the patient has adverse reactions to prednisone, or it is found to be ineffective. In situations where the efficacy of the first-line treatments is not found to be satisfactory, second-line treatments such as monoclonal antibodies (mABs) are put into action. The mABs used in the treatment of MG are generally used for treatment of different autoimmune diseases and were

not initially developed specifically for MG, however, their efficacy in treatment of MG is demonstrated to be highly promising. These mAB treatments include but are not limited to rituximab, eculizumab, and many more (Alabbad et al., 2020). They alter the immune system in such a way that the result is a suppressed immune system that ends up with less antibodies circulating that is going to affect the NMJ. Rituximab in particular is shown to be the most effective in patients with MuSK-MG when compared AChR-MG since prednisone and the first-line immunosuppressive drugs have a lower chance of having satisfactory results, causing refractory status. It is effective towards resolving muscle weakness, enabling a reduction in the dosage of other immunosuppressive and even enabling remission for extended periods of time (Beecher et al., 2018; Topakian et al., 2019; C. Zhao et al., 2021).

In cases where thymus involvement is present, such as EOMG with AChR antibodies or thymoma associated MG, a thymectomy is an additional part of the treatment plan. It serves the purpose of trying to improve the patients' quality of life by reducing symptoms, reducing the usage of immunosuppressive drugs because of the decreased severity of the said symptoms, and aid in increasing the chance of remission (Aljaafari & Ishaque, 2022; Marx et al., 2013)

Agonist antibody treatment in recent years also have shown that even before disease symptoms occur in mouse disease models derived from pathogenic MuSK MG patients, the treatment with agonist antibodies binding to MuSK has a very high chance of preventing the negative effects of pathogenic antibodies. Even after the onset of disease symptoms, administration of agonist antibody leads to full survival. One such example of this type of treatment is the agonist antibody ARGX-119, which binds to the Fz-like domain on the extracellular side of MuSK. It does not compete with pathogenic antibodies for binding to MuSK and is able to reverse the course of disease (Oury et al., 2024).

Treating MG is not only about managing the symptoms or treating the underlying cause, but it is also about ensuring proper intervention during myasthenic crises. These crises, occurring especially in severe cases of MG, require assisted ventilation caused by weakness in bulbar and respiratory muscles. Crises can be triggered by infection, medications and poor treatment of the condition as well as over straining such as vigorous exercise, causing further weakness in muscle that are already prone to weaken through continuous exertion. Even emotional stress is a contributing factor to crises (Clayton et al., 2023). It is of utmost importance to restore respiration, and to intervene with procedures to alleviate the crisis. This is accomplished typically by two methods, first by plasmapheresis, which serves the purpose of filtering the patients' blood from the pathogenic autoantibodies and cytokines that falsely activate the immune system, and second by intravenous immunoglobulins (IVIgs), which work through binding the pathogenic autoantibodies and neutralizing them, and by modulating the immune system during acute exacerbations such as crises.

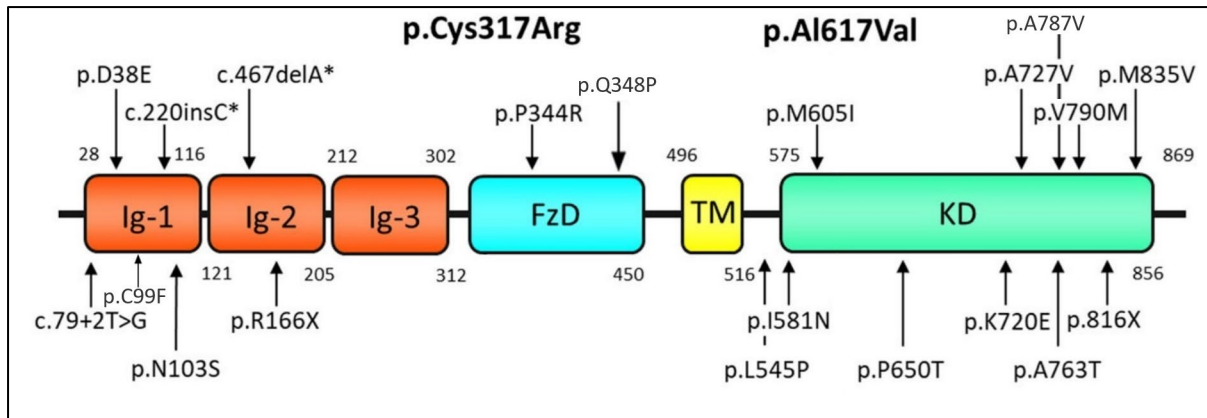


Figure 7: **Identified mutations in MuSK that result in CMS.** 22 mutations reported to date show that more than half of the mutations are found in the kinase domain of MuSK (Modified from Rodríguez Cruz et al., 2020).

The two mutations that were studied in this thesis are the N103S mutation found in the Ig1 domain in the extracellular region of MuSK described by Giarrana et al. in 2015, and the L545P mutation that is found in the juxtamembrane region on the intracellular side described by Owen et al. in 2018. As mentioned previously in the section “Formation of AChR Clusters Through Activation of MuSK”, the Ig1 domain enables interaction of MuSK with Lrp4 during activation of MuSK.

This domain is quite sensitive to changes in stability, caused by for example the absence of hydrogen bonds that would normally be present with the wildtype Asn103, but absent in the mutant with a replacement of the asparagine residue with a shorter sidechain containing serine. Without the proper stabilizing hydrogen bond in place, the domain may destabilize at the N-terminal of MuSK and may go as far as to even unfold. Without a stable Ig1 domain, interaction of Lrp4 with MuSK upon Agrin stimulation would be prevented, failing to activate MuSK and MuSK downstream signaling.

The second mutation L545P is positioned in the juxtamembrane region of MuSK, and it is very close to the Tyr553 residue that is crucial for the interaction between MuSK and Dok7. The Tyr553 residue is indispensable for allowing Dok7 to bind to MuSK, after MuSK has been phosphorylated on this tyrosine residue. Without this phosphorylation, there can be no Dok7 binding achieved, and no further phosphorylation on MuSK is observed.

4.4.2.2 Clinical presentation of congenital myasthenic syndromes

Depending on the time of onset, CMS can be further classified as such:

Neonatal presentation: Certain CMS subtypes that present during neonatal period are most often associated with respiratory insufficiency, which can manifest acutely or intermittently as apnea. Contractures can be observed at birth caused by restricted movement during pregnancy. These symptoms are usually accompanied by weakness, feeding difficulties, stridor (a high-pitched sound caused by a restriction of airways), as well as ptosis. Observation of some characteristic facial features of certain CMS subtypes may be possible, such as long face, narrow jaw and an arched palate (Ohno et al., 2023; Palace et al., 2007)

Childhood presentation: CMS that presents during childhood is usually characterized by muscle weakness and fatigue, and this situation is observed to a higher degree after physical exertion. Compared to neonatal onset, respiratory symptoms are less observed, resolved over time or may not even be present to begin with, however children may show delayed motor development, or difficulty running and going up stairs. Difficulties in chewing, swallowing or even speaking might be observed by the involvement of bulbar and facial muscles, ptosis may be present but to a lesser extent compared to neonatal onset of CMS (Engel, 2012; Palace et al., 2007)

Limb-Girdle (Late onset) CMS: Limb-Girdle pattern CMS (LG-CMS), associated with late onset, can be caused by various mutations, such as DOK7, COLQ, DPAGT1, GFPT1, ALG2, ALG14, GMPPB (Evangelista et al., 2015).

This thesis focuses on the N103S and L545P mutations in MuSK. To date, there are only two patients with the N103S and only one patient with the L545P mutation with LG-CMS that is reported in literature (Giarrana et al., 2015; Owen et al., 2018).

The first patient with the N103S mutation had respiratory failure at birth, requiring ventilation. Bilateral ptosis and external ophthalmoplegia was observed at 9 months of age. He had minor facial dysmorphism, and motor milestone delays where he could stand-up and walk without aid only at age 2.5 years as well as axial and facial weakness that led to “dropped head”. At 4 years of age, he still required assisted ventilation, however primarily during sleep and during infections. Cognitive functions and speech were not impeded (Giarrana et al., 2015).

The second patient with the N103S mutation was a 41-year-old female who experienced mild bilateral ptosis, facial weakness and difficulty walking during childhood. At 30 years of age, she presented again with muscle pain and proximal muscle weakness, as well as exertion induced weakness. Overall worsening of weakness, dysphonia and dysphagia was reported during pregnancy. She also had asymmetrical proximal upper limb, and lower limb weakness with ptosis, ophthalmoparesis, dysphonia and facial nerve weakness (Owen et al., 2018)

The first patient with the L545P mutation was a 24-year-old male who showed no symptoms until the age of 18 years. The symptoms first manifested as slowing of sports activities and worsening for 2 years until it reached a point of fatigability in day-to-day tasks. He had trouble walking upstairs, getting up from the floor and further activity caused him to fall down. He had a history of ptosis and difficulty swallowing; his symptoms were fluctuating and diurnal. It was revealed after his examination that he had central tongue atrophy, facial, upper and lower proximal limb as well as neck weakness. (Owen et al., 2018)

4.4.2.3 Diagnosis

The diagnosis of CMS is quite important in the sense that it has to be differentiated from MG since both diseases can present with similar symptoms, the major symptom being muscle weakness, especially through exertion. Since the treatment options between an autoimmune disease and a congenital disease might differ, it is of utmost importance to be able to differentiate between the two diseases.

Since the symptoms between the two diseases are similar, with muscle manifestation being prominent in both CMS and MG, it is crucial to differentiate between them, thus an antibody test to rule out the possibility of MG is important. Electrodiagnostic methods discussed in the diagnosis of MG, RNS and SF-EMG, remain to be two of the most important tools in determining that muscle contractions are affected due to a defect in the NMJ. The same thresholds of the decrement between CMAP measurements for RNS and jitter value differences for SF-EMG measurements also hold true for diagnosing CMS. Especially, SF-EMG seems to be an even better suited option for diagnosing children since it eliminates the requirement of voluntary muscle contraction, which might prove to be tough in newborns and children (Pitt, 2008; Tidswell & Pitt, 2007). However these electrodiagnostic methods are not the definitive answer for the diagnosis of the disease.

Family histories, going back 3 generations looking for reports of muscle weakness should also be taken when a CMS diagnosis is in question, given the fact that it is a hereditary disease. If family members also had symptoms resembling CMS such as muscle weakness or ptosis, it makes the diagnosis more likely.

A trial dose of an AChE inhibitor, such as pyridostigmine, can be administered, however not all types of CMS respond positively to the drug, as they can even deteriorate the patient especially in MuSK-CMS.

The most definitive, but also the costliest option is for the suspected genes to be sequenced. Techniques such as Whole Exome Sequencing (WES) and Next Generation Sequencing (NGS) aid in the identification of the mutations that are causing the pathogenicity of the disease (Ramdas & Beeson, 2021). Through sequencing and other methods of diagnosis, especially given the presentation of other symptoms, lead the way in the formation of a treatment plan for CMS.

4.4.2.4 Treatment Options

Treating CMS is akin to treating MG in the sense that the underlying problem cannot be solved, however symptoms and issues caused by the disease can be managed. Since CMS is a very heterogeneous disease, there is not a be all end all solution to treating all subtypes, one drug that is shown to be effective in one subtype might even cause deterioration in another subtype (Ramdas & Beeson, 2021). An example of this is pyridostigmine, an AChE inhibitor. While in certain subtypes of CMS it is shown to be effective, such as AChR and rapsyn deficiency (Engel et al., 2015), it's proven to be ineffective at best, deteriorative at worst especially in MuSK-CMS (Farmakidis et al., 2018; Gallenmüller et al., 2014; Ziaadini et al., 2024).

In cases where pyridostigmine is ineffective, another option is 3,4-diaminopyridine, which is a potassium channel blocker used for extending the duration of the AP in the motor neuron innervating the muscle fiber (Engel, 2007). This extension is accomplished by blocking the potassium channels in the motor neuron, which increases calcium movement into the cell during depolarization, and eventually results in the release of more ACh into the synaptic cleft. This release of more ACh can aid muscle contraction by compensating for the deficiencies in the NMJ.

Salbutamol is a different therapeutic option that works by stimulating the beta-2 adrenergic agonists for certain subtypes of CMS. The mechanism of action of salbutamol is not clearly understood, however the end result of its use is known. Regular use increases grip strength, prolongs survival in mice with various CMS conditions, and improves structural NMJ defects (McMacken et al., 2019; Webster et al., 2020). Its effects are particularly valuable in subtypes like DOK7-CMS, where impaired synaptic development and maintenance are the most important issues (Ohno et al., 2023). The role of salbutamol in CMS treatment best manifests muscle strength and endurance, though its efficiency is not uniformly effective in all subtypes.

Ephedrine is a sympathomimetic drug that has been used for more than three decades as a treatment option in certain subtypes of CMS. The exact mechanism of action of ephedrine is not known, but it is believed to stimulate the release of norepinephrine, which can result in increased muscle strength through enhanced neuromuscular transmission. It has been useful in subtypes such as COLQ and DOK7-CMS, where it can produce significant improvements in motor function (Lashley et al., 2010). The responsiveness of CMS to this treatment, however, as with the other treatments for CMS, largely depends on the mutation causing CMS, and in certain instances, the treatment may be ineffective or even harmful.

The aforementioned agonist antibody against MuSK, ARGX-119, was also shown to be effective against Dok7 CMS (Vanhouwaert et al., 2024) by rescuing diseased mice models, by enhancing MuSK phosphorylation and dimerization in instances where Dok7 was not able to. The administration of the agonist antibody ARGX-119 restores the NMJ organization, as well as the motor function to a level indistinguishable from healthy mice. Since MuSK is the cornerstone of NMJ function and organization, it is also suggested that this therapeutic method is suited not only for Dok7 CMS, but also for Agrin, MuSK and Lrp4 and many other neuromuscular diseases (Oury et al., 2021).

5. Aims

The aims of this thesis are twofold, focusing on myasthenia gravis (MG) and congenital myasthenic syndromes (CMS), two different diseases of the neuromuscular junction (NMJ).

The goal in the first part was to develop a cell-based detection method for Lrp4-MG using an in-house generated cell line that expresses the Lrp4 protein fused with mCherry upon doxycycline induction. This cell line was then incubated with patient sera samples to assess Lrp4 positivity in MG.

In the second part, the thesis aimed to investigate MuSK mutations linked with CMS, specifically N103S mutation in the Ig1 domain, and L545P mutation in the juxtamembrane domain. Muscle cells were used to investigate the effect of the mutations on myotube characteristics, such as acetylcholine receptor (AChR) clustering via transient retroviral transduction and overexpression, respectively.

6. Methods

6.1 PCR

There were two types of PCR protocols followed during the course of the experiments, the first type was used for cloning, to amplify a gene of interest. The second type was “Colony PCR”, which aimed to screen a single colony within an LB-Agar plate that has been plated after cloning to check if the specific colony contains the proper plasmid.

6.1.1 Cloning PCR

PCR reactions for cloning were performed in 20 μL , according to Table 1. Since every combination of primer was going to have different properties, a template program for the PCR reaction is provided in Table 2 as follows:

<u>Reagent</u>	<u>Volume</u>
H ₂ O	Ad 20 μL
5X GC or HF Buffer	4 μL
10 mM dNTPs	0.4 μL
Forward Primer 10 μM	1 μL
Reverse Primer 10 μM	1 μL
Template DNA	1 pg-50 ng
Phusion High Fidelity Polymerase	0.1 μL

Table 1: **Phusion HF Polymerase reaction volumes for cloning**

<u>Step</u>	<u>Temperature</u>	<u>Duration</u>
Initial Denaturation	98°C	30 seconds
Denaturation	98°C	10 seconds
Annealing	X°C	20 seconds
Extension	72°C	15-30 kb/second
Final Extension	72°C	5 minutes

Table 2: **Phusion HF Polymerase reaction program**

When the PCR reactions were complete, aliquots of the resulting products were loaded into a 1% agarose gel to be run and check for the efficiency of the PCR reaction since some modifications to the annealing temperatures or the type of buffer used might be required in order to get a more efficient PCR reaction. If the result of the reaction was satisfactory, it could be cleaned up using a kit and used downstream in cloning.

6.1.2 Colony PCR

As mentioned above, colony PCR was used for checking whether the attempted cloning worked, and plasmids had the correct sequence. To be able to do this, first, multiple colonies were picked from the plate and inoculated into 100 μL of LB+antibiotic in a 96-well plate and incubated at 37°C for 2-3 hours on a shaker. Then, 1 μL of bacteria from the wells were individually put in PCR tubes to be denatured at 95 °C for 5 minutes. A master mix for the colony PCR reaction was prepared, as can be seen in the table below. This master mix was prepared for however many colonies were picked, plus a “No Template Control”, a “Positive Control” and margin of error in pipetting.

<u>Reagent</u>	<u>Volume</u>
H ₂ O	10.75 μL
5X DreamTaq Buffer	1.5 μL
Forward Primer 10 μM	0.75 μL
Reverse Primer 10 μM	0.75 μL
10 mM dNTPs	0.15 μL
DreamTaq DNA Polymerase	0.1 μL
Total	14 μL

Table 3: **DreamTaq Polymerase Volumes for 1 reaction**

After the denaturation time, 14 μL of the prepared master mix was added to the bacteria and the tubes were placed in the PCR machine to follow the program below.

<u>Step</u>	<u>Temperature</u>	<u>Duration</u>
Initial Denaturation	95°C	1 minute
Denaturation	95°C	30 seconds
Annealing	X°C	20 seconds
Extension	72°C	2 kb/minute
Final Extension	72°C	5 minutes

Table 4: **DreamTaq Polymerase PCR program**

After the reaction was complete, all of the samples were loaded in a 1% agarose gel to check for positive colonies that possess the desired plasmid. If positives were found, they were inoculated in overnight cultures to be sent for sequencing for another level of confirmation.

6.2 Plasmid Construction & Purification

PCR-based cloning was done by amplification of the gene that was desired to be introduced into a plasmid backbone by PCR, followed by restriction enzyme digestion. Forward and reverse primers were designed to amplify the gene of interest flanked by the required restriction sites. After PCR amplification of the gene, it was purified using the Promega Wizard SV Gel and PCR Cleanup System and digested with appropriate restriction enzymes alongside the plasmid backbone to which the gene was ligated. Digestions were performed following the specific enzyme's requirements for both temperature and duration of the restriction reaction. Ligation was carried out using T4 DNA Ligase at room temperature for 2 hours. After ligation, transformation was performed as explained in the “Bacterial Transformation” subsection. Once

colonies were present on the plate, colony PCR was conducted as explained in the “Colony PCR” subsection, and if positives were found, they were sent for sequencing. If the sequencing results indicated that the plasmid had been successfully constructed, an overnight culture was prepared to purify the plasmid from. The following day, the culture was used for plasmid purification following the kit’s guidelines, and depending on the amount of plasmid desired, a Miniprep or a Midiprep kit was used. When the plasmid purification was completed, its concentration was measured using a Nanodrop instrument, and a control digestion was performed using a restriction enzyme to ensure that the plasmid was digested as expected. All the constructed plasmids are described in the “Plasmids” subsection. PCR based cloning was used for the following plasmids using the mentioned reaction and program templates:

Generated Plasmid	Backbone	Template	Primer Forward	Primer Reverse	Annealing Temperature	Forward Sequence	Reverse Sequence
pLXSG MuSK N103S	pLXSG (EcoRI x XhoI)	RSV MuSK N103S	MuSK_EcoRF (EcoRI)	MuSK_Xho/Sal_R (SalI)	50-55 °C	ATATGAATTC GCTAGCATG AGAGAGCTC	TTTACTC GAGTCGA CCTTAGA CGCCTAC CGTTCCC
pLXSG MuSK L545P	pLXSG (EcoRI x XhoI)	RSV MuSK L544P	MuSK_SnaBI_EcoRI_F (EcoRI)	MuSK_Xho/Sal_R (SalI)	56-60 °C	TAAATACGTA GAATTCAAA CAGTCATTAG TGGACGACT CTATTG	TTTACTC GAGTCGA CCTTAGA CGCCTAC CGTTCCC
PiggyBac MuSK WT HA	PB_TRE_MCS_Ubqc_rta_Ires (EcoRI x MluI)	pcDNA MuSK WT HA	MuSK_EcoRF (EcoRI)	PB_MuSK_T-MluI-Rev (MluI)	69-71 °C	ATATGAATTC GCTAGCATG AGAGAGCTC	AAAACGC GTCTTTC CGCCTCA GAAGC
PiggyBac MuSK N103S HA	PB_TRE_MCS_Ubqc_rta_Ires (EcoRI x MluI)	pcDNA MuSK N103S HA	MuSK_EcoRF (EcoRI)	PB_MuSK_T-MluI-Rev (MluI)	69-71 °C	ATATGAATTC GCTAGCATG AGAGAGCTC	AAAACGC GTCTTTC CGCCTCA GAAGC
PiggyBac MuSK L545P HA	PB_TRE_MCS_Ubqc_rta_Ires (EcoRI x MluI)	pcDNA MuSK L544P HA	MuSK_EcoRF (EcoRI)	PB_MuSK_T-MluI-Rev (MluI)	69-71 °C	ATATGAATTC GCTAGCATG AGAGAGCTC	AAAACGC GTCTTTC CGCCTCA GAAGC

Table 5: Details of plasmids generated using PCR cloning

6.3 Competent DH10 *E. coli* Bacteria Generation

To be able to generate a supply of competent DH10 *Escherichia coli* the first step was to streak them on an antibiotic free agar plate from a batch that was previously made. The plate was incubated at 37°C overnight. The following day, a colony was picked and was once again incubated overnight in 3 mL of antibiotic free LB medium at 37°C on an orbital shaker. After overnight growth, 2 mL of the culture was inoculated into 200 mL of antibiotic free LB medium and incubated at 37°C again on an orbital shaker until the OD600 measurement of the culture reached a value of 0.3-0.6, which took around 2-3 hours. When the desired OD600 measurement was achieved, all of the culture was centrifuged at 5000xg for 5 minutes at 4°C.

The supernatant was removed, and the pellet was resuspended using freshly prepared TSB solution. The competent cells were then aliquoted into tubes and incubated in ice for 30 minutes. After 30 minutes, they were flash frozen using liquid N₂ and stored at -80°C until they were needed.

6.4 Bacterial Transformation

The first step in bacterial transformation was to take a tube of frozen competent DH10 *E. coli* cells from -80°C and place it on ice until it completely thaws. In the meantime, transformation mixes were prepared, there were two types of mixes that could be prepared as seen in the table below:

<u>Reagent</u>	<u>Plasmid Transformation</u>	<u>Ligation Transformation</u>
DNA	0.1-10 ng	All of ligation mixture
5X KCM	10 µL	20 µL
ddH ₂ O	Ad 50 µL	Ad 100 µL
Competent Bacteria	50 µL	100 µL

Table 6: Transformation mixture ingredients for either plasmid or ligation transformation

When the bacteria were completely thawed, they were added to one of these mixtures depending on the type of transformation that was being performed. Then, the mixture was incubated on ice for either 15 minutes for plasmid or 30 minutes for ligation transformation. When the incubation time was completed, the tubes were put on a heat block at 42°C for 2 minutes in order to heat-shock the bacteria. They were then placed on ice until room temperature LB was added to them, 900 µL for plasmid and 950 µL for ligation transformation. The tubes were put on a heat block to be incubated at 37°C with a 300 rpm shake setting and were incubated for 15 minutes for plasmid and 30 minutes for ligation transformation for them to recover. After the recovery time, 100–200 µL of the culture was plated on a selective LB-agar plate that contained the proper antibiotic for plasmid transformation. The ligation transformations were centrifuged at 10,000xg for 2 minutes, and the resulting pellet was resuspended in 100 µL of LB, and everything was plated on a selective LB-agar plate. In either case, the plates were incubated overnight at 37°C for colonies to form.

6.5 Bacterial Glycerol Stock

Bacterial glycerol stocks were made after an overnight culture to take a sample of the cells to be streaked on a plate if they were once again needed in the future. This was accomplished by taking 800 µL of the overnight culture and mixing it with 200 µL of 100% glycerol. The tube was then stored at -80°C until they were needed again.

6.6 Cell Culture

During the course of this thesis, the following cell lines were used:

HeLa PiggyBac Lrp4-GFP: Previously generated at Herbst Lab (unpublished results). HeLa cells expressing Lrp4-GFP in a doxycycline inducible manner.

HeLa PiggyBac Lrp4-mCherry*: HeLa cells expressing Lrp4-mCherry in a doxycycline inducible manner.

MuSK^{-/-} muscle cells (C3): Muscle cells generated by Herbst & Burden, 2000, from MuSK knockout mice.

Wild-type muscle cells (K3): Immortalized wild-type muscle cells expressing MuSK (Mazhar & Herbst, 2012).

K3 PiggyBac MuSK WT-HA*: K3 muscle cells expressing MuSK WT-HA in a doxycycline inducible manner.

K3 PiggyBac MuSK N103S-HA*: K3 muscle cells expressing MuSK N103S-HA in a doxycycline inducible manner.

K3 PiggyBac MuSK L545P-HA*: K3 muscle cells expressing MuSK L545P-HA in a doxycycline inducible manner.

Cell lines marked with * are cell lines generated within the scope of this thesis.

6.6.1 Thawing Cells

Cells were thawed by taking appropriate cryotubes out of -80 °C or liquid N₂ and placing the tubes in lukewarm water. After the contents of the cryotube were completely thawed, they were transferred into a 15 mL Falcon tube where 4 mL of pre-warmed growth medium was very gently added. The Falcon tube was centrifuged for 5 minutes at 300xg. The supernatant was removed, and an appropriate amount of pre-warmed growth medium was used to resuspend the cell pellet, and the cells were plated.

6.6.2 Splitting Cells

Cells are split when the desired confluency is achieved. The confluency that is targeted for splitting for myoblasts was 30%, whereas the confluency that was targeted for HeLa and Phoenix cells were 70-80%. When the cells were observed to be in these confluency percentages, they were first washed with sterile PBS, then trypsinized to detach them from the plate. Trypsinization was done by adding trypsin-EDTA into the plates followed by incubation in 37°C 5% CO₂ incubator for 3 minutes. They were observed under a microscope to make sure that all of the cells have detached. The detached cells were then collected using growth medium and centrifuged for 5 minutes at 300xg. After centrifugation and removal of the supernatant, using appropriate dilutions, generally 1:3 for myoblasts and 1:8 for HeLa and Phoenix cells, the cells were plated. If the confluency of the HeLa and Phoenix cells were higher to begin with, they could also be split with up to a 1:10 ratio.

6.6.3 Freezing Cells

Cells could be frozen when they reach a certain confluency, 30-40% for myoblasts and 70-80% for HeLa and Phoenix cells. The cells were washed, trypsinized and centrifuged as explained in the subsection “Splitting Cells”. The cells were resuspended in growth medium and additionally FBS and DMSO are added as cryoprotectants to a final concentration of 10% each. The cells were then immediately placed in styrofoam racks at -80°C for at least one week in order to lower the temperature in a controlled manner and then moved to boxes for storage. If need be, they could be stored in liquid N₂ for long term storage.

6.6.4 Cell Counting

Cells that were going to be counted were subjected to the same steps of washing, trypsinization and centrifugation as explained in the section above. The pellets were resuspended in growth medium and 10 µL of the resulting suspension is released into the Neubauer chamber. The chamber is made up of 4 big (1 mm x 1 mm) squares on the outer quadrants and 16 even smaller (0.25 mm x 0.25 mm) squares within each of those quadrants. The cells within the limits of the 4 outer quadrants were counted and averaged. This resulting number was multiplied by 10.000 in order to get a cell count per milliliter of the cell suspension used.

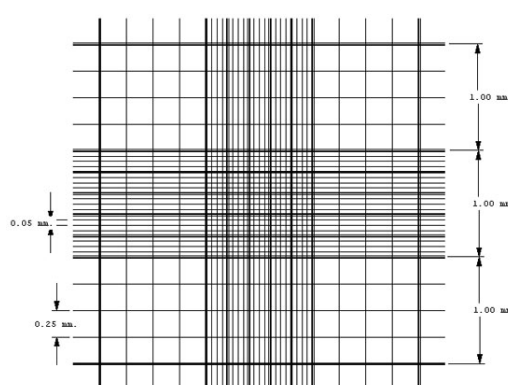


Figure 8: Neubauer Chamber

6.6.5 Transfections

Transfections, regardless of the size of the plates, were done following the same steps. Appropriate amounts, as seen in the table below, of DNA were diluted in an appropriate volume of serum-free medium. The transfection reagent was vortexed for 10 seconds, and appropriate volumes of it were added to the diluted DNA-medium mixture. This final mixture was once again vortexed for 10 seconds and then incubated for 15 minutes at room temperature. After the incubation period, the mixture was added onto the cells gently in a drop-wise fashion in order not to break the formed reagent-DNA complexes.

	Plasmid	Medium	Transfection Reagent
12-well	1 µg	100 µL	2 µL TurboFect
6-well	2 µg	200 µL	4 µL TurboFect
6-cm	4 µg	400 µL	6 µL TurboFect
10-cm	6 µg	800 µL	8 µL TurboFect/

Table 7: Transfection template

The plates were gently rocked in order to homogenously mix the added complexes. After 5–6 hours of incubation, the growth medium was replaced to avoid toxic effects on cells.

6.6.6 Retroviral Transduction of C3 Myoblasts

The process of retroviral transduction of C3 myoblasts utilizes the help of a system called the “Phoenix System” (GP Nolan, 1997) . Using this system, viruses can be produced by transfecting the Phoenix cells with retroviral plasmids that are desired to be introduced into the C3 myoblasts. The plasmids, however, must contain 5’ and 3’ long terminal repeats at the beginning and end of the gene of interest in order to be successfully integrated into the genome of the target C3 cells. This has to be accomplished by cloning the gene of interest into an empty pLXSG plasmid, which also coincidentally contains an EGFP gene under the control of a SV40 promoter that can be used to assess transfection efficiency of the Phoenix cells, as explained previously.

The first step in this process was to plate Phoenix cells in such a way that they were going to be 70% confluent the day after plating. The day after plating, Phoenix cells were transfected with the desired plasmids and the PSI2 helper plasmid, as explained in the “Transfections” subsection. The ratios between the desired pLXSG and the PSI2 plasmid is 1:3, where 1 part is the PSI2 helper plasmid, and 3 parts are the pLXSG plasmid. The following day, the Phoenix cells were moved to the 33°C 5% CO₂ incubator for more efficient viral production. The same day, C3 myoblasts were counted and plated on gelatine coated plates so that they were going to be ready to start differentiation in 2 days. On the next day, the virus containing medium from Phoenix cells was collected and centrifuged for 5 minutes at 1500 rpm. The supernatant was filtered into a new tube using a 0.45 µm filter and polybrene at 1:1000 dilution was added to the supernatant. The growth medium from C3 myoblasts was removed and the viral supernatant was added to the plates and incubated for 4 hours. After the incubation period, cells were washed using sterile PBS and fresh growth medium was added. The next day, differentiation was started as explained in the “Myotube Differentiation” subsection. If and when the myoblasts differentiate into myotubes, acetylcholine receptor clustering was performed as explained in “Acetylcholine Receptor Clustering” subsection.

6.6.7 Myotube Differentiation

In order to be able to do experiments with C3 or K3 cell lines, myoblasts had to be differentiated into myotubes. To accomplish this, multiple plates of the required myoblasts were trypsinized and counted as explained in the “Cell Counting” subsection. The myoblasts were plated in such a way that the cells were going to be 100% confluent either the next day of plating or the next day after that. When they were 100% confluent, the growth medium was exchanged for differentiation medium, which was made up of only DMEM, 10% HS and 1% Pen-Strep. The plates were then put in 39°C 5% CO₂ incubator instead of 33°C 5% CO₂ and they were kept in that incubator until all of the myoblasts were differentiated into myotubes. Since this generally took a couple of days, the differentiation medium was renewed every 2 days. When myoblasts were fully differentiated, they were ready to be used in further experiments.

6.6.8 Myotube Stimulation, Lysis & Immunoprecipitation

K3 myoblasts that have been used to generate stable cell lines were differentiated as explained in the “Myotube Differentiation” subsection. They were induced with doxycycline for 48 hours. When the induction time was complete, and the myoblasts were fully differentiated into myotubes, they were lysed by first placing the plates on ice and gently washing them with PBS. Lysis was accomplished by preparing RIPA supplemented with protease inhibitors. This RIPA+Inhibitor cocktail was released onto the cells, and they were incubated on a shaker for 15 minutes at 4°C. After the lysis period, the cells were scraped using rubber scrapers and all of the lysates were collected in tubes which were then centrifuged at full speed for 15 minutes at 4°C. The supernatants were transferred into new tubes and aliquots for total cell lysates were taken and frozen after the addition of loading dye at -20°C until they were further required. Into the rest of the lysates, antibodies were added in their proper dilutions, and they were rotated at 4°C for two hours. After their rotation time, either Protein G or Protein A agarose beads were added, depending on the species of the antibody used for immunoprecipitation and they were rotated overnight at 4°C. The following day, the tubes were centrifuged for 1 minute at 9000xg, the supernatant was discarded, and the beads were washed twice with RIPA buffer with centrifugation. After the last wash, the supernatant was removed once again, loading dye was added and the samples were frozen at -20°C until they were ready to be analyzed.

If need be, on the day of the lysis, the cells were starved by changing their medium to serum-free DMEM for one hour and then stimulated using 5 nM final concentration of C-Agrin 4.8.

6.7 Generation of PiggyBac Cell Lines

PiggyBac is a transposon-transposase mobile element system that works by the “cut and paste” mechanism to ensure the gene of interest, the transposon, is inserted into “TTAA” sites in the target genome (S. Zhao et al., 2016). The transposon is inserted into genomes of cells when the transposase enzyme is also present. This is done by co-transfection of a plasmid encoding the transposase enzyme and another plasmid containing the transposon, flanked by inverted terminal repeats that are recognized by the enzyme. The promoter responsible for the expression of the transposon is under the control of doxycycline, a semi-synthetic member of

the tetracycline class. With the addition of doxycycline into the growth medium of cells, expression of the desired transposon can be achieved.

6.7.1 Generation of HeLa PiggyBac Lrp4-mCherry

70% confluent wildtype HeLa cells were transfected using TurboFect with the two mentioned plasmids, the transposon plasmid that contains the sequence of Lrp4-mCherry, as can be seen in Figure 12, and with the transposase plasmid. The ratio between the transposase and transposon plasmids was 1:3, (i.e. 0.5 μ g:1.5 μ g). Antibiotic selection was started two days after transfection, using puromycin with a final working concentration of 5 μ g/mL. Two days later, selective pressure was removed since all control cells were dead, and the transfected cells were left to recover. After having enough cells, a doxycycline test was performed with a final working concentration of 1 μ g/mL to see whether the cells were producing Lrp4-mCherry by checking under a fluorescent microscope.

6.7.2 Generation of K3 PiggyBac MuSK Variants

50% confluent wildtype K3 myoblasts were transfected using Turbofect following the same setup for the generation of HeLa PiggyBac Lrp4-mCherry, however instead of one cell line being generated, three were being generated with plasmids that can be seen in Figure 9, Figure 10 and Figure 11. One day after transfection, antibiotic selection was started with 5 μ g/mL of puromycin. 3 days after starting antibiotic selection, the cells were moved from 6-well plates into 12-well plates because of the number of dead cells. One day after replating, all control cells died, the selective pressure was removed, and cells were left to recover. After having enough cells, a doxycycline test was performed by lysis with a final working concentration of 100 ng/mL to see the production of various MuSK constructs, followed by an AChR clustering assay.

6.8 Acetylcholine Receptor Clustering

Acetylcholine Receptor (AChR) clustering was done by first counting C3 or K3 myoblasts and plating them in 0.2% gelatine coated 35-mm plates. The coating process was done by adding enough gelatine to cover the surface of the plate and incubating the plate for at least 15 minutes at 37°C 5% CO₂ incubator and washing with sterile PBS after the incubation period. The myoblasts were differentiated as explained in the “Myotube Differentiation” subsection. When the myoblasts were differentiated into myotubes, C-Agrin was added to the plates at a final concentration of 10 ng/mL and the cells were stimulated for either 7 hours or overnight in the incubator. When the stimulation time was completed, the medium from the plates were discarded and the cells were washed once with PBS. Then, using 2% PFA, the cells were fixed for 10 minutes at room temperature. The plates were washed 3 times with PBS and then Alexa-555 conjugated α -bungarotoxin (α -BTX) in 3% BSA made with PBS at 1:2500 dilution was added to the plates, and they were incubated in the dark, at room temperature for 30 minutes. After 30 minutes of incubation, the plates were washed with PBS twice, for 3 minutes each. 7 μ L of mounting medium of Mowiol 4-88 is added on top of the plate and the cells are covered using a square coverslip. Plates were stored at 4°C until the mounting medium was dry and then they were visualized with fluorescence microscopy for analysis.

6.9 Western Blot

Western blots were performed by first preparing two polyacrylamide gels, a 7.5% PAA running gel and a 5% stacking gel. When gels had been poured and they had polymerized, samples that had already been mixed with 4X loading dye were denatured at 95°C for 10 minutes and they were then centrifuged for 2 minutes at 10,000xg and loaded into the PAA gels. The gels were first run at 110V until the samples reached the running gel, afterward the voltage was increased to 140V. The gels were run until either the loading dye started running out from the bottom of the running gel, or depending on what was going to be analyzed, until the appropriate marker remained in the gel. In order to be able to analyze the proteins, the gels had to first be transferred to membranes. Nitrocellulose membranes were used for western blots unless otherwise stated. The membrane and the gels were shaken for equalization purposes in BSN buffer before the transfer process could start. This process was done using Bio-Rad Trans Blot Turbo Transfer System. The system was composed of cassettes into which first, 8 layers of filter towels were placed, they were wet using BSN buffer and air bubbles were rolled out. Onto the towels, a nitrocellulose membrane was placed where the proteins in the gel were going to be transferred to. Onto the lid of the cassette, another 8 layers of filter towels were placed that when closed, would directly overlap with the layers in the cassette. The filter towels on the lid were also wet using BSN buffer and air bubbles were rolled out. The lid was closed on top of the cassette and locked, inserted into the machine and the gel was transferred to the membrane for 11-12 minutes. After the transfer was complete, the membrane was ready for further processing. It could be blocked using various solutions depending on what antibodies were going to be used to probe the membrane, such as 5% milk, RotiBlock, 5% BSA all of which were prepared in TBS buffer and the blocking process took 1 hour at room temperature and was done on a shaker. When the blocking process was done, the membranes were washed on a shaker, 3 times for 5 minutes each, to completely get rid of the blocking solution. The primary antibody solution was added on top of the membranes, and they were incubated, unless otherwise stated, overnight at 4°C and on a shaker. The following day, the antibodies were collected, and the membranes were washed with TBS-Tween, 3 times for 5 minutes each. A secondary antibody solution depending on which species the primary antibody was from was prepared and the membranes were incubated with the solution for 1 hour at room temperature. After the incubation period, the membranes were once again washed with TBS-T and then they were ready for visualization using chemiluminescence. This was accomplished by taking the membranes out and placing them on a clear piece of folio and introducing a 1:1 mixture of horse radish peroxidase and its substrate luminol on top of them and incubating for 5 minutes at room temperature. Afterwards, they were visualized using Bio-Rad Chemidoc Visualization System. If need had been, the membranes could have been incubated again with a different set of primary antibodies. To do this, the membranes were stripped using 10% acetic acid for 45 minutes on a shaker at room temperature. After the stripping process, the membranes were washed with TBS until the smell of the acetic acid was gone from them and then, the blocking process started over, and the steps described above were repeated until they were visualized.

6.10 Immunofluorescence Staining on Coverslips

Immunofluorescence cell staining, hereby referred to as “IF” for simplicity’s sake, was done by first plating HeLa-Lrp4 cells in such a way that the day following plating, they would reach ~70% confluency. The cells were plated on gelatin-coated circular coverslips that were sterilized with UV. The next day, the coverslips were moved each into one well of a 12-well plate, and the cells were induced with 1 µg/mL doxycycline and were placed at 33°C, 5% CO₂. After 48 hours of induction with doxycycline, the IF staining process began and could be done in three different ways.

6.10.1 Live Staining

The coverslips were placed on the lid of a 6-cm plate that was covered with parafilm and a wet paper towel was placed around the lip of the lid. 50 µL of primary antibody solution that was prepared in growth medium was placed on top of the coverslips and the coverslips were incubated in 33°C, 5% CO₂ for 1 hour. After the incubation period, the coverslips were placed back into 12-well plates and they were washed with PBS, 3 times each for 5 minutes. When all washing steps were completed, the cells were fixed using 2% PFA at room temperature for 10 minutes and afterwards washed twice with PBS. After washing steps, the cells were blocked using 2% BSA made in PBS for 30 minutes at room temperature. The cells were washed once with PBS to get rid of the blocking solution and they were incubated with proper secondary antibodies for 1 hour at room temperature in the dark. Once the incubation period was done, they were washed with PBS, 3 times for 5 minutes each and then they were mounted on microscope slides using Mowiol 4-88. They were stored at 4°C until the Mowiol 4-88 was completely dry then, they were visualized with fluorescence microscopy.

6.10.2 Fix & Stain

The medium from the wells in which the coverslips were in was removed, and they were washed with PBS. Then, the cells were fixed using 2% PFA at room temperature for 10 minutes and afterward were washed twice with PBS. They were blocked using 2% BSA made in PBS for 30 minutes at room temperature, and they were incubated with primary antibody solutions made in the same blocking solution in a 15-cm plate. The plate was covered with aluminum foil on all sides to block all light entry, and the inside walls were covered with wet paper towels in order to prevent the antibody solution from drying out. When the incubation period was over, the coverslips were moved back to the 12-well plates and they were washed with PBS, 3 times each for 5 minutes. Afterward, they were incubated with proper secondary antibody solutions for 1 hour at room temperature and in the dark. They were once again washed with PBS, 3 times each for 5 minutes and mounted on a microscope slide with Mowiol 4-88.

6.10.3 Fix, Permeabilize & Stain

The steps followed for this were practically the same except after fixation and before primary antibody incubation, the cells were permeabilized using 0.1% Triton-X made in PBS for 5 minutes at room temperature. All the steps before and after the permeabilization were the same as the “Fix & Stain” procedure.

6.11 Patient Sera Staining

In order to analyze whether individual patient serum samples contain autoantibodies against Lrp4 in potential cases of myasthenia gravis, the samples were used during the staining process. The staining protocol followed for the analysis was either the “Fix & Stain” or “Fix, Permeabilize & Stain”, as explained above. The difference in the case of using patient sera was that the samples were diluted 1:40 in staining buffer and used as primary antibodies during staining. Since the sera have the potential of containing antibodies against Lrp4 and the samples were from humans, α -human 488 secondary antibodies were used during the following steps of the staining process. Aside from using the patient sera, commercial Lrp4 antibodies have also been incorporated into the staining process to achieve a double staining as a control. The secondary antibodies detecting the commercial primary Lrp4 antibodies were chosen with a Cy5 fluorophore to distinguish between the sera and commercial staining.

6.12 Quantifications

6.12.1 Western Blot Quantification

Western Blots were quantified using Bio-Rad ImageLab 6.0 and Microsoft Excel. ImageLab was used to measure the signal of the lanes in the blots. This measured signal was exported to Microsoft Excel to do calculations.

6.12.2 Patient Sera Immunocytochemistry Quantification

Quantification of patient sera immunocytochemistry was done in ImageJ and Microsoft Excel. All three channels (mCherry, 488 and Cy5) of the coverslips were individually thresholded and areas with fluorescent cells for each channel were selected. Areas of overlap were measured by using the Region of Interest tool. The area measurements were exported to Microsoft Excel for calculation of overlaps between channels.

6.12.3 AChR Assay Quantification and Statistical Analysis

6.12.3.1 MuSK^{-/-} Muscle Cells

Using ImageJ, all the myotubes and possible AChR clusters present in each image were manually outlined and their areas were measured. The data was exported to Microsoft Excel for calculations. Two tailed t-Test was performed on the calculations to look for significant differences.

6.12.3.2 Wild type Muscle Cells overexpressing MuSK WT-HA, N103S-HA or L545P-HA

Using ImageJ, obtained images were used to run a macro (Rodríguez Cruz et al., 2020) that automatically detects and measures the areas of AChRs. All of the myotubes present in images were also outlined and their areas were measured. Data was exported to Microsoft Excel for calculations. Two tailed t-Test was performed on the calculations to look for significant differences.

7. Materials

7.1 Antibodies

<u>Antibody</u>	<u>Species</u>	<u>Dilution</u>	<u>Source</u>
α -Acetylcholine Receptor (IB)	<u>Mouse</u>	<u>1:500</u>	<u>BD Biosciences 610988/-9</u>
α -GAPDH (IB)	<u>Mouse</u>	<u>1:1000</u>	<u>Cell Signalling 14C10</u>
α -Goat HRP (IB)	<u>Donkey</u>	<u>1:5000</u>	<u>JIR 705-035-147</u>
α -HA (IB)	<u>Mouse</u>	<u>1:1000</u>	<u>Cell Signalling 6E2</u>
α -HA (IP)	<u>Rabbit</u>	<u>1:100-1:300</u>	<u>Sigma H6908</u>
α -HA (IP)	<u>Mouse</u>	<u>1:100</u>	<u>Sigma 12CA5</u>
α -HA 12CA5-E2 (IP)	<u>Mouse</u>	<u>1:100</u>	<u>Sigma 12CA5-2</u>
α -Human 488 Alexa Fluor (S)	<u>Rabbit</u>	<u>1:1000</u>	<u>JIR 109-545-003</u>
α -Insulin Receptor (IB)	<u>Rabbit</u>	<u>1:500</u>	<u>BD Biosciences 611276/-7</u>
α -Insulin Receptor β (IB)	<u>Mouse</u>	<u>1:500</u>	<u>BD Biosciences 610109</u>
α -Integrin β I (IB)	<u>Rabbit</u>	<u>1:1000</u>	<u>Cell Signalling 34971</u>
α -Mouse 488 Alexa Fluor (S)	<u>Donkey</u>	<u>1:1000</u>	<u>JIR 715-485-150</u>
α -Mouse Cy5 (S)	<u>Donkey</u>	<u>1:1000</u>	<u>JIR 715-495-150</u>
α -Mouse HRP (IB)	<u>Goat</u>	<u>1:5000</u>	<u>JIR 115-035-062</u>
α -MuSK (IB)	<u>Goat</u>	<u>1:500</u>	<u>AF562</u>
α -MuSK (IP)	<u>Rabbit</u>	<u>1:500</u>	<u>S. Burden Lab 94277</u>
α -N207/27 Lrp4 (IB, S)	<u>Mouse</u>	<u>1:20</u>	<u>Addgene</u>
α -N207/27 Lrp4 (IB, S)	<u>Mouse</u>	<u>1:2</u>	<u>Home made</u>
α -Phospho Tyrosine (IB)	<u>Mouse</u>	<u>1:1000</u>	<u>BD Biosciences 61000</u>
α -Phospho Tyrosine (IB)	<u>Mouse</u>	<u>1:1000</u>	<u>Santa Cruz Biotechnology SC-7020</u>
α -Phospho Tyrosine (IB)	<u>Mouse</u>	<u>1:2000</u>	<u>Cell Signaling 9411</u>
α -Rabbit HRP (IB)	<u>Goat</u>	<u>1:5000</u>	<u>JIR 111-035-144</u>
α -Tubulin (IB)	<u>Mouse</u>	<u>1:5000</u>	<u>Cell Signaling 3873</u>

Table 8: List of antibodies, their species, dilution, and source

S: Staining, IP: Immunoprecipitation, IB: Immunoblot

7.2 Buffers & Media

7.2.1 Bjerrun Schafer-Nielsen (BSN) Buffer

<u>Reagent</u>	<u>Final Concentration</u>
Tris-Base	48 mM
Glycine	39 mM
Methanol	20%

Table 9: BSN Buffer recipe

All reagents were dissolved in H₂O according to the final concentrations.

7.2.2 HeLa & Phoenix Growth Media

The growth media of HeLa and Phoenix cells was made up of 10% FBS and 1% Pen/Strep antibiotic mixture made in high glucose DMEM.

7.2.3 Myoblast Growth Media

The growth media of myoblasts was made up of 10% FBS, 10% HS, 1% Pen/Strep and Interferon- γ made in high glucose DMEM.

7.2.4 Myoblast Differentiation Media

The differentiation media of myoblasts was made up of 10% HS and 1% Pen/Strep made in high glucose DMEM.

7.2.5 5X KCM

<u>Reagent</u>	<u>Final Concentration</u>
1M KCl	0.5 M
1M CaCl ₂	0.15 M
1M MgCl ₂	0.25 M

Table 10: 5X KCM recipe

All the ingredients were dissolved in H₂O and sterilized using 0.45 μ m filter.

7.2.6 LB Agar

<u>Reagent</u>	<u>Amount</u>
LB Medium	12.5 grams
Agar-Agar	7.5 grams
Antibiotic (50 mg/mL)	1 mL
H ₂ O	500 mL

Table 11: LB Agar recipe

The ingredients except the antibiotic were dissolved in H₂O and autoclaved. When they were cooled down, 1 mL of the desired antibiotic was added and poured in a sterile fashion into plates. The plates were kept at room temperature until they were solidified, and the moisture had evaporated, then they were moved to 4°C for storage.

7.2.7 LB Broth

<u>Reagent</u>	<u>Amount</u>
LB Medium	12.5 grams
H ₂ O	500 mL

Table 12: **LB Broth recipe**

LB medium and water were mixed, and they were autoclaved. After it cooled down to room temperature, it was stored at 4°C.

7.2.8 10X PBS

<u>Reagent</u>	<u>Final Concentration</u>
NaCl	1.37 M
KCl	27 mM
Na ₂ HPO ₄	43 mM
KH ₂ PO ₄	14 mM

Table 13: **10X PBS recipe**

All reagents were mixed in H₂O according to their final concentrations.

7.2.9 RIPA

<u>Reagent</u>	<u>Final Concentration</u>
NaCl	150 mM
Tris-HCl (pH 8.0)	50 mM
NP-40 Alternative	1%
Sodium deoxycholate	0.5%
SDS	0.1%

Table 14: **RIPA Buffer recipe**

All reagents were mixed in H₂O according to their final concentrations.

7.2.10 Staining Buffer

<u>Reagent</u>	<u>Final Concentration</u>
NaCl (1 M Stock)	140 mM
HEPES (1 M Stock)	20 mM
CaCl ₂ (1 M Stock)	1 mM
MgCl ₂ (1 M Stock)	1 mM
KCl (1 M Stock)	5 mM

Table 15: **Staining buffer recipe**

The reagents were mixed with water, the pH was adjusted to 7.4 and then the solution was sterilized using a 0.45 µm filter.

7.2.11 10X TBS

<u>Reagent</u>	<u>Amount</u>
Tris	24 grams
NaCl	88 grams
H ₂ O	1 liter

Table 16: **10X TBS recipe**

All reagents were dissolved in H₂O and the pH was adjusted to 7.6.

7.2.12 10X TBS-Tween

<u>Reagent</u>	<u>Final Concentration</u>
Tris-Cl pH 8.0	100 mM
NaCl	1.5 M
Tween	0.5%

Table 17: **10X TBS-Tween recipe**

All reagents were dissolved in H₂O according to their final concentrations.

7.2.13 TSB Solution

<u>Reagent</u>	<u>Final Concentration</u>
Polyethylene Glycol (PEG)	10%
DMSO	5%
MgCl ₂	10 mM
MgSO ₄	10 mM

Table 18: **TSB Solution recipe**

All of the reagents were mixed with LB broth, and the solution was made fresh for generation of competent *E. coli* bacteria.

7.3 Chemicals, Consumables & Kits

Reagent	Manufacturer
10X Buffer EcoRI	Thermo Fisher Scientific
10X Buffer R	Thermo Fisher Scientific
10X Buffer Tango	Thermo Fisher Scientific
10X FastDigest Buffer	Thermo Fisher Scientific
10X NEB r3.1	New England Biolabs
10X rCutSmart	New England Biolabs
10X T4 DNA Ligase Buffer	Thermo Fisher Scientific
5X DNA Loading Dye	Homemade
5X DreamTaq Buffer	Thermo Fisher Scientific
5X HF/GC Buffer	Thermo Fisher Scientific
Acetic Acid	Carl Roth
Acrylamide	Carl Roth
Agar-agar	Carl Roth
Agarose	Carl Roth
Agarose Beads	Thermo Fisher Scientific
Alexa-555 α -Bungarotoxin	Molecular Probes
Ampicillin	Carl Roth
Apal Fast Digest	Thermo Fisher Scientific
Bacterial Plates	Greiner Bio-one
BamHI-HF	New England Biolabs
BglII	New England Biolabs
BioRad ECL	Bio-Rad Laboratories
BSA	Sigma-Aldrich
BspHI	New England Biolabs
CaCl ₂	Carl Roth
Coverslips	Roth/Marienfeld
Cryotubes	Sarstedt
DMEM	Sigma-Aldrich
DMSO	Carl Roth
dNTPs	Thermo Fisher Scientific
Doxycycline	Fisher/VWR
DreamTaq DNA Polymerase	Thermo Fisher Scientific
EcoRI	Thermo Fisher Scientific
Epprouvettes	Carl Roth
Ethanol	Merck
Ethidium Bromide	Carl Roth
Falcon Tubes	Sarstedt
FastAP Thermosensitive Alkaline Phosphatase	Thermo Fisher Scientific
FBS	Sigma-Aldrich
Gelatine	Carl Roth
GeneJET MiniPrep Plasmid Kit	Thermo Fisher Scientific
GeneRuler 1 kb DNA Ladder	Thermo Fisher Scientific
Glycerol	Thermo Fisher Scientific
Glycine	Carl Roth
HincII-FD	Thermo Fisher Scientific
HindIII-FD	Thermo Fisher Scientific

Immobilon ECL	Sigma-Aldrich/Millipore
Kanamycin	Carl Roth
KCl	Carl Roth
KpnI	New England Biolabs
LB Medium	Carl Roth
MgCl ₂	Fluka
Micropipette Tips	Greiner Bio-one
Microscope Slides	Thermo Fisher Scientific
Midiprep Plasmid Kit	QIAGEN
Milk Powder	Carl Roth
MluI	Thermo Fisher Scientific
Mowiol 4-88	Carl Roth
NaN ₃	Carl Roth
NaCl	Carl Roth
NcoI	Thermo Fisher Scientific
Nitrocellulose Membranes	Amersham
Opti-MEM	Gibco
Orthovanadate	Sigma-Aldrich
Parafilm	Amcor
Paraformaldehyde (PFA)	Fluka
Phenylmethylsulfonyl Fluoride (PMSF)	Sigma-Aldrich
Phusion High Fidelity DNA Polymerase	Thermo Fisher Scientific
Plates (96-well, 12-well, 6-well, 6-, 10-cm)	Sarstedt
Precision Plus Protein Dual Color Standards	Bio-Rad Laboratories
Protein A/G Beads	Thermo Fisher Scientific/Roche
Puromycin	Carl Roth
Reaction Tubes	Greiner Bio-one
RotiBlock	Carl Roth
Sall	Thermo Fisher Scientific
Serological Pipettes	Sarstedt
SmaI	Thermo Fisher Scientific
Streptavidin-Agarose Beads	Thermo Fisher Scientific
Sulfo-NHS-LC-Biotin	Thermo Fisher Scientific
SuperSignal ECL	Thermo Fisher Scientific
T4 DNA Ligase	Thermo Fisher Scientific
Trypsin-EDTA	Sigma-Aldrich
Tubes	Sarstedt
TurboFect	Thermo Fisher Scientific
Wizard SV Gel and PCR Cleanup System	Promega Corporation
XhoI	Thermo Fisher Scientific

Table 19: List of chemicals, consumables, kits, and their manufacturers

7.4 Equipment

<u>Equipment</u>	<u>Manufacturer</u>
4°C and RT Shaker	GFR/VWR
Bacterial Incubator	VWR
BioRad ChemiDoc™ Touch Imaging System	Bio-Rad Laboratories
BioRad Mini-PROTEAN Tetra Vertical Electrophoresis Cell	Bio-Rad Laboratories
BioRad PowerPac™ HC High-Current Power Supply	Bio-Rad Laboratories
BioRad Trans-Blot® Turbo™ Transfer System #1704150	Bio-Rad Laboratories
Cell Culture Incubators (33°C, 37°C, 39°C)	Thermo Fisher Scientific/Binder
Centrifuges	Eppendorf/VWR
Heat Blocks	Eppendorf
Micropipettes	Eppendorf/Gilson
Microscopes	TissueFaxes/Motic/Zeiss
Nanodrop	Thermo Fisher Scientific
Neubauer Chamber	Assistent
Orbital Shaker	VWR
Thermal Cycler	VWR

Table 20: List of equipment, and their manufacturers

7.5 Plasmids

7.5.1 Plasmid maps

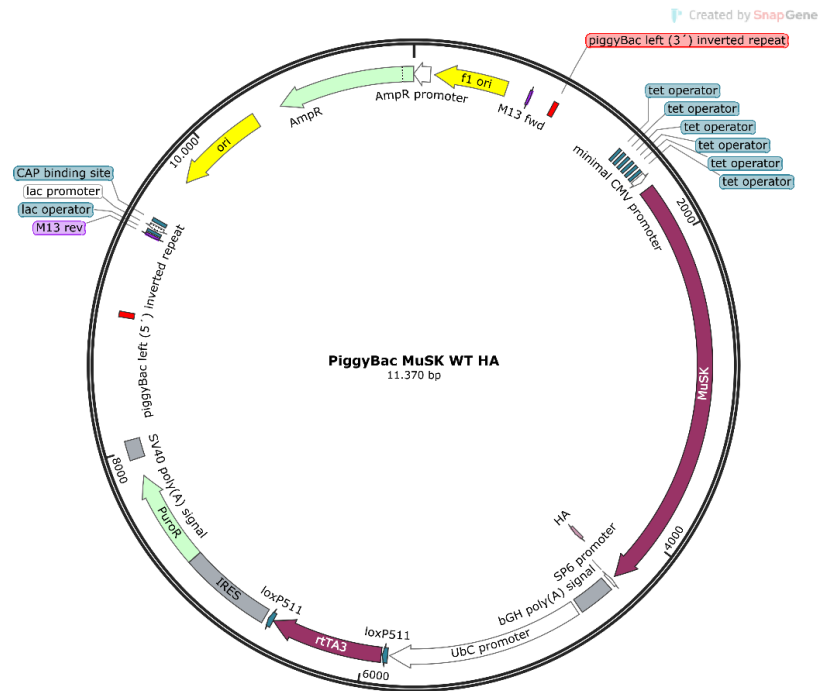


Figure 9: Plasmid map of PiggyBac MuSK WT HA

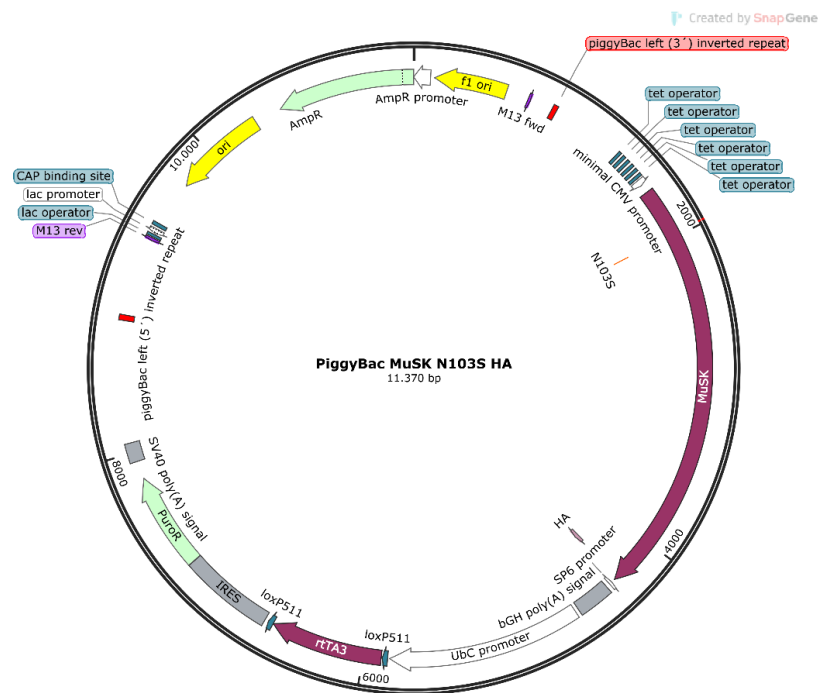


Figure 10: Plasmid map of PiggyBac MuSK N103S HA

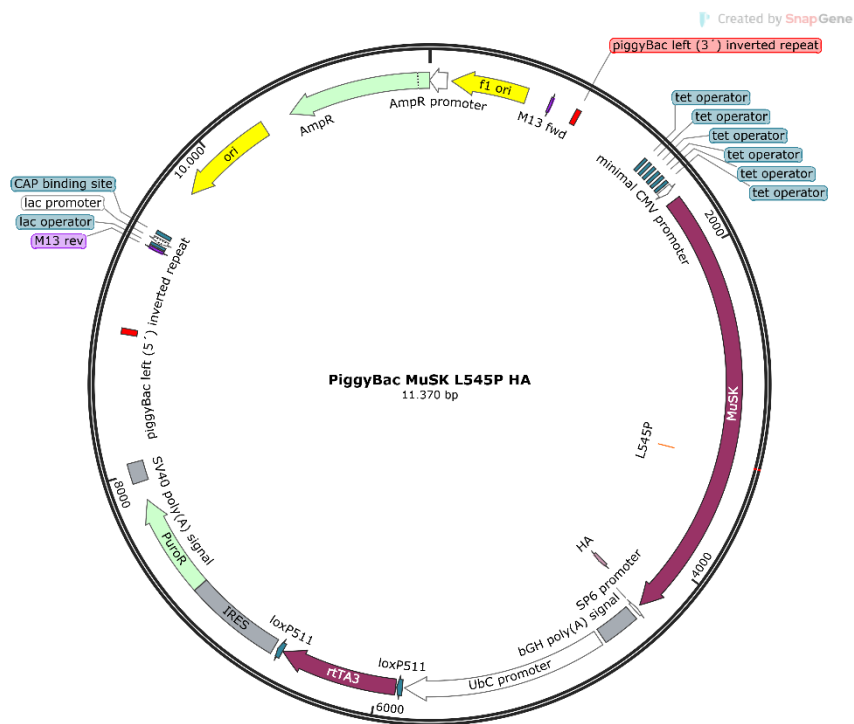


Figure 11: Plasmid map of PiggyBac MuSK L545P HA

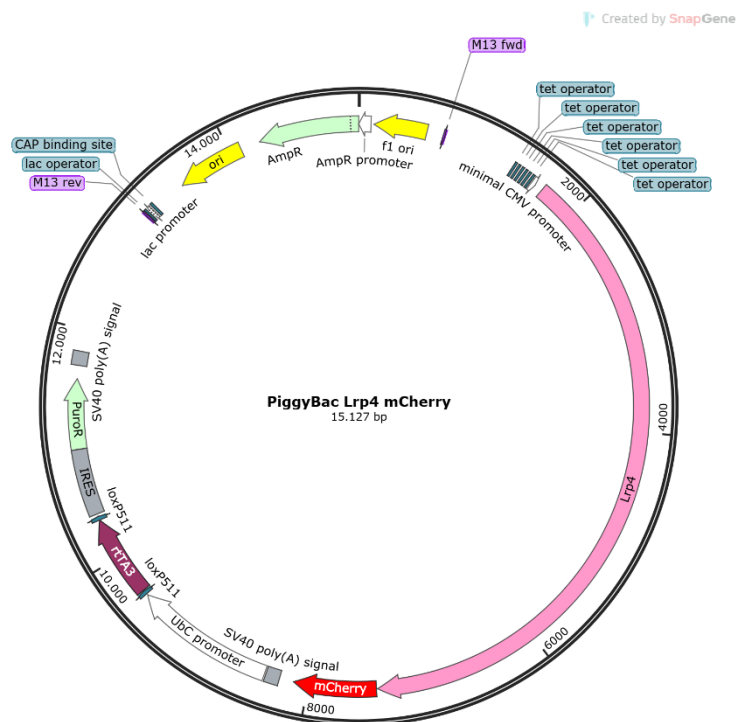


Figure 12: Plasmid map of PiggyBac Lrp4 mCherry



Figure 13: Plasmid map of pLXSG Empty

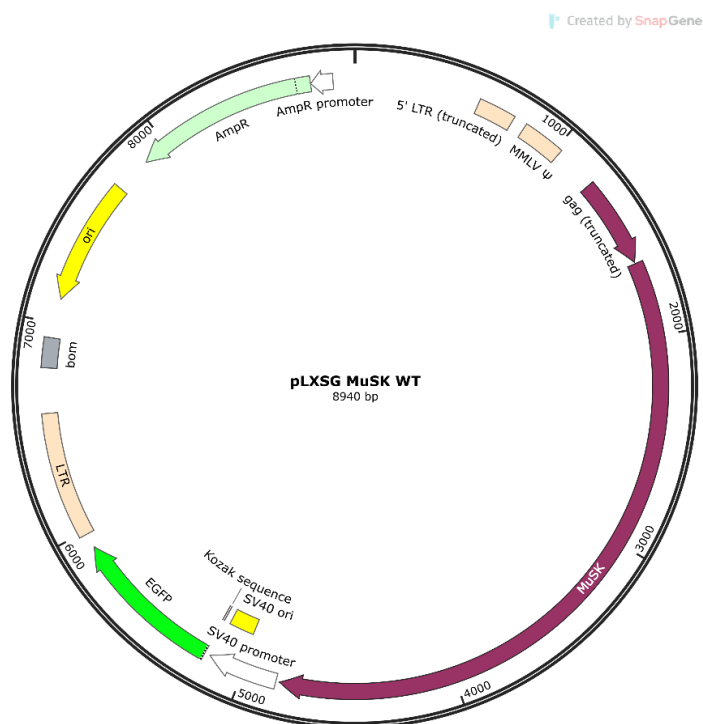


Figure 14: Plasmid map of pLXSG MuSK WT



Figure 15: Plasmid map of pLXSG MuSK N103S



Figure 16: Plasmid map of pLXSG MuSK L545P

7.5.2 List of plasmids

Full name of plasmid	Source	Insert	Used name
PB TRE MCS Ubqc rtta Ires	Herbst Lab	-	PiggyBac
PiggyBac Transposase	Gift from Julia Arand	-	Transposase
PiggyBac MuSK WT HA	This thesis	MuSK WT-HA	WT-HA
PiggyBac MuSK N103S HA	This thesis	MuSK N103S-HA	N103S-HA
PiggyBac MuSK L545P HA	This thesis	MuSK L54P-HA	L54P-HA
PiggyBac Lrp4-mCherry	This thesis	Lrp4-mCherry	PB Lrp4-mCherry
pLXSG Empty	Gift from Kewal Ramani	-	Empty
pLXSG MuSK WT	Herbst Lab	MuSK WT	MuSK WT
pLXSG MuSK N103S	This thesis	MuSK N103S	MuSK N103S
pLXSG MuSK L545P	This thesis	MuSK L545P	MuSK L545P

Table 21: List of plasmids used

7.6 Primers

Primer	Sequence
MuSK EcoRF	ATATGAATTCGCTAGCATGAGAGAGCTC
MuSK SnaBI EcoRI F	TAAATACGTAGAATTCAAACAGTCATTAGTGGACGACTCTATTG
MuSK Xho/Sal R	TTTACTCGAGTCGACCTTAGACGCCTACCGTTCCC
PB MuSK T-MluI-Rev	AAAACGCGTCTTTCCGCCTCAGAAGC

Table 22: List of primers used

8. Results

The aim of this thesis was to contribute to the understanding of myasthenia gravis and congenital myasthenic syndromes. Specifically, (1) a novel method for the detection of Lrp4-positive myasthenia gravis was developed and (2) the effect of two pathogenic MuSK mutations on the formation of AChR clusters in myotubes was analyzed.

8.1 Characterization of HeLa PiggyBac Lrp4-mCherry cell line expressing Lrp4 upon doxycycline induction

For detecting the existence of potential Lrp4 autoantibodies in patient serum samples, HeLa cells that express Lrp4 in a doxycycline-dependent manner were generated.

HeLa cells were co-transfected with a PiggyBac transposase plasmid and the Lrp4-mCherry plasmid (Methods 6.7.1 Generation of HeLa PiggyBac Lrp4-mCherry). After transfection, the cells that have been successfully transfected were selected using the antibiotic puromycin.

After expansion of the selected cells, they were plated and induced with doxycycline to check for total and surface expression of Lrp4 while also trying different conditions to see if the incubation temperature of the cells after induction had any effect on the level of Lrp4 expression. To determine the surface expression living cells underwent surface biotinylation to label the proteins on the surface. After labelling and lysis of the cells, parts of the lysates were used for streptavidin pulldown for analyzing the surface expression of Lrp4.

Figure 17 shows the comparison of surface and total expression levels of Lrp4 in HeLa PiggyBac Lrp4-GFP cell line upon doxycycline induction at 33°C and 37°C. Looking at the surface pulldown blots, it is clear that at 33°C, Lrp4 is more expressed compared to cells at 37°C. Total cell lysate shows higher Lrp4 levels at 37°C compared to 33°C, indicating that more of the Lrp4 that is expressed is stuck on the inside of the cells rather than being transported to the membrane.

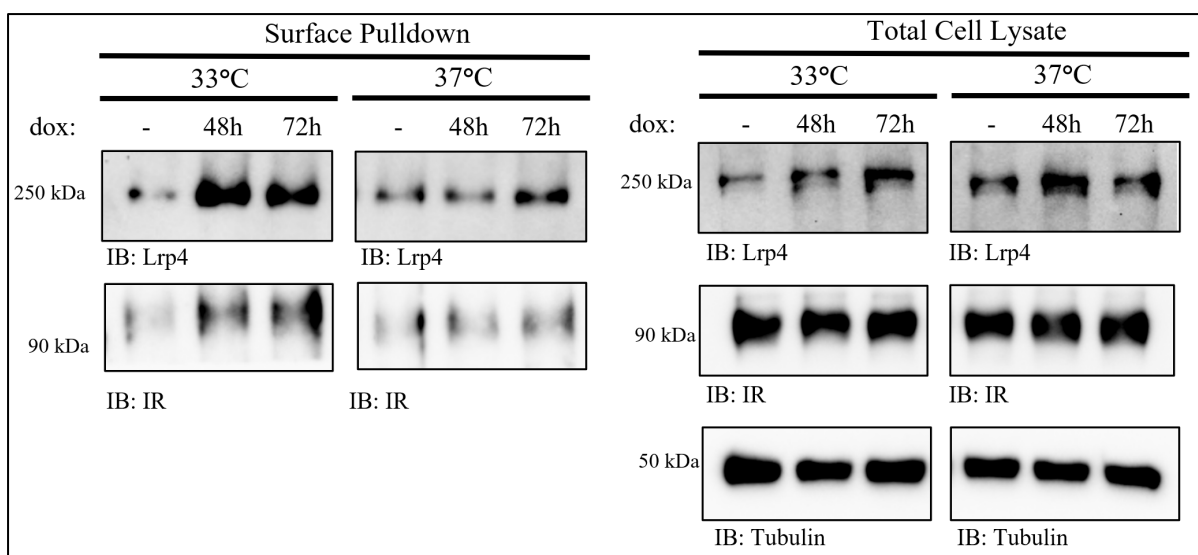


Figure 17: **Comparison of surface and total expression levels of Lrp4 in HeLa PiggyBac Lrp4-GFP cell line at 33°C and 37°C, with 48 and 72 hours of induction.** Western blots showing Lrp4, Insulin Receptor (IR) and Tubulin expressions of cells induced with doxycycline for either 48- or 72 hours and incubated at either 33°C or 37°C. -: No induction. 48h: Induction for 48 hours. 72h: Induction for 72 hours.

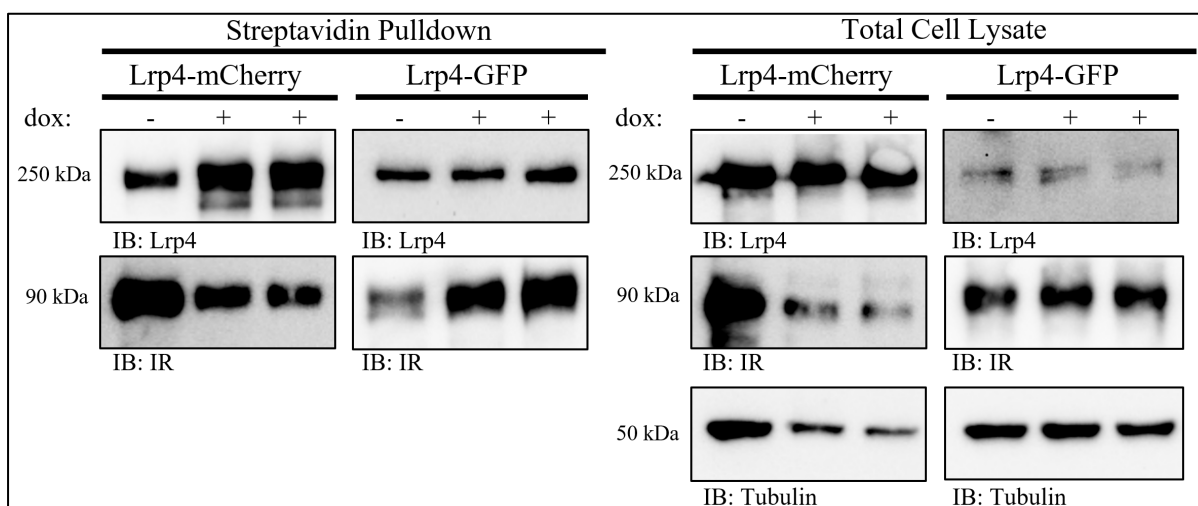


Figure 18: **A comparison of HeLa PiggyBac Lrp4-mCherry and PiggyBac Lrp4-GFP cell lines.** Blots showing Lrp4, Insulin Receptor (IR) and Tubulin expression of HeLa Lrp4-mCherry and HeLa Lrp4-GFP cell lines. Streptavidin pulldown blots indicate the surface expression levels of the respective proteins. -: Without doxycycline induction. +: With doxycycline induction.

The generated cell line HeLa PiggyBac Lrp4-mCherry was shown to successfully express Lrp4-mCherry upon doxycycline induction, as shown in Figure 18. The expressed Lrp4-mCherry was confirmed to be transported to the membrane by surface biotinylation and streptavidin pulldown. Comparing the expression levels of Lrp4-mCherry and Lrp4-GFP, it was seen that the former was expressed more compared to the latter. Insulin receptor detection was used as both a confirmation for the efficacy of the pulldown and as a control for quantifications of the blots.

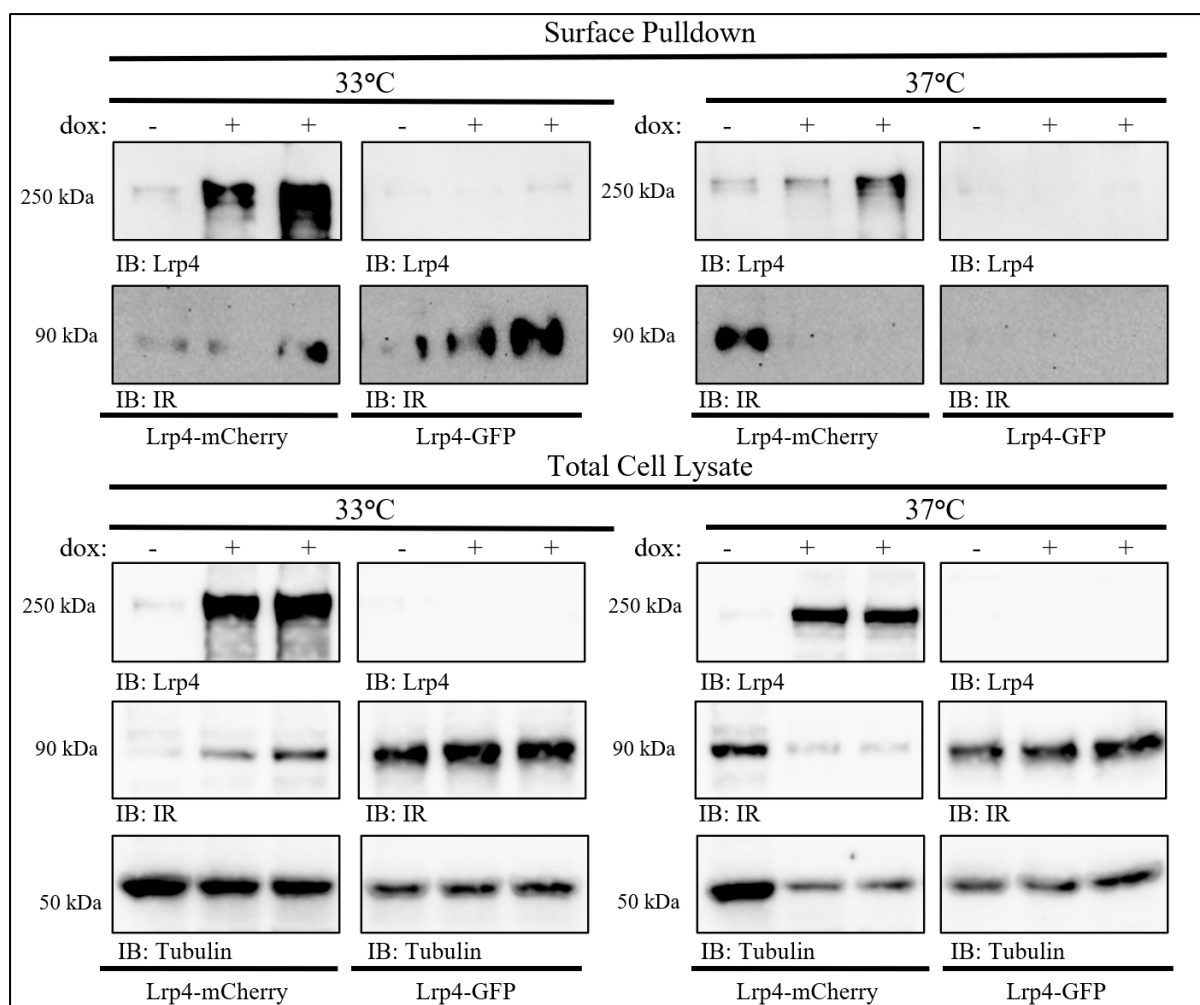
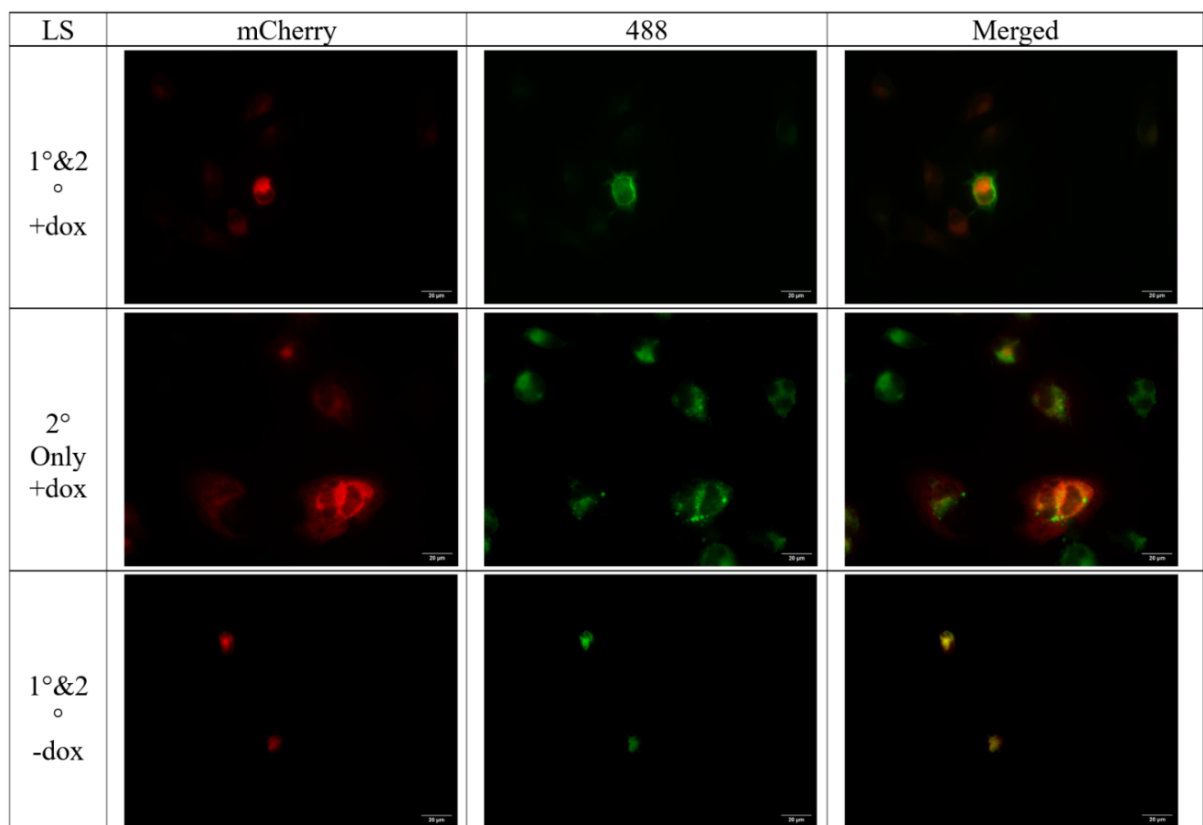
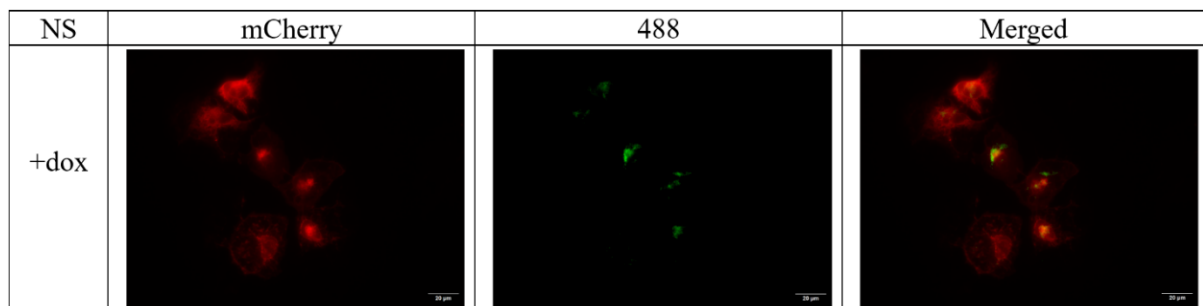


Figure 19: A comparison between HeLa PiggyBac Lrp4-mCherry and Lrp4-GFP cell line at 33°C and 37°C. Western blots done by probing blots with Lrp4, Insulin receptor (IR), and Tubulin for both streptavidin pulldown samples and total cell lysates samples. The HeLa PiggyBac Lrp4-GFP cell line shows clear inability to express Lrp4 both on the surface of the cells and in the total cell lysate samples even with doxycycline induction regardless of the temperature of incubation after induction.

As can be seen in Figure 18 and Figure 19, the newly generated cell line HeLa PiggyBac Lrp4-mCherry expressed Lrp4 more robust compared to the Lrp4-GFP cell line. Also comparing the expression levels of Lrp4-GFP between Figure 18 and Figure 19, the cells have a remarkably decreased ability to reliably express Lrp4 upon doxycycline induction. The HeLa PiggyBac Lrp4-GFP cell line lost its ability to express Lrp4 upon doxycycline induction after reaching passage number 13, thus, they were discarded and not used in any further experiments.

8.2 Analysis of Lrp4 expression in HeLa PiggyBac Lrp4-mCherry

The HeLa PiggyBac Lrp4-mCherry cell line was first used for immunocytochemistry experiments using commercial antibodies as a proof of concept to showcase that staining was possible in terms of differentiating between Lrp4 expression on the surface versus intracellular. For this, three different conditions were used: (1) live staining (LS), (2) staining after fixation of cells (FS), and (3) staining after cell fixation and permeabilization (FPS). The details of the staining processes are explained in the “Methods” section, under the “Immunofluorescence Staining on Coverslips” subsection. As indicated in Figure 20, the stainings included samples stained with primary (1°) and secondary (2°) antibodies in both the presence and the absence of doxycycline induction, as well as control samples, and with only secondary antibodies with doxycycline induction.



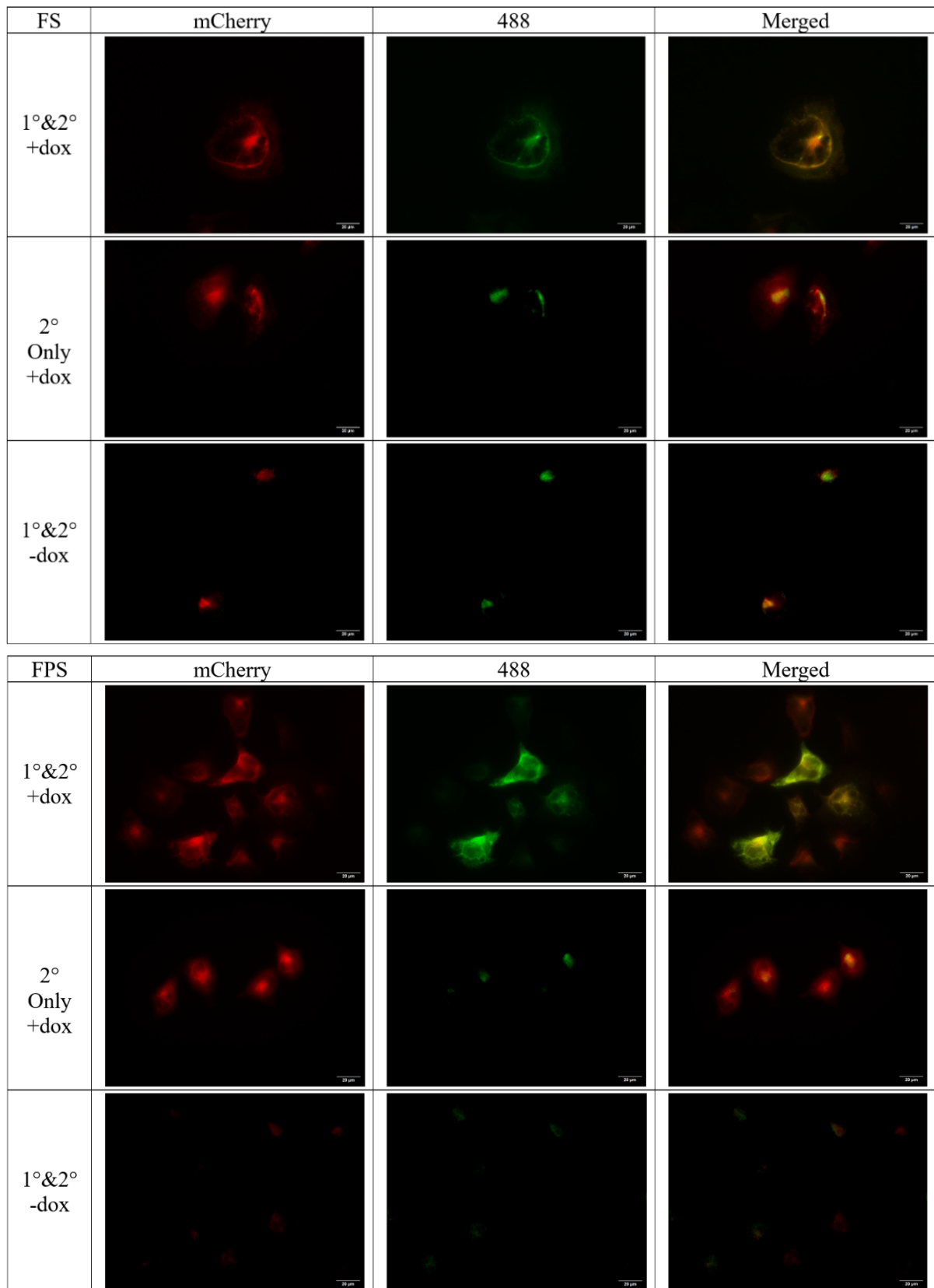


Figure 20: **Immunocytochemistry of HeLa PiggyBac Lrp4-mChery cells with different conditions of staining.** Total Lrp4 expression is monitored via the fused mCherry (red). Surface Lrp4 was detected via antibody against an extracellular Lrp4-epitope followed by secondary antibodies conjugated to Alexa488 (green) **NS**: No staining. **LS**: Live stain. **FS**: Fix and stain. **FPS**: Fix, permeabilize and stain. Scale bar: 20 μ M

Three different methods were used to ascertain which type of staining works best with the cells. Comparing the results, it can be seen that all of the not induced (-dox) samples in all conditions as well as the 488-channel of the “No Stain” and “2° Only” samples had very low signal, indicating that the autofluorescence coming from the cells was very low.

Both in terms of performing the stainings and the results, live staining proved to be the least effective among the three, and since FS and FPS gave similar results compared to each other, the patient sera staining experiment was chosen to be done using these two methods.

8.3 Patient sera analysis for potential Lrp4-positive myasthenia gravis diagnosis

Myasthenia gravis is an autoimmune disease that, as explained previously, is characterized by the presence of autoantibodies targeting proteins of the NMJ. As a diagnostic method for Lrp4-positive MG, a cell-based assay using the generated HeLa PiggyBac Lrp4-mCherry cells was proposed as an improvement to the current methods of diagnoses.

All of the patient sera samples were obtained by the courtesy of Inga Koneczny, samples included both healthy control sera samples as well as potentially Lrp4-positive sera samples. However, none of the samples at hand were 100% positive for Lrp4 autoantibodies that would lead to a confirmed diagnosis of Lrp4-MG and a definitive result for the sensitivity and the specificity of the proposed cell-based assay method of detection.

The immunocytochemistry experiment of detecting autoantibodies described in this section was done by incubating cells both with individual patient serum samples, as well as a control staining, through the use of a commercial Lrp4 antibody (N207/27). This double staining served as a control for the experiment in terms of showing that the staining would work. After staining with the primary antibodies, either patient serum samples or the commercial antibody, the cells were incubated with appropriate secondary antibodies, α -human 488 AlexaFluor for patients and α -mouse Cy5 for the commercial antibody. The coverslips on which the cells were grown on were mounted onto microscope slides using Mowiol 4-88 to be imaged with TissueGnostics TissueFAXS fluorescence microscope.

Figure 21 and Figure 22 shows representative images from the patient sera staining experiments. Experiments were done with $\pm$ doxycycline induction conditions and either permeabilizing or not permeabilizing the cells before stainings were performed, respectively. There are two different patient stainings shown in the figures for both conditions, as well as healthy control. The mCherry column shows the signal from the cells, the 488 column shows the signal coming from the anti-human secondary antibody that was aimed to bind to the potential Lrp4 antibodies found in patient sera samples. The Cy5 column shows the signal of a commercial antibody against Lrp4 used as a positive control.

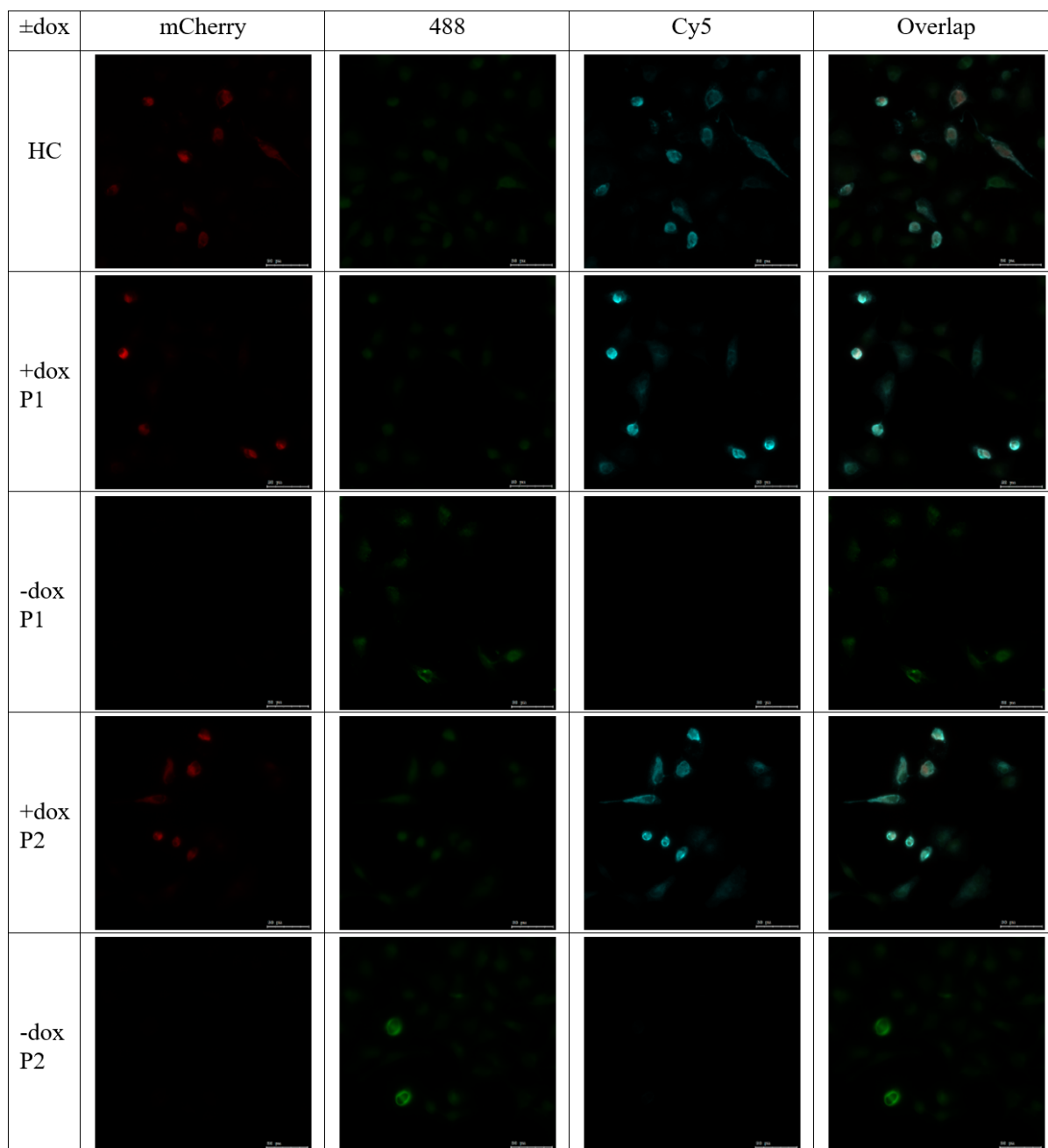


Figure 21: Representative images of patient sera staining using HeLa PiggyBac Lrp4-mCherry cell line with ±doxycycline induction. HC: Healthy control P1: Patient 1 P2: Patient 2. Images show the total Lrp4-mCherry signal in the mCherry column (red), the serum signal detected via α -human secondary antibodies conjugated Alexa 488 (green) and the control staining done with a commercial α -Lrp4 antibody followed by secondary antibodies conjugated to Cy5 (cyan). The overlap column shows the merged images from all three channels. The experiment was set up in a way that the same patient sera samples were investigated both in the presence and the absence of doxycycline induction. Scale bar: 50 μ M.

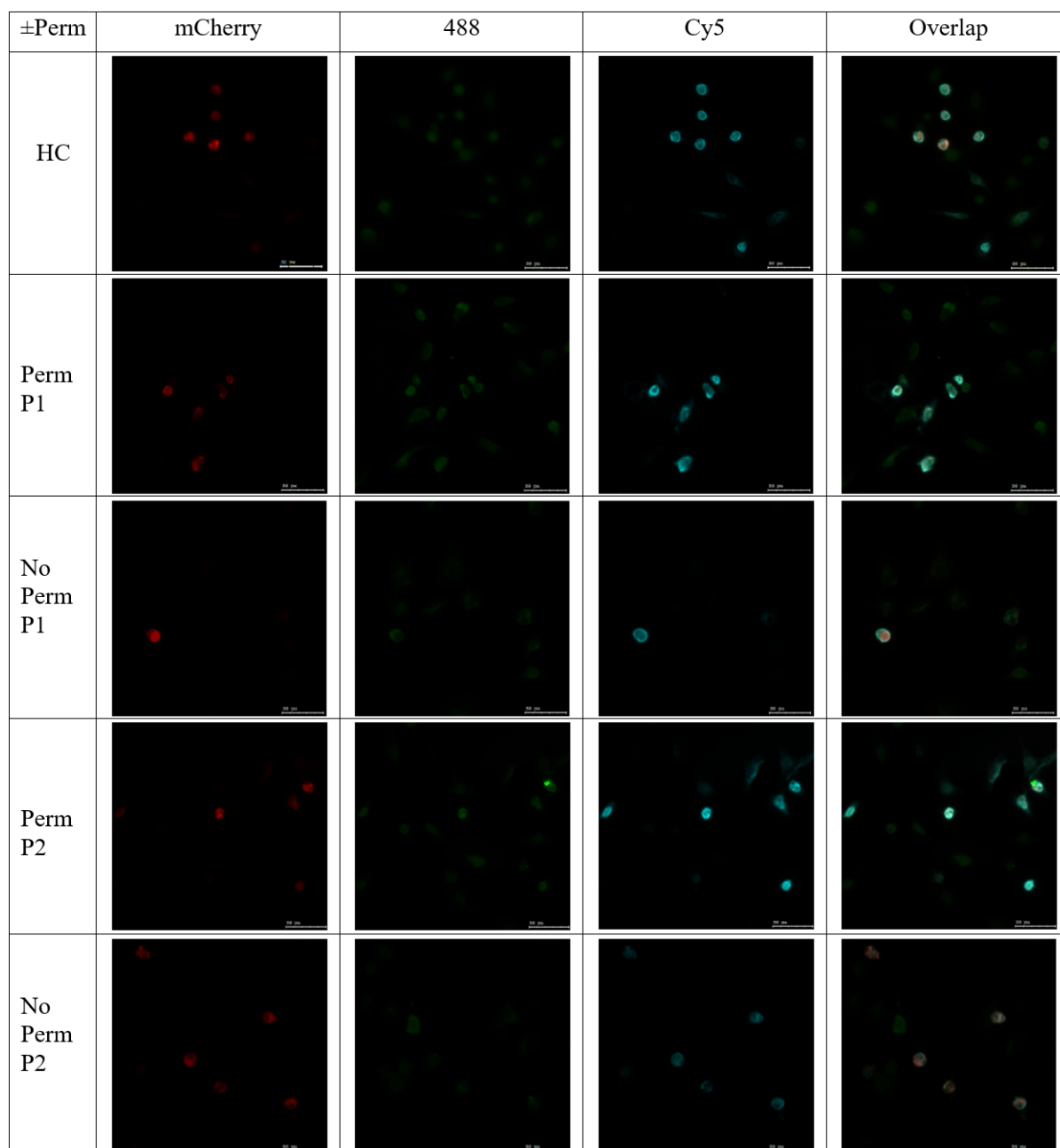


Figure 22: **Representative images of patient sera staining using HeLa PiggyBac Lrp4-mCherry cell line with permeabilization or no permeabilization. HC: Healthy control P1: Patient 1 P2: Patient 2** Images show the total Lrp4-mCherry signal in the mCherry column (red), the serum signal detected via α -human secondary antibodies conjugated Alexa 488 (green) and the control staining done with a commercial α -Lrp4 antibody followed by secondary antibodies conjugated to Cy5 (cyan). The overlap column shows the merged images from all three channels. The experiment was set up in a way that the same patient sera samples were investigated both in the presence and the absence of doxycycline induction. Scale bar: 50 μ M.

A weak patient sera signal, regardless of doxycycline treatment, or their state of permeabilization, was coming from all of the cells on the coverslips. This can especially be observed in the -doxycycline stainings in Figure 21, where there was no mCherry and thus no Cy5 fluorescence, as expected in the absence of doxycycline induction. There was however a clear 488 nm signal even though there was nothing for the potential Lrp4 antibodies in the patients' sera to bind to on the surface of the cells. This issue was not caused by the unspecific

binding of the secondary α -human 488 antibody, as confirmed by the lack of fluorescence when using only secondary antibody (data not shown).

Comparing the different conditions of permeabilization, the Cy5 signal observed on samples with permeabilization was of higher intensity, as expected. This was caused by staining not only the membrane bound Lrp4, but also ones that were still intracellular and not transported to the membrane.

8.4 Analysis of patient sera staining overlap with Lrp4-specific signals under $\pm$ doxycycline and $\pm$ permeabilization conditions

Sample	Cy5-488/Cy5	mCherry-Cy5/mCherry
Healthy Control	0,842%	95,315%
Patient 1 +dox	1,028%	96,832%
Patient 1 -dox	0,676%	5,846%
Patient 2 +dox	0,254%	45,530%
Patient 2 -dox	0,486%	0,959%

Table 23: **Percentage of overlap between channels for $\pm$ doxycycline condition.**

Sample	Cy5-488/Cy5	mCherry-Cy5/mCherry
Healthy Control +perm	0,000%	24,303%
Healthy Control -perm	10,228%	87,198%
Patient 1 +perm	0,687%	64,120%
Patient 1 -perm	6,690%	47,191%
Patient 2 +perm	2,943%	57,428%
Patient 2 -perm	1,017%	44,251%

Table 24: **Percentage of overlap between channels for $\pm$ permeabilization condition.**

Quantification of the images were done as explained in the section “Patient Sera Immunocytochemistry Quantification”. The obtained areas of fluorescent cell overlaps were normalized to either the total area of the Cy5 signal (commercial Lrp4 antibody) or the total area of the mCherry signal (Lrp4-mCherry). This resulted in obtaining percentages of Cy5 positive cells which are also positive for patient autoantibodies, as well as percentage of mCherry positive cells, indicative of Lrp4 expression, which are also Cy5 positive, as a control for the staining. As shown in Table 23 and Table 24, patient sera did not reveal specific Lrp4 staining and was indistinguishable from health controls.

8.5 Investigation of the effect of MuSK N103S & L545P mutations on AChR clustering in MuSK^{-/-} myotubes using transient retroviral transduction

The formation and maintenance of AChR clusters is crucial for proper muscle function. In order to test whether two mutations in MuSK, which have been implicated in CMS play a role in the clustering of AChRs, myoblasts that are deficient in MuSK were retrovirally transduced to express MuSK wildtype, N103S or L545P, differentiated into myotubes and stimulated with Agrin (as explained in the “Methods” section). Following stimulation, myotubes were stained using Alexa-555 conjugated α -bungarotoxin that binds AChRs in an irreversible manner. Images of myotubes were taken with Zeiss 40x objective either all the myotubes within the coverslip were visualized or a minimum of 30 random myotubes were obtained. All of the images taken were manually quantified using ImageJ, quantifying for the area of both the myotubes and the clusters.

For the investigation of congenital myasthenic syndromes, two different cell lines of myoblasts were used to analyze the effects of 2 different MuSK mutations, namely the N103S and the L545P mutations. Both cell lines, one of which was MuSK^{-/-}, hereafter referred as C3 myoblasts/myotubes, and generated using wildtype myoblasts overexpressing various MuSK constructs, hereafter referred as K3 myoblasts/myotubes, have been used to study AChR clustering.

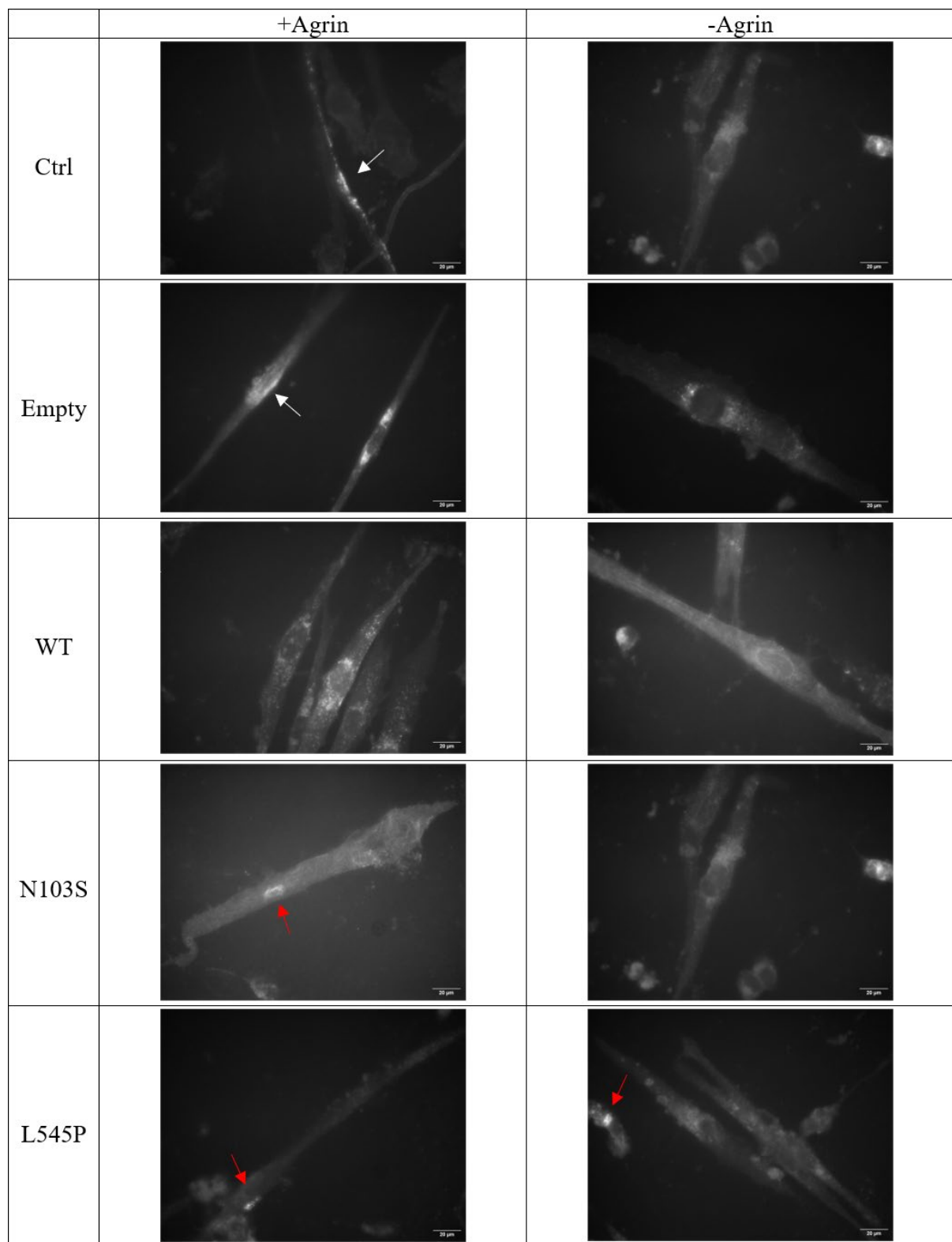


Figure 23: **AChR clustering assay of MuSK deficient C3 myotubes transduced with various MuSK constructs.** **Ctrl:** No infection **Empty:** Infected with viruses carrying no construct. **WT:** Infected with viruses carrying the wildtype MuSK construct. **N103S:** Infected with viruses carrying the MuSK N103S construct. **L545P:** Infected with viruses carrying the MuSK L545P construct. Analysis of images were done with ImageJ by manually measuring every myotube and every cluster present in images. The left column indicates Agrin stimulated myotubes and the right column indicates myotubes not stimulated with Agrin. Red arrows indicate the formed clusters, white arrows indicate background staining. Scale bar: 20 μ m

Figure 23 shows myotubes transiently transduced with MuSK variants, WT, N103S or L545P and stained for AChR clusters. Myotubes formed very few and small patches that were clearly recognized as AChR Clusters (red arrows). Many myotubes demonstrated microclusters (control and WT, +Agrin) or diffuse intracellular background staining (white arrow). Of note, myoblasts differentiated very poorly into myotubes. The red arrows in the images are the clusters that have formed on the myotubes in either the presence or the absence of Agrin stimulation.

Two box plots showing the number of AChR clusters per 10.000 μm^2 myotube area can be seen in Figure 24. The left panel shows the experiments conducted with Agrin stimulation, and the right panel shows the experiments conducted without Agrin stimulation.

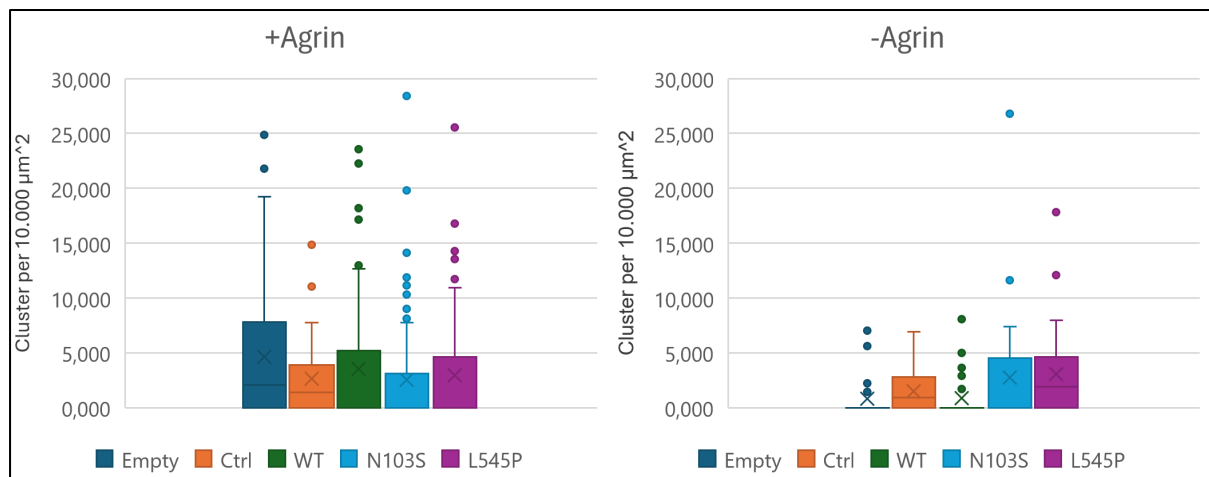


Figure 24: Graphs showing the results of AChR clustering assay of C3 myotubes transiently transduced with various MuSK variants. Empty: Infected with viruses carrying no construct. **Ctrl:** No infection. **WT:** Infected with viruses carrying the wildtype MuSK variant. **N103S:** Infected with viruses carrying the MuSK N103S variant. **L545P:** Infected with viruses carrying the MuSK L545P variant. Calculations were done by dividing the total number of clusters in every image by the total area of the myotubes in the same image and multiplying by 10.000 to get the number of clusters per 10.000 μm^2 .

Number of individual images:

+Agrin: Empty: 85 Ctrl: 29 WT: 77 N103S: 104 L545P: 103

-Agrin: Empty: 20 Ctrl: 28 WT: 32 N103S: 24 L545P: 30

Under +Agrin condition, all variants except the mutants showed higher clustering numbers compared to the -Agrin condition, consistent with the Agrin being the key component in the clustering of AChRs. Although the mean number of clusters formed under +Agrin condition was lower in the mutant variants compared to the wildtype, the difference did not achieve significance in two tailed t-Test ($p > 0.05$). In the -Agrin condition, where wildtype resulted in very few clusters, the two mutant variants formed more clusters compared to the +Agrin condition. As mentioned above, the reason for the discrepancy in the number of images obtained may be caused by the cells not being able to differentiate.

8.6 Generation and characterization of myotubes stably overexpressing wildtype, N103S or L545P MuSK

K3 wildtype myotubes that overexpress wildtype-HA, N103S-HA or L545P-HA MuSK in an inducible manner, were generated by co-transfecting the PiggyBac transposase together with the appropriate construct of MuSK in order for the construct to get integrated into the genome of the cells. After selecting for the cells that have successfully integrated the new MuSK construct into their genomes using puromycin, they were plated, differentiated, and induced with doxycycline. To determine the optimal duration of induction, all 3 generated cell lines were first tested with 48 and 72 hours of induction before lysis and analysis, as can be seen in Figure 25.

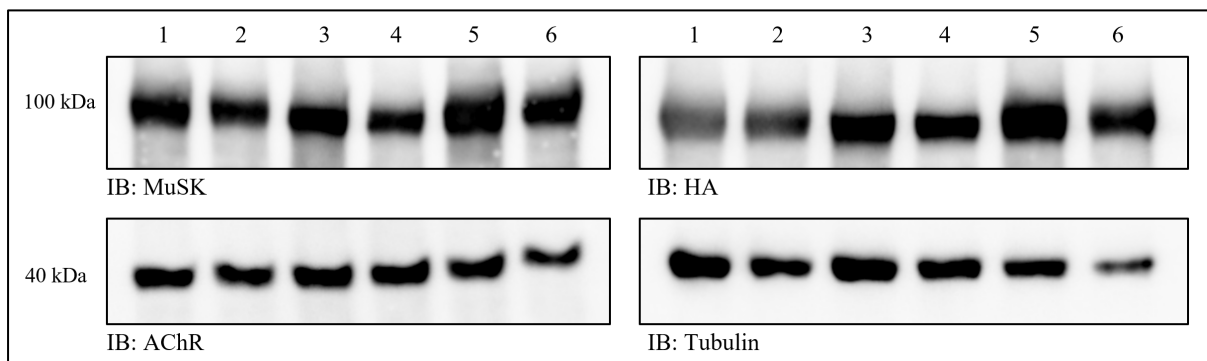


Figure 25: MuSK expression in generated cell lines: K3 PiggyBac Wildtype HA, N103S HA, and L545P HA. The myoblasts were differentiated into myotubes, and induction with doxycycline was performed either 48 or 72 hours before lysis and Western blotting. **1:** K3 PiggyBac Wildtype HA 48h. **2:** K3 PiggyBac Wildtype HA 72h. **3:** K3 PiggyBac N103S HA 48h. **4:** K3 PiggyBac N103S HA 72h. **5:** K3 PiggyBac L545P HA 48h. **6:** K3 PiggyBac L545P HA 72h.

When the blots were probed with an anti-MuSK antibody, the resulting signal was the combination of both the endogenous MuSK that was produced normally by the cells and the overexpressed, HA-tag containing MuSK. Whereas when the blot was probed using an HA antibody, it only showed the overexpressed MuSK as opposed to the combination of both. Comparing lanes between the 48- and 72-hour samples within each cell line, there was clear indication that the expression levels of MuSK and MuSK-HA decrease as the duration of induction increases from 48 to 72 hours. This is caused by the toxicity of doxycycline. Prolonged exposure caused cells to die, shown by the decrease in tubulin signal. Taking this observation into account, further experiments were performed with 48 hours of induction.

To add to these findings, the morphology and number of myotubes also showed a difference between the different induction durations, supporting the observed toxicity of doxycycline on cells during the 72 hours of induction. When the cells have been induced for 72 hours, there

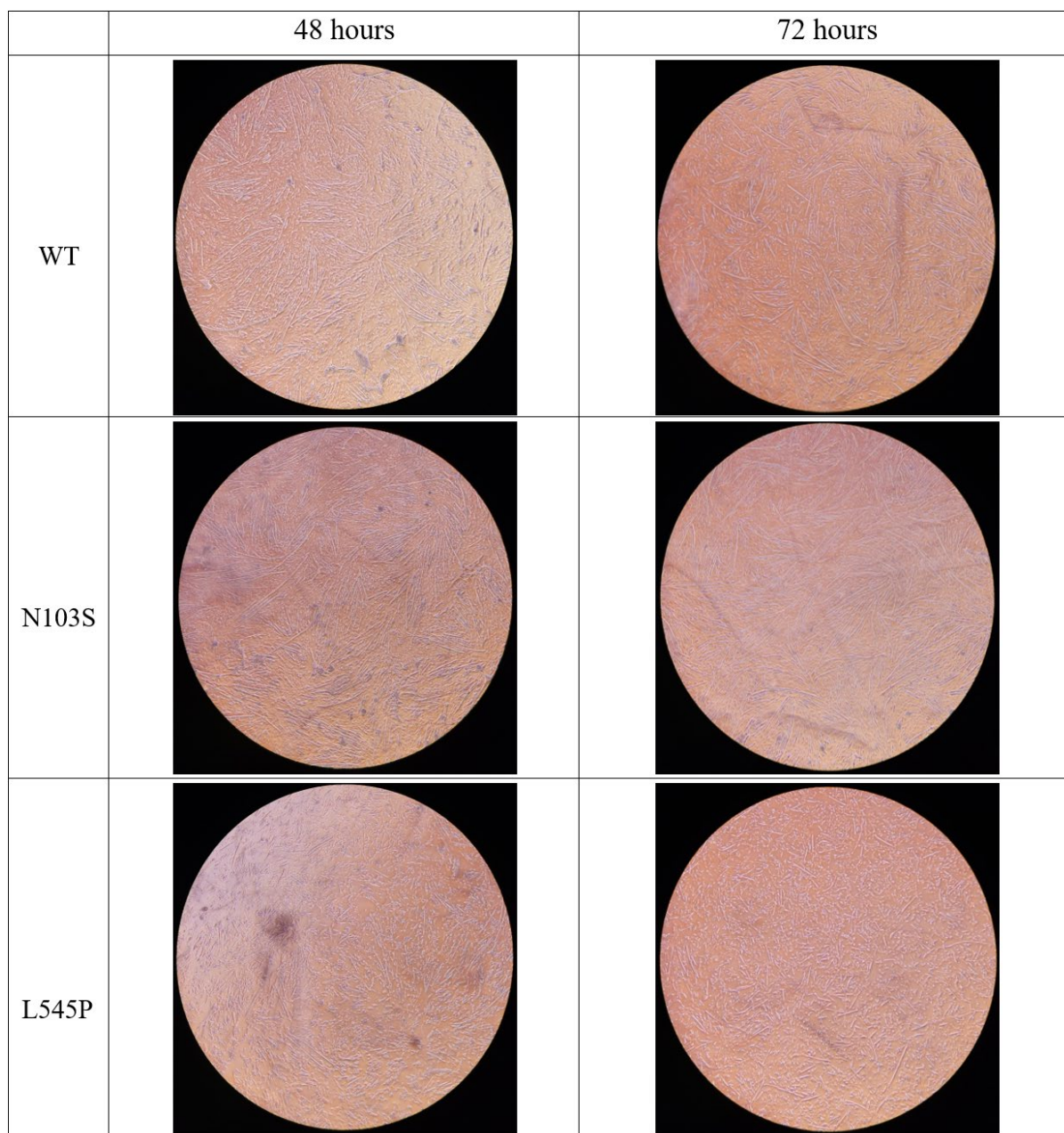


Figure 26: **Light microscope images from K3 PiggyBac Wildtype HA, N103S HA and L545P HA morphologies.** The images show the differing numbers and morphologies of the myotubes at the day of lysis for both 48- and 72-hour doxycycline induction durations.

were fewer myotubes observed in the plates with more empty space around the myotubes present, as can be seen in Figure 26.

After the ideal induction time was determined, considering the toxicity of doxycycline on the cells, tests were performed to determine the optimal doxycycline concentration for induction. 10, 100 and 1000 ng/mL concentrations were chosen to be tested by lysis and analysis by Western blotting.

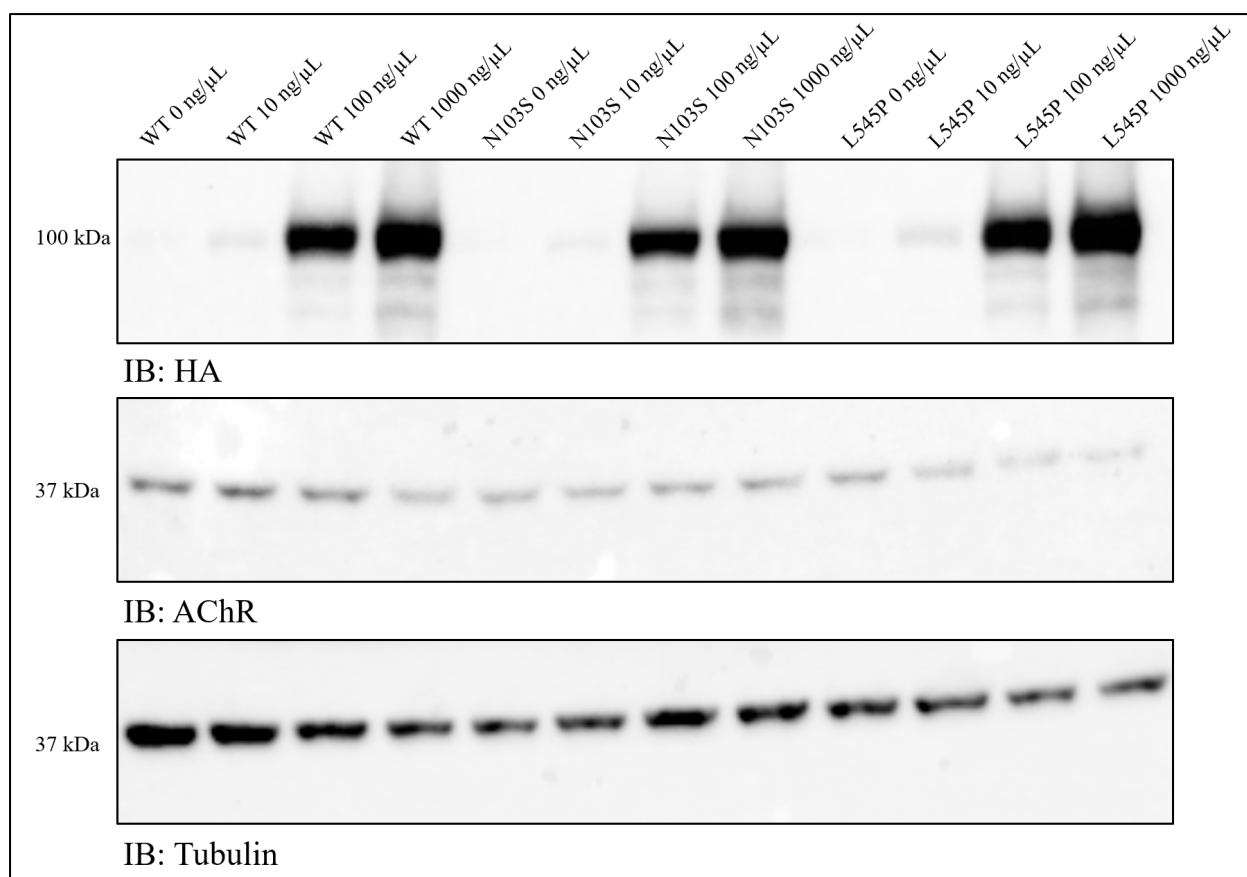


Figure 27: **MuSK expression upon doxycycline concentration gradient of K3 PiggyBac Wildtype HA, N103S HA and L545P HA.** 0, 10, 100 and 1000 ng/μL concentrations of doxycycline has been used to assess expression levels of the different MuSK variants through 48 hours of induction, followed by lysis and Western blotting. The blots were probed using anti-HA, anti-AChR and anti-Tubulin antibodies.

The trend for overexpression of MuSK was the same between all three of the generated cell lines, as expected with no induction, there wasn't any expression of ectopic MuSK. 10 ng/mL induction with doxycycline was not enough to overexpress MuSK-HA at a satisfactory level, whereas starting from 100 ng/mL concentration, expression became quite high and highest with the 1000 ng/mL concentration. However, taking into account the toxicity that was observed at 1000 ng/mL concentrations and the marginal increase in the expression of MuSK-HA, further experiments were conducted with 100 ng/mL concentration for induction.

8.7 Investigation of MuSK phosphorylation in myotubes overexpressing MuSK mutants N103S or L545P

Phosphorylation of MuSK on its various tyrosine residues is crucial for the activation and function of the protein, as discussed in detail in the "Introduction" section. To see whether the mutations have any effect on the phosphorylation of MuSK, MuSK activation in response to Agrin was tested in K3 PiggyBac MuSK Wildtype HA, N103S HA and L545P HA cell lines. The cells were plated, differentiated and induced using 100 ng/mL doxycycline for 48 hours, as deemed optimal from experiments described above, and lysed. The lysates were divided in order to separately immunoprecipitated MuSK using anti-HA antibodies and anti-MuSK antibodies. The samples that were immunoprecipitated using the anti-HA antibodies pull down

only the overexpressed ectopic MuSK construct because of the HA-tag, and the MuSK antibodies pull down only the endogenous MuSK since the epitope that the antibodies bind is blocked because of the HA-tag in the overexpressed construct. The blots were probed against phospho-Tyrosine (pY), MuSK, AChR and GAPDH (Figure 28).

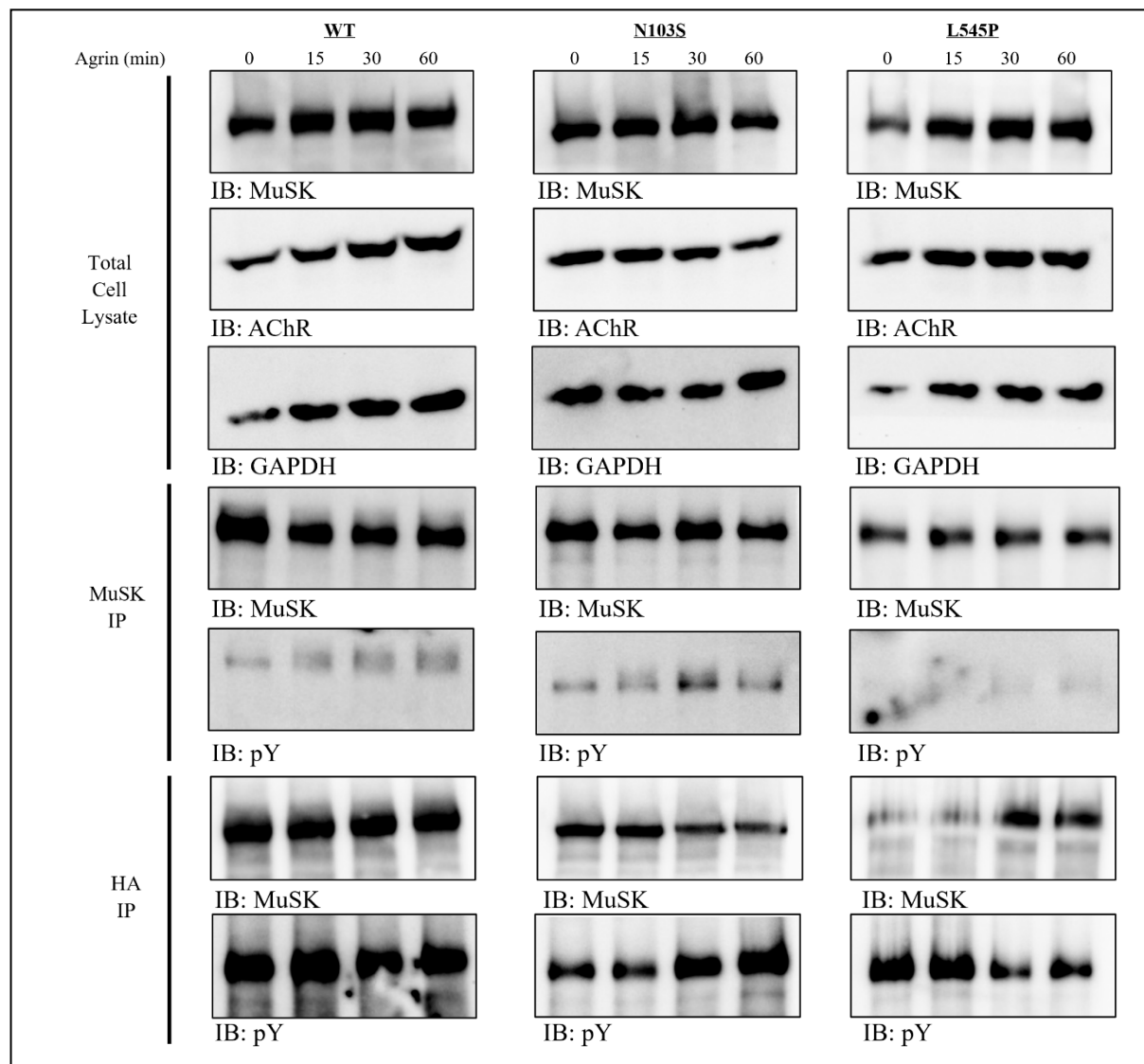


Figure 28: **Phosphorylation of Wildtype HA, N103S HA and L545P HA overexpressed in K3 muscle cells.** Blots show both total cell lysate and immunoprecipitation samples probed with anti-MuSK, anti-AChR, anti-GAPDH, anti-HA and anti-pY antibodies. Blots corresponding to samples of total cell lysates, MuSK immunoprecipitation or HA immunoprecipitation are marked appropriately on the left side of the blots. The left column indicates the blots of Wildtype HA, middle column indicates N103S HA, and the right column indicates L545P HA.

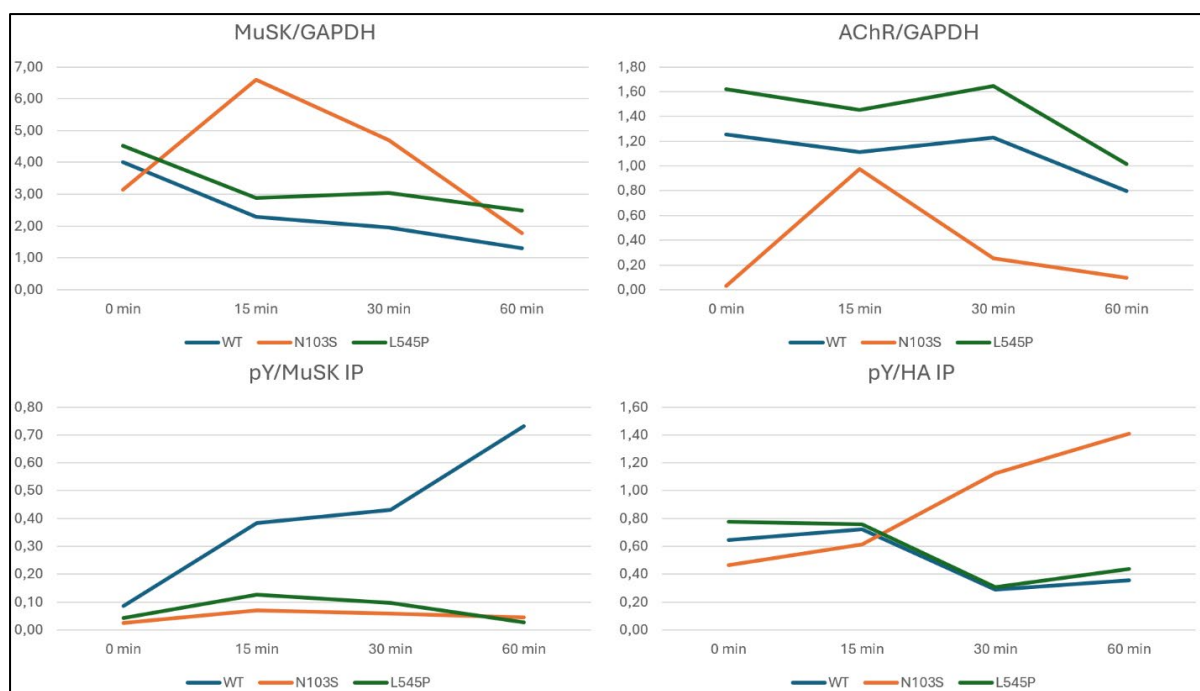


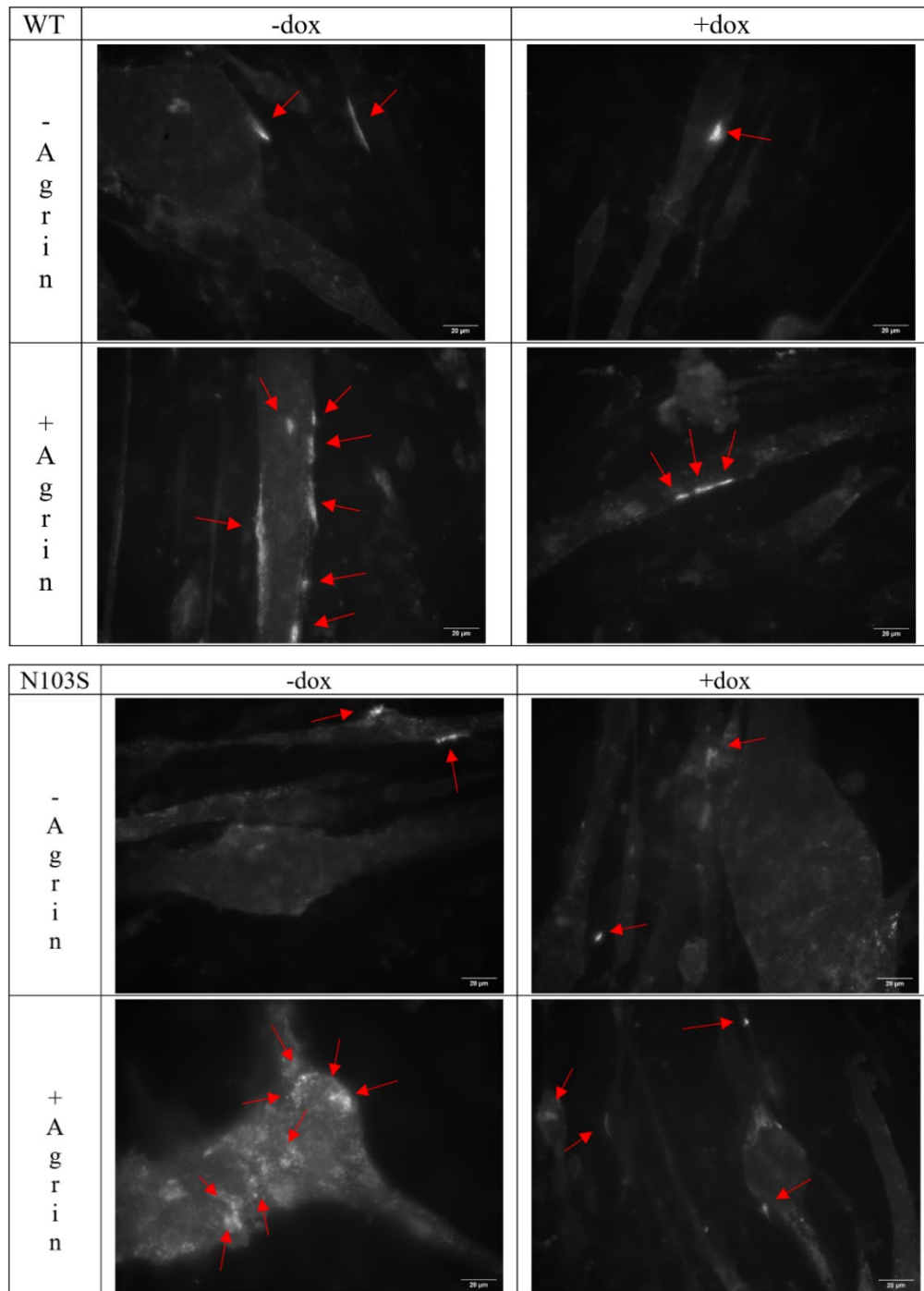
Figure 29: Quantification of phosphorylation from Western blots. The quantifications of pY (phospho-Tyrosine) blots were done using the corresponding MuSK blot. The pY signal coming from the HA IP were normalized to the MuSK blot from the HA IP, the same is for the pY signal of the MuSK IP. The quantification of the signals was done using Bio-Rad Image Lab 6.0 software. Due to some unquantifiable lanes in the blots, the calculations were done using the average of two experiments following the same protocol.

The quantification of MuSK phosphorylation was done by averaging the signal from two sets of experiments with the unquantifiable lanes omitted. Looking at the phosphorylation graphs in Figure 29, the different MuSK variants, overexpressed and endogenous, have different phosphorylation kinetics. The quantifications of pY graphs were done by normalizing the pY signal by its respective immunoprecipitation blot. Comparing between the two IP's, it was observed that for the case of MuSK IP, where only the endogenous MuSK is pulled down, WT shows the highest total phosphorylation, then the L545P variant, followed close by the N103S construct. The phosphorylation peaks, however, are observed to be different, where the peak of phosphorylation is reached at 60 minutes for the WT, and at 15 minutes for both the N103S and the L545P construct. The overexpressed MuSK variants, on the other hand, shows peak phosphorylation at different time points. The peak of WT-HA is seen to be occurring at 15 minutes, the N103S-HA has its peak at 60 minutes, and the L545P-HA has the most phosphorylation when not treated with Agrin.

However, given that there were weak and unquantifiable lanes in the blots, e.g. HA IP blot of L545P, the results should be treated with caution.

8.8 Analyzing the effect of MuSK N103S & L545P mutations on AChR clustering

MuSK activation regulates AChR clustering. Thus, an AChR clustering assay was performed using the generated cell lines, K3 PiggyBac Wildtype HA, N103S HA and L545P HA. Because of the overexpression of MuSK in the myotubes, it is expected that the expressed variants will act dominantly over the endogenous MuSK expressed in K3 myotubes.



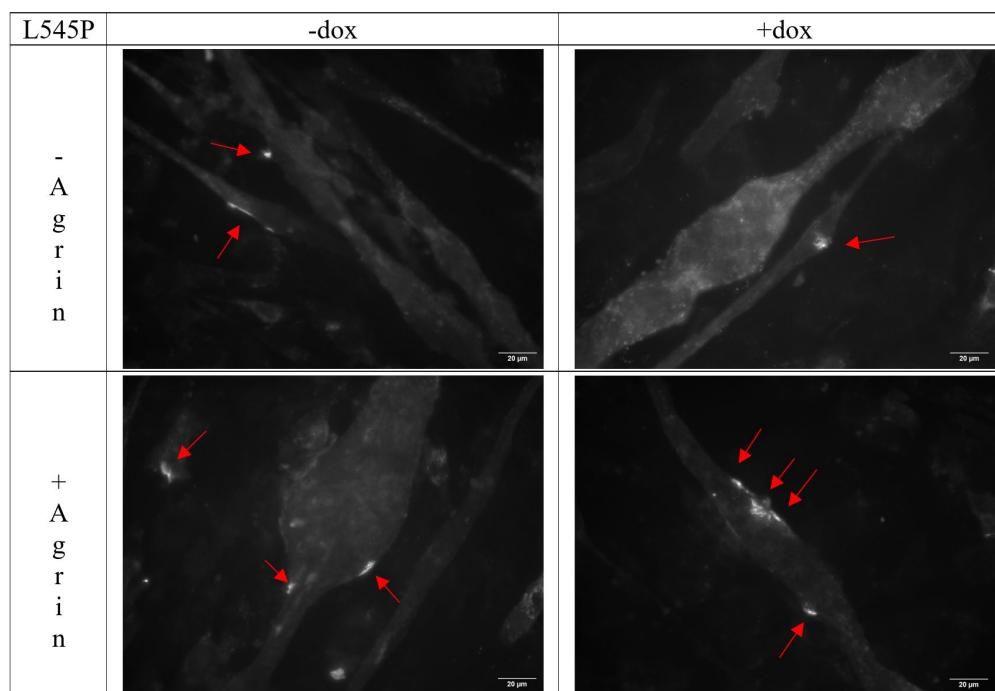


Figure 30: AChR clustering of K3 PiggyBac Wildtype HA, N103S HA and L545P myotubes with various conditions. The myoblasts were differentiated into myotubes, induced for 48 hours with doxycycline and stimulated with 10 ng/mL Agrin overnight, the day before staining. The staining was done using Alexa-555 conjugated α -bungarotoxin after fixation of myotubes. Left columns show -doxycycline, right columns show +doxycycline, and top rows show -Agrin, bottom rows show +Agrin. The analysis for AChR clusters was done with ImageJ using the C2 Beeson macro (Rodríguez Cruz et al., 2020). Scale bar: 20 μ m

Myoblasts were plated and differentiated as explained in the section “Myotube Differentiation”. 48 hours before AChR staining was to be performed, 100 ng/mL final concentration of doxycycline was added to the cell in order to promote overexpression. One day before the staining was performed, the differentiated myotubes were stimulated with 10 ng/mL Agrin overnight. The staining was performed as explained in the “Acetylcholine Receptor Clustering” section.

Based on the results of the AChR clustering assay shown in Figure 30 and Figure 31, it can be seen that the addition of doxycycline, independent from the presence of Agrin, decreases the number of clusters formed on the myotubes. This is observed in all conditions, except when doxycycline treatment was applied with Agrin stimulation for the L545P mutants, where the number of AChR clusters per 10.000 μ m² has increased. Addition of Agrin on the other hand, increases the number of AChR clusters formed, independent of the presence of doxycycline, highlighting the importance of the presence of Agrin as the driving force in the formation of clusters in these conditions for all three of the cell lines. Statistical analysis (two-tailed t-Test) revealed that there was no significant difference among any of the tested conditions ($p > 0.05$).

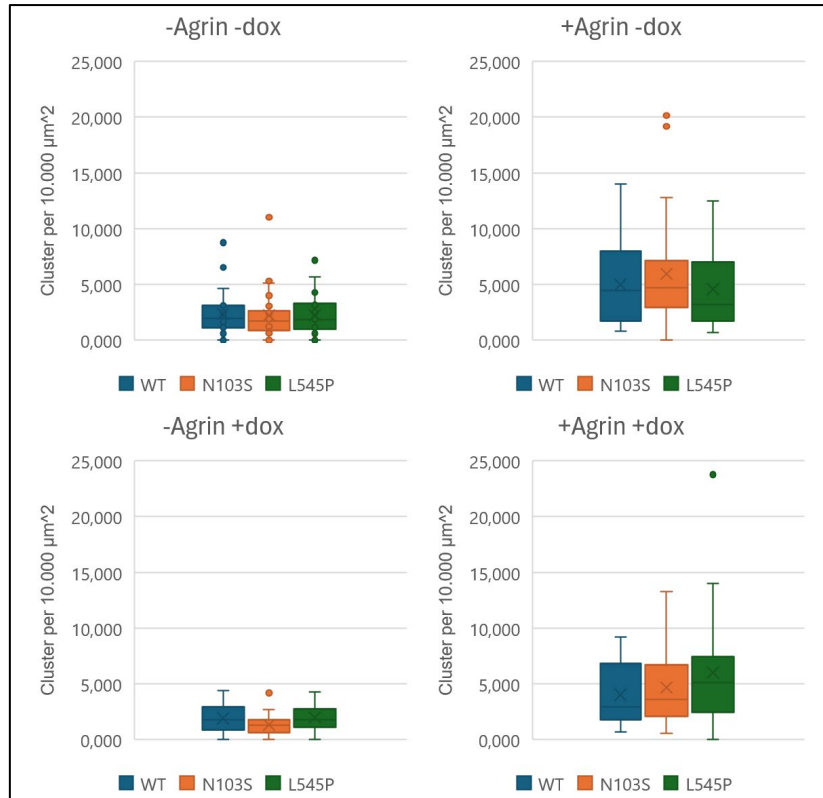


Figure 31: **Quantification of AChR clustering assay of K3 PiggyBac Wildtype HA, N103S HA and L545P HA.** Calculations were done by dividing the total number of clusters in every image by the total area of the myotubes in the same image and multiplying by 10.000 to get the number of clusters per 10.000 μm^2 .

9. Discussion

This thesis is comprised of two parts, the first part aimed to develop a cell-based assay for the detection of Lrp4-positive myasthenia gravis (MG). For this a generated doxycycline inducible cell line that expresses Lrp4-mCherry on the cell surface was generated. This expression of Lrp4 on the cell surface enables stainings with patient serum samples to be analyzed using fluorescence microscopy to determine whether the sample in question is positive or negative for MG.

The second part of the thesis focused on congenital myasthenic syndromes (CMS), specifically the N103S mutation in the extracellular Ig1 domain, and the L545P mutation in the juxtamembrane region of MuSK. Investigation included analyzing the impact the mutations have on the formation of acetylcholine receptor (AChR) clusters, and the activation levels of MuSK when stimulated with the neuronally derived heparan sulfate glycan Agrin.

9.1 Detection of Lrp4-positive MG using HeLa PiggyBac Lrp4-mCherry

Lrp4 autoantibodies that result in MG have been discovered recently and the condition is a rare disease (Higuchi et al., 2011). It is important to differentiate between subtypes of MG to better tailor a treatment plan since there is not a universal treatment option for all subtypes. The issue with diagnosing Lrp4 MG is that to date, there is no method of reliably diagnosing the subtype. Currently, enzyme-link immunosorbent assay (ELISA) or cell-based assays (CBAs) are used for testing for Lrp4-MG, and there are only two commercially available test for CBA, manufactured by Neurocode, USA and Athena Diagnostics, USA. Between ELISA and CBAs, the latter is more sensitive while having comparable specificity. The CBAs used to date have always been done with HEK293 cells in a transient manner where before a sample is tested, cells need to be transfected. Permeabilization is not recommended, though some studies have chosen to utilize it (Pevzner et al., 2012), as it decreases the sensitivity of the test by potentially detecting intracellular Lrp4 which is misrepresentative of the disease conditions. These introduce complexity, potential failure points and experimental variations such as efficiency of transfection and false positives. Concerns about the sensitivity and specificity have been declared by several researchers (Heber & Li, 2023; Racke et al., 2022)

To establish an improved CBA for the diagnosis of Lrp4 autoantibodies, we used an approach whereby HeLa cells expressing tetracycline-inducible Lrp4-mCherry were successfully generated. The expression of Lrp4-mCherry on the cell membrane was confirmed by both fluorescence microscopy and biochemically using surface biotinylation. Surface biotinylation was performed to confirm that the expressed Lrp4 was transported to and present on the cell membrane rather than being stuck intracellularly.

The concept of Lrp4-surface staining was confirmed by using commercial antibodies against the extracellular region of Lrp4, meaning that testing patient sera samples should also be possible. However, the test between healthy controls and patient sera revealed that none of the samples were positive for Lrp4-MG (Figure 21 and Figure 22), the calculation of overlap between the channels resulted in the same conclusion. The measurements of the cells with

fluorescent signal were done with ImageJ, and the calculations were done in Microsoft Excel. The interpretation of results was hampered since none of the patients' sera were validated for the presence of Lrp4 autoantibodies. This made it difficult in terms of differentiating between potential Lrp4-MG positive and negative samples since we had no validated positive control to compare to. Without the inclusion of a known positive control of an Lrp4-MG positive patient sample, it will not be accurate to say whether patient sera testing was successful or not feasible.

Literature is divided in half regarding the efficacy of cell-based assays (CBAs) for the diagnosis of Lrp4-MG. While some studies are not able to find Lrp4 antibodies in double seronegative MG (dSN-MG) patients in their study cohorts (Gallardo et al., 2014; Hoffmann et al., 2023; Klein et al., 2022; Rodríguez Cruz et al., 2015), others revealed that the incidence of Lrp4 antibodies was found to be ranging from 4% of the dSN-MG patients (Li et al., 2017), 8% in both dSN-MG patients and 8% in MuSK seropositive MG patients (Cossins et al., 2012), 14.5% of dSN-MG patients (Marino et al., 2015) and up to 53.8% of the dSN-MG cohort (Pevzner et al., 2012). These different results of the studies indicate the variability of the results based on many factors, such as the size of the cohort tested, the demographic of people since some countries have a higher prevalence compared to others, and the methods used. All the studies that have utilized CBAs for Lrp4-MG diagnosis have used HEK293 cells with transient transfection and some with permeabilization. These data support the need for a reliable and standardized diagnostic assay.

I focused in this thesis on the generation of HeLa cells that expresses Lrp4-mCherry stably upon doxycycline induction on the cell surface. Lrp4 being expressed on the cell surface is a better representative of the disease conditions since it corresponds to the physiologically functional state of the protein, compared to the detection of potentially misfolded and misrepresentative Lrp4 when cells are permeabilized. This problem needs to be overcome by only detecting the surface Lrp4 because the misfolded intracellular Lrp4 might not have right confirmation for the epitope where normally the disease-causing autoantibodies would bind, or the conformational changes might create unspecific epitopes where there will be false positives. When further optimized and confirmed with known positive sera samples, this method has the potential to be a simple and reliable method for diagnosing Lrp4-MG.

Overall, this proposed approach requires more optimization, however, apart from the already mentioned issues such as positive control, after all the issues are fixed it looks promising to be a relatively easy method to test for Lrp4-MG. Comparing between the three different staining methods, that are live staining, staining of fixed and unpermeabilized samples, and finally staining of fixed and permeabilized samples, the latter two are easier to perform, and less prone to handling errors of live staining, so in a diagnostic laboratory setting samples would be suggested to get processed following fixation, but no permeabilization given the issues that might arise from permeabilization.

One drawback of the approach turned out to be a decrease of Lrp4-mCherry expression with increasing passage number of the cells. I observed that the fluorescence intensity of the cells decreased when kept in culture for a long time. For diagnosis it will be important that cells provide a constant diagnostic output. Cells can be regenerated and further enhanced by selecting for high expressers using FACS. With only a co-transfection of the PiggyBac Lrp4-mCherry plasmid and the PiggyBac transposase plasmid, puromycin selection can be performed for 2 days, after which the cells would be ready for testing using doxycycline for

induction, and fluorescence microscopy and western blot for confirmation of Lrp4-mCherry expression.

Interpretation of the results is an important aspect of coming to a conclusion about the diagnosis of a specific sample. I would suggest the results be interpreted by two independent investigators, in a blinded manner and based on a points system, as done in previous research (Gallardo et al., 2014; Rodríguez Cruz et al., 2015; Li et al., 2017; Cossins et al., 2012; Marino et al., 2015; Pevzner et al., 2012). A macro can be created in image processing software, such as ImageJ, to correspond to the results of the investigators in order to streamline the diagnosis process and when in doubt, the samples can be reviewed manually by investigators. Reproducibility of the results is another important aspect for the reliability of this approach and thus, it is important that the cells used do not have diminished Lrp4 expression and should always be checked.

Only a couple of days are required from plating the cells to interpreting the results of fluorescence microscopy images. This, combined with the fact that this is a quick and low-cost method makes it a desirable, viable and feasible option for the detection of Lrp4-MG in a diagnostic setting.

9.2 Impact of congenital myasthenic syndromes causing mutations N103S&L545P in MuSK

CMS are genetic disorders caused by mutations in the proteins of the NMJ. There are many mutations that result in CMS; however, the focus of this thesis was two mutations in MuSK: N103S in the Ig1 domain and the L545P in the juxtamembrane region. Since MuSK plays an essential role in the formation and maintenance of the NMJ, mutations may impair its ability in these processes, leading to clinical symptoms of CMS. It is important to shine light on how these mutations affect the stability and function of the NMJ to better develop therapeutic treatments to combat the disease and potentially counteract its effects. In this thesis, it was hypothesized that the mutations N103S and L545P would impair AChR clustering ability of myotubes, as well as changing the phosphorylation kinetics of the mutants compared to wild type. Considering the locations of the mutations, N103S would impact the interaction between MuSK and Lrp4, whereas the juxtamembrane positioning of the L545P mutation would impact the interaction between MuSK and Dok7.

9.2.1 AChR clustering in MuSK^{-/-} muscle cells transiently expressing wildtype, N103S & L545P MuSK

Since MuSK function is critical for AChR clustering, the efficiency of AChR clustering is a read-out for MuSK activity. Therefore, a change in AChR clustering ability of myotubes is correlated with the function of MuSK. If the mutations have an effect on MuSK function, then it would result in observing differences in AChR clustering between WT and mutant MuSK.

The mutations N103S and L545P in MuSK that cause CMS were investigated using transient retroviral transduction of MuSK^{-/-} muscle cells. These cells were obtained from MuSK^{-/-} mice and lack endogenous MuSK expression (Herbst & Burden, 2000). Myoblasts were retrovirally

transduced in a transient manner to express either wildtype, N103S or L545P MuSK. Myotubes were stimulated with Agrin to study their effects on AChR clustering (Figure 23). MuSK^{-/-} myotubes are good model systems to study the role of MuSK mutations during AChR clustering (Herbst & Burden, 2000). However, inconsistencies observed throughout the experiments resulted in not being able to form a clear picture of the effectiveness of the method and the results. Enough replicates could not be obtained for all conditions, so doing statistical analysis on the results to determine whether there are significant differences or not was unfortunately not possible.

A major obstacle throughout these experiments was the failure of myoblasts to differentiate properly, resulting in the death most of the myoblasts instead of fusing to form multinucleated myotubes, and thus the resulting myotubes were shorter, smaller, and thinner than expected. Despite varying conditions such as confluencies at the day of plating, using earlier passages and experimenting with different cell stocks, consistently differentiating myotubes were not achieved. It was established that the reason that they were not differentiating was not the retroviral infection, since the control cells that were not subjected to viral supernatants also failed to differentiate as expected. Figure 23 shows representative images of the mentioned myotubes, and Figure 24 shows the individual evaluation of the images obtained from the analysis of the AChR staining experiments. Discrepancies between the different conditions regarding the number of replicates present is that the number of experiments that could successfully be performed differed between the conditions, caused by the myoblasts' inability to differentiate into myotubes. The graphs in Figure 24 represent a compilation of all of the successfully performed and analyzed experiments.

Comparing the data between the different conditions very clearly demonstrated that the experiments are inconclusive since control myotubes formed more clusters compared to WT. Since control myotubes were not transduced and therefore lack any MuSK expression, there should have been nothing enabling the formation of AChR clusters. Besides problems with differentiation, other technical issues have to be considered. The transduction rates of the myotubes were also not known. During transfection of Phoenix cells, varying percentages of the cells were observed to be expressing GFP, which provides a direct readout of the transfection efficiency of the cells. However, it was not possible to determine the transduction rate of the myotubes. To add to this issue, the expression level of MuSK in the transduced cells was also not determined.

9.2.2 MuSK phosphorylation in wild-type muscle cells overexpressing wildtype-HA, N103S-HA & L545P-HA MuSK

An alternative to the above-described approach was the use of wild-type myoblasts to overexpress mutant MuSK using the inducible PiggyBac expression system. This approach was based on the hypothesis that the overexpression of mutant MuSK would compete with the endogenous MuSK, thereby having a dominant-negative effect.

Phosphorylation of MuSK is a crucial step in its activation. After phosphorylation of Tyr553, Dok7 is recruited to promote dimerization and phosphorylation of further residues such as Tyr750, Tyr754 and Tyr 755. Only when all of these residues are phosphorylated, is MuSK then fully activated. Under experimental circumstances, MuSK has its phosphorylation peak at

15 minutes of Agrin stimulation, and gradually loses its phosphorylation over the course of 4 hours (Camurdanoglu et al., 2016).

To determine the effect of the MuSK mutants on MuSK activation, I studied MuSK phosphorylation in response to Agrin. The experimental setup allowed to separate between the overexpressed MuSK, wildtype, N103S and the L545P mutants, which have an HA-tag on their C-terminals, and endogenous MuSK constructs during immunoprecipitations and Western blots. The HA-tag blocks the epitope where the anti-MuSK antibody binds, further allowing discrimination between endogenous MuSK and the overexpressed MuSK I used this to examine endogenous and exogenous MuSK phosphorylation separately (Figure 28 and Figure 29).

The experiment was done 2 times, and some of the data points revealed technical issues such as unquantifiable lanes in Western blots and the quantification is therefore based on the average of the two experiments performed, without the low-quality data points. The low number of data points, the low signal strength in some data points and technical problems in others might explain the inconsistent phosphorylation kinetics. For instance, endogenous MuSK in WT-HA expressing cells showed a constant increase in phosphorylation during Agrin stimulation whereas endogenous MuSK in N103S-HA and L545P-HA showed little phosphorylation that peaks at 15 minutes. Similarly, the overexpressed MuSK N103S protein revealed different kinetics compared to WT-HA and L545P-HA. Considering the above-mentioned issues, I conclude that the observed differences in the phosphorylation kinetics between the wildtype and the mutant constructs are inconclusive. These results highlight the need for further experimentation with increased replicates and eliminated technical issues to have a better understanding of how the mutations alter the phosphorylation kinetics.

Interestingly, the phosphorylation of the overexpressed MuSK is much stronger than that of the endogenous MuSK. In particular, overexpressed MuSK is strongly phosphorylated even in the absence of Agrin. Since the amount of MuSK expressed increased dramatically, the total phosphorylation also naturally increased caused by the increase in total MuSK expression. The anti-pY antibody used in the experiments is not able to differentiate between which residues of MuSK are phosphorylated between Tyr553, Tyr750, Tyr754 and Tyr755; and the observed phosphorylation of MuSK under no Agrin stimulation can be the effect of having high levels expression. In further experiments, the concentration of doxycycline used for induction can be adjusted to prevent a high level of phosphorylation under no Agrin stimulation while still achieving the desired dominant negative effect.

MuSK dimerizes as it elicits its function, and it would be expected that the endogenous MuSK and the overexpressed MuSK also heterodimerize, however this is not the observed case. If they heterodimerized, we would see much increased phosphorylation in the pY blots of the endogenous MuSK obtained through MuSK immunoprecipitation. It could also be the fact that they heterodimerize, but the ratio between the homodimerization of endogenous MuSK and heterodimerization could be very low. To get to the bottom of this issue, further experiments are suggested to be conducted including no induction conditions, as well as the wildtype myotubes in addition to the generated cell lines to get a better understanding of the ratio between homo- and heterodimerization.

It is currently not possible to come to a conclusion about the results of phosphorylation experiments with the data at hand, and more experiments need to be done to get a clear understanding of how the mutations affect the phosphorylation.

9.2.3 AChR Clustering in wildtype muscle cells overexpressing wildtype-HA, N103S-HA & L545P-HA

MuSK activation induces a signaling cascade resulting in AChR clustering. Based on our hypothesis that overexpressing MuSK would lead to a dominant negative effect on the endogenous MuSK, I analyzed the effects of the mutations on the cells ability to form AChR clusters (Figure 30 and Figure 31). The lowest number of clusters formed with -Agrin and +doxycycline, as opposed to -Agrin and -doxycycline which was the expected case. This could have been caused by the toxicity of doxycycline, where it might also affect the formation of AChR clusters through an unknown mechanism. From the phosphorylation level of mutant MuSK in the presence of Agrin (0 minutes), it would be expected to observe clusters in -Agrin +dox conditions however this was observed to be not the case. Likewise, the highest number of clusters were expected from +Agrin and +doxycycline since presence of Agrin is the driving factor in forming clusters, and the addition of doxycycline would lead to more MuSK production. However, the highest number of clusters were formed with +Agrin and -doxycycline, except for the L545P mutant where they were the highest with +Agrin and +doxycycline. Overall, comparing Agrin stimulated and not stimulated conditions, it is still the presence of Agrin that drives the formation of clusters and not overexpression to a major extent.

In the absence of doxycycline but presence of Agrin, N103S and L545P mutants formed lower number of clusters compared to WT, and the heterodimerization mentioned previously might play a role in this instance given that even with no induction, some expression is to be expected. This result would indicate an impairment in the function of the endogenous MuSK and could be coming from heterodimerization at very low expression of the mutants. However, under overexpression conditions where doxycycline was present, comparable or higher clustering levels could be explained by the compensation through overexpression of MuSK, although the physiological impact of this remains unclear.

9.2.4 Conclusion

Overall, experiments with MuSK^{-/-} muscle cells presented challenges, such as problems in differentiation, reproducibility and reliability. In contrast, the K3 wild-type muscle cells overexpressing MuSK-HA reliably differentiated into myotubes, and formed clusters as expected. However, a protein expression comparison is required between endogenous MuSK and the overexpressed MuSK for further studies for both better understanding how heterodimerization affects both phosphorylation and cluster formation, as well as the change they elicit in AChR cluster formation. Doxycycline concentration can be finer tuned to minimize its toxic effects while still enabling the dominant negative effect over endogenous MuSK.

Taken all together and moving forward, although the MuSK^{-/-} myotubes posed challenges, they are still the better model system for investigating CMS since they have the potential to better represent the effects of mutations compared to wild-type myotubes because they lack the endogenous MuSK the wild-type myotubes possess.

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