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Interaction Studies on a Novel Member of the Guanine Nucleotide
Exchange Factor Family of RIN Proteins

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ZUSAMMENFASSUNG

Die neuromuskuläre Synapse (NMS) ist ein geeignetes Modell-System zur Untersuchung der molekularen Mechanismen während der Synaptogenese. Für die Bildung und Aufrechterhaltung der NMS ist das postsynaptische Transmembranprotein, MuSK (Muscle-specific kinase) wichtig. MuSK ist eine Rezeptortyrosinkinase, die überwiegend in ausgereiften NMS konzentriert ist. Agrin, ein von Motoneuronen synthetisiertes Proteoglycan, wird in die synaptische Basallamina ausgeschüttet, wo es postsynaptische Differenzierung initiiert und dadurch auch die Phosphorylierung, die zytoskeletale Verankerung und die Aggregation von Acetylcholin-Rezeptoren (AChR) veranlasst. Der Agrin-Rezeptor, Lrp4 (LDL-receptor-related protein 4) ist ein Mitglied der LDLR Familie und bildet einen Komplex mit MuSK. Aufgrund dieser Komplexbildung führt die Aktivierung von Lrp4 durch Agrin auch zur Phosphorylierung von mehreren Tyrosinen in der intrazellulären Domäne von MuSK. Diese MuSK-Phosphorylierungen führen zur Aktivierung von MuSK, welche essenziell für die präsynaptische und postsynaptische Differenzierung ist. MuSK, Lrp4 oder Agrin „knock-out“ Mäuse sind nicht lebensfähig und sterben unmittelbar nach der Geburt, an respiratorischer Insuffizienz, da sie keine NMS bilden können. Außerdem sind AChR und weitere synaptische Proteine in diesen „knock-out“ Mäusen gleichmäßig entlang der Muskelfasern verteilt. Wie die Signale, die zur Aggregation von AChR führen, von aktiviertem MuSK weitergeleitet werden, ist größtenteils unbekannt und erfordert weitergehende Untersuchungen. Daher ist die Identifizierung und Charakterisierung von MuSK-Bindungspartnern von großer Bedeutung.

Für die Identifizierung bis dato unbekannter Bindungspartner von MuSK wurde ein „Yeast-Two-Hybrid (Y2H) Screen“ durchgeführt. Dafür wurde die zytoplasmatische Domäne von MuSK als „bait“ (Köder) und eine Muskel-cDNA Bibliothek als „prey“ (Beute) verwendet. Dabei wurde ein Protein, vorläufig Cl-6 genannt, gefunden, das spezifisch mit MuSK interagiert und ein neues Gen repräsentiert. Eine Analyse der Sequenz ergab, dass Cl-6 in seinem C-terminalen Teil über eine Vps9 Domäne verfügt, die homolog zur katalytischen Domäne des „guanine nucleotide exchange factors“ (GEF) Vps9p ist. Zusätzlich besitzt Cl-6 noch eine Src homology 2 (SH2) Domäne, die für die Interaktion mit phosphorylierten Tyrosin Resten benötigt wird, und eine RIN homology (RH) Domäne, die ein charakteristisches Merkmal für die Familie der RIN Proteine ist. Alle drei bekannten RIN Proteine besitzen wie Cl-6 auch eine SH2 und eine Vps9 Domäne. Die Vps9 Domäne ist in allen drei RIN Proteinen aktiv und wirkt als GEF für Rab5. Es konnte gezeigt werden, dass die RIN Proteine in Endozytose- und vesikuläre Transportprozesse involviert sind. Daher scheint es auch wahrscheinlich, dass Cl-6 eine Rolle in vesikulären Transportmechanismen spielen könnte. Wegen der spezifischen Bindung von Cl-6 an MuSK, lässt sich weiters auf eine mögliche Beteiligung von Cl-6 in MuSK-Transportprozessen schließen.

Im Rahmen dieser Diplomarbeit wurde die Interaktion von Cl-6 mit MuSK und Rab5 untersucht und zusätzlich die Effekte einer Herunterregulierung von Cl-6 in Muskelzellen beobachtet. Es konnte gezeigt werden, dass die Interaktion von Cl-6 mit MuSK abhängig von der RH Domäne des Cl-6 Proteins ist, was die Ergebnisse eines vorangegangenen „Y2H screens“ bestätigt. Die Vps9 Domäne wurde als Interaktionsdomäne für Rab5 gefunden und es konnte gezeigt werden, dass Cl-6, wie auch RIN1, spezifisch mit der dominant-negativen Mutante, S34N, von Rab5 interagiert. Schließlich wurde mit Hilfe von stabil transfektierten Muskelzelllinien gezeigt, dass eine spezifische Herunterregulierung von Cl-6 mittels „RNA interference“ (RNAi), zu einer verminderten Aggregation der AChR führt. Dieses Ergebnis

führt zu der Schlussfolgerung, dass Cl-6 eine wichtige nachgeordnete Komponente im Agrin/Lrp4/MuSK Signalweg ist, welche einen Einfluss auf die Zusammenlagerung der AChR an der NMS hat. Mithilfe dieser Zelllinien können nun auch die Funktion von Cl-6 selbst und weitere Konsequenzen der Herunterregulierung der Proteinexpression von Cl-6 noch genauer in Augenschein genommen werden.

ABSTRACT

The neuromuscular synapse (NMS) is used as a simple model system to study molecular mechanism during synaptogenesis. The formation and maintenance of the NMS requires the postsynaptic transmembrane protein, MuSK (muscle-specific kinase). MuSK is a receptor tyrosine kinase, which is located predominantly at the NMS of mature muscle. Agrin is a proteoglycan, which is synthesized by motor neurons and gets secreted into the synaptic basal lamina, where it initiates postsynaptic differentiation and leads to the phosphorylation, the cytoskeletal linkage and the aggregation of acetylcholine receptors (AChRs). The receptor of Agrin, Lrp4 (LDL-receptor related protein 4), which is a member of the LDLR family, builds a complex with MuSK. Therefore, the activation of Lrp4 by Agrin leads to the phosphorylation of multiple tyrosine residues in the intracellular domain of MuSK. Due to phosphorylation, MuSK is activated, which is essential for presynaptic and postsynaptic differentiation. MuSK, Lrp4 or Agrin knock-out mice are nonviable and die shortly after birth due to respiratory failure since they fail to form NMS. In addition, AChRs and other synaptic proteins are uniformly distributed along the muscle fibers in these knock-out mice. How activated MuSK relays the signals that lead to the aggregation of AChR, is still unclear and requires additional studies. Therefore, the identification and characterization of MuSK binding partners is extremely important.

A yeast-two-hybrid (Y2H) screen was performed to identify so far unknown MuSK binding partners. For this purpose the cytoplasmic domain of MuSK has been used as bait, and a mouse muscle cDNA library as prey. Thereby, a protein, currently termed Cl-6, has been identified, which specifically interacts with MuSK and represents a novel gene. Sequence analysis revealed that Cl-6 possesses a Vps9 domain, which is homologous to the catalytic domain of the guanine nucleotide exchange factor (GEF) Vps9p, in its C-terminal part. Additionally, Cl-6 contains a Src homology 2 (SH2) domain, which interacts with phosphorylated tyrosine residues, and a RIN homology (RH) domain, which is a characteristic feature of the family of RIN proteins. Moreover, all of the three RIN proteins also have a SH2 and a Vps9 domain. The Vps9 domain is active in all three RIN proteins and serves as GEF for Rab5. It has been shown, that the RIN proteins are involved in endocytosis and transport processes. Therefore, it seems likely that Cl-6 plays a role in vesicular transport processes. The specific binding of Cl-6 with MuSK further suggests that Cl-6 might be involved in transport processes of MuSK.

In the context of this diploma thesis the interaction of Cl-6 with MuSK and Rab5 has been studied and additionally, the effects of Cl-6 downregulation in muscle cells have been investigated. It could be shown, that the interaction of Cl-6 with MuSK depends on the RH domain of Cl-6, which confirms the results of a previously performed Y2H screen. The Vps9 domain has been found as interaction domain for Rab5 and it could be shown, that Cl-6, like RIN1, interacts preferentially with the dominant-negative mutant, S34N, of Rab5. Finally, it could be shown, with the help of stable transfected muscle cell lines that a specific downregulation of Cl-6 via RNA interference (RNAi) results in reduced clustering of AChRs. This result, leads to the conclusion, that Cl-6 represents an important component in the downstream signaling of the Agrin/Lrp4/MuSK signaling pathway, which influences the clustering of AChRs at the NMS. By means of these cell lines, the function of Cl-6 itself and further consequences of its downregulation can be investigated.

1. Introduction

1.1. The Neuromuscular Synapse

The site, where a motor neuron makes a contact with a skeletal muscle fiber, is called a neuromuscular synapse (NMS). The precise interaction between these two is critical for the transmission of chemical signals to muscle fibers, which leads to muscle fiber contraction. Each muscle fiber is innervated by a single motor axon. Therefore, the NMS is the preferred model system to study neuronal networks and the formation of synapses. But not only because of its relative simplicity, but also because of its large size and its unparalleled accessibility when compared to synapses in the brain, which are about 100 times smaller and much harder to access *in vivo* (Figure 1), the NMS is the best studied electrical synapse in vertebrates (Sanes and Lichtmann, 2001).

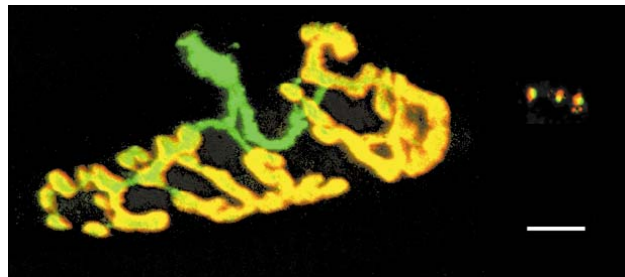


Figure 1. The Neuromuscular Synapse. The NMS of an adult mouse (on the right) in comparison to three synapses of cultured mouse hippocampal neurons (on the left) shown at the same scale. The postsynaptic membrane is stained in red and the nerve terminal in green. (Picture modified from Sanes and Lichtmann, 2001)

Additionally, the NMS is a particularly favourable synapse for studying synapse formation, for the following reasons: (1) it is geometrically simple; (2) developing and regenerating synapses can be experimentally manipulated *in vivo* with relative ease; (3) synapses between motor neurons and muscle cells can be studied in cell culture; (4) synaptic proteins can be readily purified from Torpedo electric organ, which is an abundant and homogenous source of neuromuscular-like synapses; and (5) gene expression can be altered and studied in detail in transgenic and mutant mice (Burden, 1998). Furthermore, α -bungarotoxin, the main component present in the deadly venom of *Bungaris multicinctus*, allows irreversible binding to muscle AChRs with high specificity (Berg et al., 1972).

NMSs are not only the model system for synapse formation, but are also important targets of research, since they play a role in many neuronal and muscular dysfunctions like myasthenia gravis, Lambert Eaton syndrome, congenital myasthenic syndrome and the large group of muscle dystrophies.

1.1.1. Organization and Function

The NMS is build up by an axon terminal that extends from the nerve body and is surrounded by a Schwann cell that produces an insulating myelin sheath around it, and a motoric endplate from a muscle fiber (Figure 2).

Acetylcholine (ACh) is an important neurotransmitter that is released by motor neurons at the NMS after excitation of the motor neuron form the axonal terminal. ACh is responsible for the transduction of a nerve-derived signal into muscle contractions and binds reversibly to acetylcholine receptors (AChRs), which are highly concentrated on the postsynaptic membrane of mature NMSs. For the formation of such mature synapses a series of complex interactions between motor neurons, muscle fibers and Schwann cells is needed (Burden, 2002).

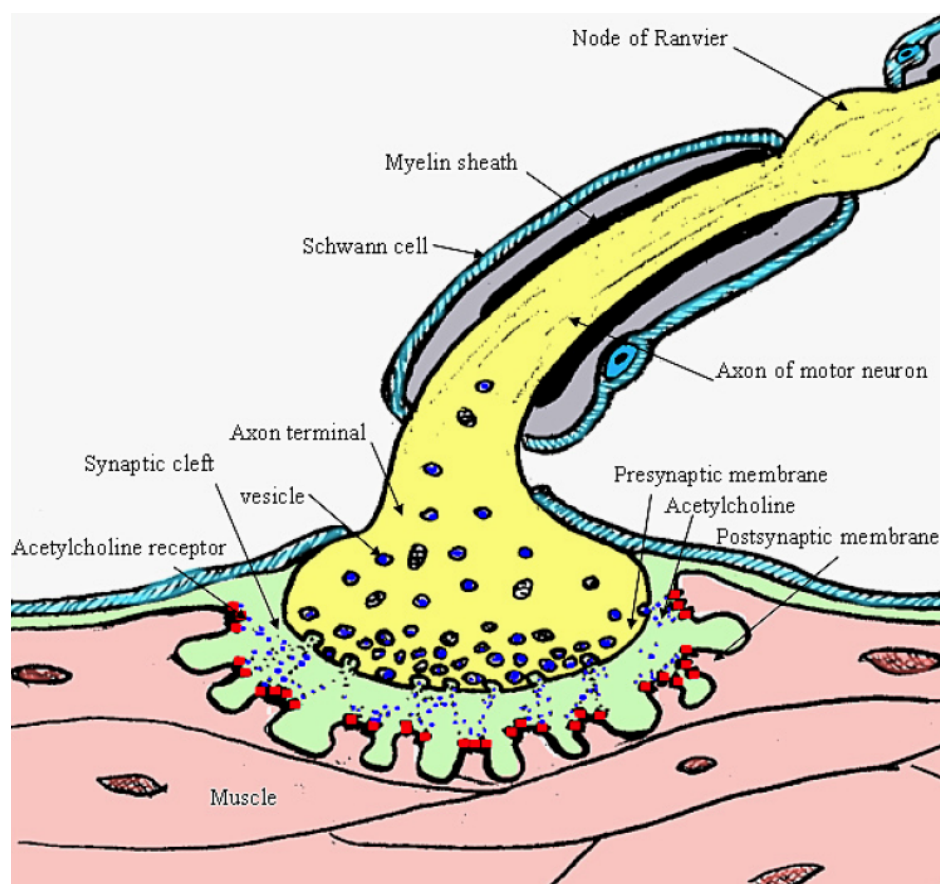


Figure 2. The Neuromuscular Synapse. Cartoon showing the organization of the NMS (Gloria Ikonge, Diploma thesis)

1.1.2. Hallmarks of Synapse Formation

The formation of the NMS is a highly complex process which involves several critical steps. It occurs during early embryonic development or after denervation of a muscle, when the nerve needs to re-establish the contact to the muscle. Characteristic for a fully mature NMS are the shallow depressions of the muscle cell membrane, which is invaginated further into

deep and regular folds, termed postjunctional folds (Figure 3) (Burden, 1998). In addition, the molecules in the presynaptic and postsynaptic membranes are precisely organized, which leads to an even higher differentiation of the NMS. This means that specific proteins are not equally distributed within the membranes, but are concentrated in spatial narrow regions. AChRs, MuSK and Rapsyn are localized to the crests of these postjunctional folds, whereas other proteins, including sodium channels, are enriched in the troughs of the postjunctional folds (Hall and Sanes, 1993). But also the nerve terminal is spatially organized. Synaptic vesicles are clustered, adjacent to poorly characterized specializations of the presynaptic membrane, termed active zones. These active zones, which are the sites of synaptic vesicle fusion, are organized at regular intervals and are aligned precisely with the mouths of the postjunctional folds (Burden, 1998). Moreover, changes like the clustering of nuclei to synaptic sites associated with an alteration in the expression level of synapse-specific genes contribute to the establishment of a fully mature synapse (Cohen et al., 1997). All together, these findings imply sophisticated mechanisms to regulate the formation and maintenance of this fully mature structure throughout the life of an organism.

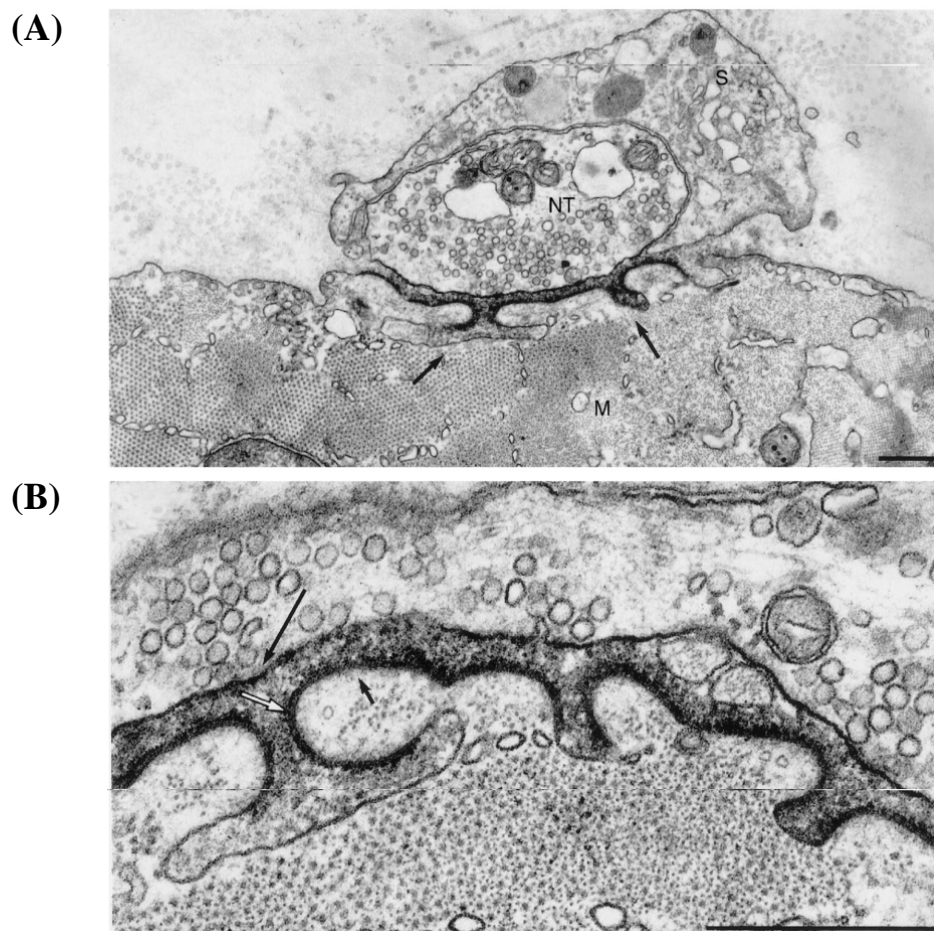
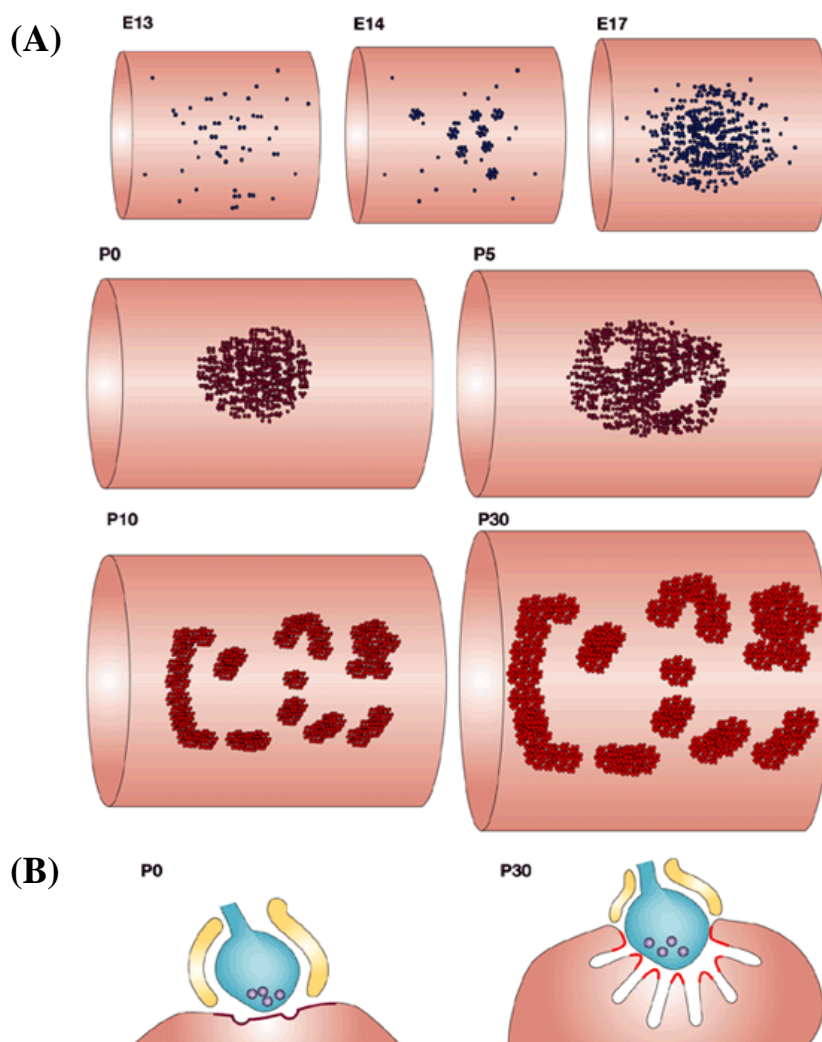


Figure 3. Electron-Microscopical Picture of the NMS. (A) The prominent postjunctional folds (indicated by black arrows) of the muscle membrane are situated opposite the active zone of the nerve terminal. (B) AChRs are concentrated at the crests of these folds, indicated by a white arrow in. NT, nerve terminal; M, Muscle; S, Schwann cell (Picture modified from Burden, 1998).

Multinucleated skeletal muscle fibers form during embryonic development (starting at ~E11 in the mouse) by fusion of precursor myoblasts. Shortly after that (E12-E13 in the mouse),

motor axons grow into this developing muscle and form a main intramuscular nerve, which in mammals extends through the central region of the muscle, perpendicular to the long axis of the myotubes. Individual motor axons start branching and terminate adjacent to the main intramuscular nerve, resulting in a narrow, distinct endplate zone, marked by presynaptic nerve terminals, clusters of AChRs, and elevated levels of AChR gene expression, in the middle of the muscle (Burden, 2002). Until E14.5 only few AChR-rich clusters are detectable and their densities are mostly aneural, which means that they are not closely apposed by nerve terminals, but located in a central region of the muscle (Lin et al., 2001; Yang et al., 2001). Following innervation of skeletal muscle fibers by the nerve, there is a redistribution of these clusters until they are trapped in a narrow, distinct region termed endplate zone (Luo et al., 2003). This process is completed at E16-E18.



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Figure 4. Maturation of the Postsynaptic Apparatus. (A) AChR microcluster coalesce to form a loose aggregate. Late in embryogenesis, the aggregate consolidates to form a plaque: its borders sharpen, its length decreases, and AChR density increases. Postnatally, the plaque becomes perforated to eventually form a pretzel-like array of branches. The branches then expand in an intercalary fashion as the muscle grows. Change in AChR colour denotes the switch from γ - to ϵ -containing AChRs. (B) As these changes occur, the plaque is intended to form a gutter, then invaginated to form folds. AChRs are concentrated at the crests of the folds.

E, embryonic day; P, postnatal day (Sanes and Lichtmann, 2001).

Although these neonate NMSs are functional even before birth, the postsynaptic membrane of neonates is very different from that of the adult. During maturation of the neonate synapse into the adult mature synapse, several changes can be observed (Figure 4). (1) The shape of the NMS changes from a simple oval plaque into a pretzel-like set of branches. (2) The initially flat junctional membrane changes into an invaginated surface with gutters and folds. (3) There is a quantitatively and qualitatively change in the composition of the basal lamina, that overlies the AChR-rich membrane and of the cytoskeletal apparatus that underlies it. (4) The composition of AChR changes from an embryonic form, containing γ -subunits, into an adult form, containing ϵ -subunits. Together with this change in subunit composition, also the properties of the channel change. There is a decrease in the channel opening times, whereas the channel conductance and its Ca^{2+} permeability increase. (5) Ion- and ligand-gated channels, along with their associated cytoskeletal elements, segregate into discrete alternating domains. Equally significant changes take place on the presynaptic site of the maturing NMS. There the nerve terminals become more efficacious, motor axons myelinate, and axonal branches are removed, leaving just one axon at each NMS (reviewed in Sanes and Lichtmann, 2001).

1.2. Agrin, MuSK and Lrp4 – The Key Players of Synapse Formation

Motor nerve terminals at the NMS release a protein called Agrin (Nitkin et al., 1987). Agrin activates a receptor complex on myotubes. This complex consists of a transmembrane receptor tyrosine kinase called MuSK (muscle-specific kinase) (Valenzuela et al., 1995) and Lrp4 (LDL-receptor-related protein 4) (Kim et al., 2008; Zhang et al., 2008). The activation of this complex leads to the phosphorylation and activation of MuSK, which triggers postsynaptic differentiation and induces the aggregation of synapse-specific components at the site of innervation. Especially, the clustering of AChRs at the motor endplate to a density of up to 20 000 receptors per μm^2 (in comparison with extrasynaptic regions with ~ 10 receptors per μm^2) can be seen as a hallmark of synapse formation (Figure 5) (Burden, 2002; Hughes et al., 2006).

This increased density of AChRs is achieved by three distinct processes: (1) clustering of diffusely distributed AChRs in the postsynaptic membrane; (2) transcriptional activation of AChR subunit genes in subsynaptic nuclei; and (3) transcriptional repression of AChR subunit genes in nonsynaptic myonuclei (Hughes et al., 2006).

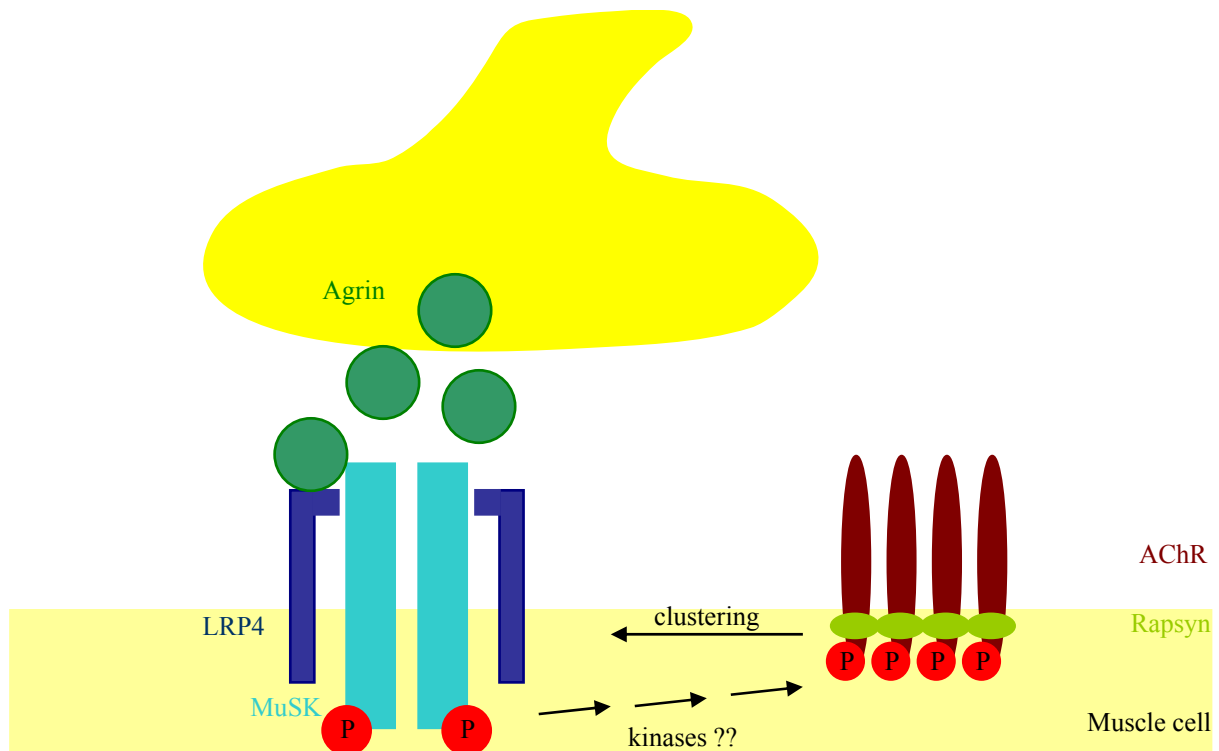


Figure 5. Model for MuSK Signaling. This cartoon shows the activation of MuSK through Agrin. Lrp4 is responsible for Agrin-dependent MuSK activation. This activation leads then to the clustering of AChRs at the postsynaptic membrane. The process of how this is actually achieved remains unknown.

1.2.1. Agrin

An extracellular matrix protein was found by McMahan and colleagues that contained an activity to stimulate AChR aggregation. They used the electric organ of *Torpedo californica* as a source for their study, because it has a concentration of cholinergic synapses far greater than that of skeletal muscle (Smith et al., 1987). Due to this clustering ability, the protein was named Agrin, from the Greek word ‘agrein’ meaning ‘to assemble’ (Nitkin et al., 1987). In all of the species from which Agrin has been cloned, the gene encodes a protein of more than 2 000 amino acids with a predicted molecular mass of 225 kDa. Agrin is extensively N- and O-glycosylated at the amino-terminal half and this increases the apparent molecular mass of Agrin to ~600 kDa (Figure 6). Due to the fact, that at least three of this carbohydrate attachment sites function as docking sites for heparin sulphate glycosaminoglycan side chains, Agrin has been assigned to the family of heparin sulphate proteoglycans (Tsen et al., 1995). In addition, Agrin contains nine follistatin-like domains which bear homology to Kazal-type protease inhibitor domains in its amino terminal half and four epidermal growth factor (EGF)-like signaling domains and three laminin G-like domains in the carboxy-terminal region (Rupp et al., 1991; Tsim et al., 1992). The C-terminal region is sufficient to induce AChR clustering in cultured myotubes (Ruegg and Bixby, 1998), whereas the amino terminal region is thought to be responsible for the association of Agrin with the extracellular matrix (McMahan, 1990; Tsen et al., 1995).

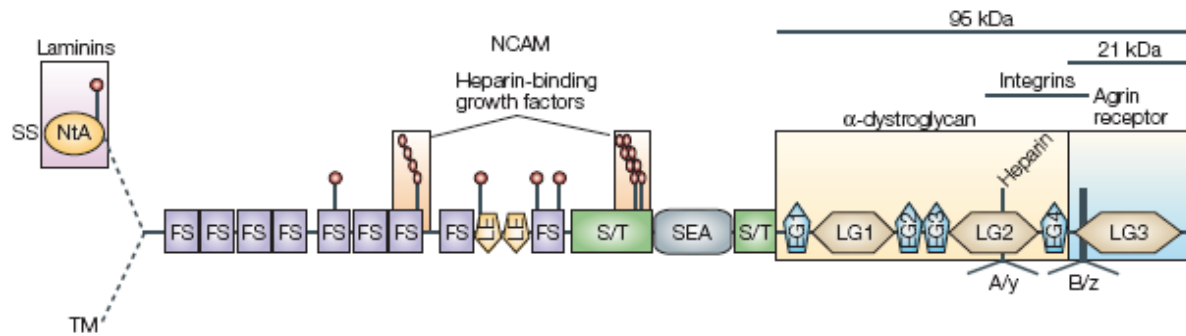


Figure 6. Structural Domains of Agrin. The schematic representation of Agrin shows its structural domains, the most important interaction partners and their binding regions indicated above the domain structure. In addition, the sites for alternative mRNA splicing (A/y, B/z) are shown below the domain structure. Usage of alternative first exons results in the synthesis of Agrin isoforms, that are either NtA- (N-terminal Agrin) domain-containing and secreted or that remain membrane associated due to a single transmembrane (TM) region. The secreted Agrin isoform is the one localized to the NMS, and the signal sequence (SS) is the region responsible for its release.

EG, epidermal growth factor-like domain; FS, follistatin-like domain; LE, laminin EGF-like domain; LG, laminin globular domain; NCAM, neuronal cell adhesion molecule; S/T, serine/threonine-rich region; SEA, sea urchin sperm protein, enterokinase, Agrin domain (Bezakova and Ruegg, 2003).

Agrin is produced by motor neurons, transported in motor axons and released at synaptic sites, where it organizes postsynaptic differentiation (McMahan, 1990; Ruegg and Bixby, 1998) and also regulates the distribution of other synaptic proteins, including acetylcholine esterase (AChE), Rapsyn, utrobin, neuregulin (NRG) and NRG receptors (ErbBs) (Apel et al., 1995; McMahan, 1990; Meier et al., 1997). This Agrin hypothesis, which was formulated by McMahan (McMahan, 1990) is supported by the following findings. (1) Agrin knock-out mice can not form functional postsynaptic structures and die immediately after birth due to respiratory failure (Gautam et al., 1996). (2) Agrin treatment of cultured muscle cells induces postsynaptic-like differentiation (Wallace, 1989). (3) Injection of expression plasmids or purified recombinant Agrin into non-synaptic regions of innervated rodent muscle establishes a fully functional, mature postsynaptic apparatus *in vivo* and activates synapse-specific gene transcription (Bezakova et al., 2001; Jones et al., 1997). This reveals that Agrin is one of the essential key factors involved in NMS formation.

The *Agrin* mRNA can be detected in various tissues and cells of the body, including central nervous system (CNS), peripheral nervous system (PNS), skeletal muscle, lung, kidney, Schwann cells and glia cells (Bezakova and Ruegg, 2003). Due to alternative splicing, several isoforms of Agrin occur, that differ in their tissue distribution and AChR clustering efficiency (Ferns et al., 1993; Ferns et al., 1992; Ruegg et al., 1992). Differential splicing at the 5' end results in two different isoforms named SS-NtA-Agrin and TM-Agrin which differ in their localization, since only the SS-NtA-Agrin isoform can bind to laminins in the extracellular matrix. While SS-NtA-Agrin is the predominant isoform at the NMS, the TM-Agrin is abundantly expressed in the CNS. Two important splice sites in the C-terminus are called Y and Z sites. Insertion of small splice-inserts at these sites gives rise to isoforms that contain 0 or 4 amino acids at the Y site, and 0, 8, 11, or 19 amino acids at the Z site. The insert at the Y site is required for heparin binding and modulates the binding of Agrin to α -dystroglycan (Gesemann et al., 1996), whereas the inserts at the Z site are crucial for Agrin-induced AChR aggregation (Burgess et al., 1999). On cultured myotubes, only the isoforms that contain an insert at the Z-site (Z+) induce AChR aggregation and this activity is absent when the insert at

the Z-site (Z-) is missing (Ruegg and Bixby, 1998). This splicing is also regulated in a tissue-specific manner. The main source for the 'active' Agrin-Z⁺ isoforms (those that can induce aggregation) is neurons, including motor neurons, whereas transcripts from all non-neuronal cells, except Schwann cells, encode 'inactive' Agrin-Z- isoforms (Bezakova and Ruegg, 2003). The isoform of Agrin, containing the four amino acid insert at the Y site in addition to the Z⁺ is more active in clustering of AChRs compared to Agrin lacking the Y insert (Glass et al., 1996).

1.2.2. MuSK

The muscle-specific kinase (MuSK) is a receptor tyrosine kinase specifically expressed in skeletal muscle. MuSK is expressed at low levels in proliferating myoblasts, but its expression is significantly upregulated after differentiation of myoblasts into myotubes and during muscle development. In mature muscle, MuSK is downregulated, and remains concentrated only at synaptic sites (Valenzuela et al., 1995).

All receptor tyrosine kinases (RTKs) contain an extracellular ligand binding domain, a single transmembrane helix and a cytoplasmic domain that contains a conserved protein tyrosine kinase core. Ligand binding to the extracellular part of the RTKs induces the dimerization of the receptors and results in autophosphorylation of their cytoplasmic domains (Schlessinger, 2000), which is responsible for activation of intracellular signaling cascades.

The extracellular domain of mammalian MuSK contains four immunoglobulin-like (Ig-like) domains and a cysteine rich domain between the third and fourth Ig-like domain. The cytoplasmic domain is most similar to the kinase domains of the Trks, which are the receptors for neurotrophins (Valenzuela et al., 1995). It consists of the juxtamembrane region and the tyrosine kinase domain (Figure 7). Activation of MuSK leads to the phosphorylation of six of the nineteen tyrosine residues in the intracellular domain (Watty et al., 2000). From these six phosphotyrosines, especially Y553 within the juxtamembrane region seems to be especially important, since mutation of this tyrosine residue abrogated the ability of Agrin to induce clustering or tyrosine phosphorylation of AChRs in cultured myotubes. This tyrosine appears to have a dual function in MuSK signaling, since it is required for fully activation of MuSK kinase activity, as well as for the recruitment of signaling component(s) that functions downstream of MuSK (Herbst and Burden, 2000). Furthermore it turned out that the juxtamembrane region of MuSK, including a single phosphotyrosine docking site, even in the context of a different kinase domain, is sufficient to activate the multiple pathways that lead to presynaptic and postsynaptic differentiation *in vivo* (Herbst et al., 2002).

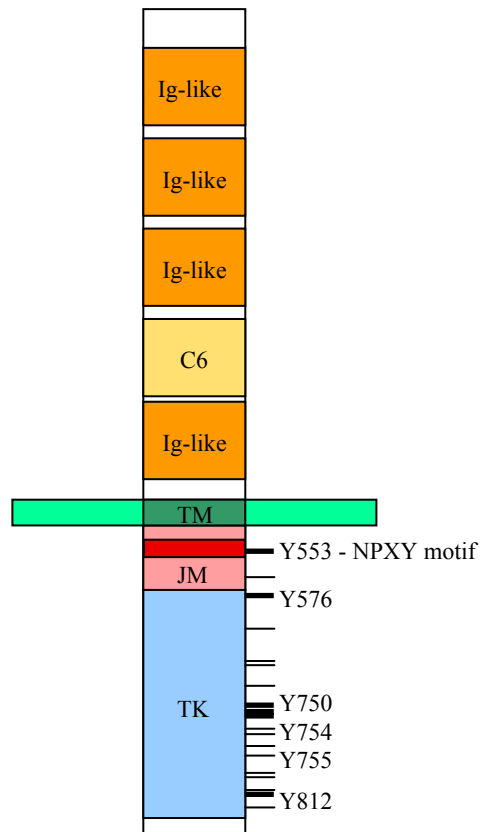


Figure 7. Schematic Drawing of the MuSK Protein. MuSK contains four immunoglobulin-like (Ig-like) domains and one cysteine-rich (C6) domain in its extracellular portion which, is separated from the intracellular part by a single transmembrane domain (TM). The cytosolic domain can be divided into a juxtamembrane (JM) domain and a kinase domain (TK). Tyrosines in these two domains become phosphorylated in response to Agrin. Tyrosine 553, which lies within an NPXY motif in the JM domain, has been shown to be essential for AChR clustering.

MuSK is unusual among RTKs (Glass et al., 1997) in two ways: (1) Agrin can stimulate the rapid phosphorylation of MuSK, but needs an accessory complex, because it can not bind MuSK directly (DeChiara et al., 1996; Glass et al., 1996); (2) ligand-dependent activation is insufficient to mediate its effects (Glass et al., 1997; Jones et al., 1997). Therefore, Sanes and colleagues proposed a model in which the ectodomain of MuSK not only mediates ligand-dependent activation of a complex signal transduction pathway, but also directs ligand-independent localization of a multi-molecular AChR-containing complex to the postsynaptic membrane (Zhou et al., 1999).

Mice deficient in MuSK lack normal NMSs. They are immobile, can not breathe and die at birth due to respiratory failure (DeChiara et al., 1996), like *Agrin* mutant mice (Gautam et al., 1996). This similar phenotype of *MuSK* and *Agrin* mutant mice, at least at birth, formed the basis that MuSK is a component of an Agrin receptor complex (Burden, 2002). Earlier in development (E14), however, AChR clusters are evident and concentrated in the central region of muscle from *Agrin* mutant mice (Lin et al., 2001; Yang et al., 2001), whereas AChR clusters are absent from *MuSK* mutant mice at all stages of development. This indicates that AChR clusters can form in the absence of Agrin, but not in the absence of MuSK. Furthermore, it has been shown, that Agrin counteracts the 'antisynaptogenic' influence of ACh and is therefore necessary for the maintenance of AChRs but not for their clustering (Misgeld et al., 2005).

1.2.3. Lrp4

It has been known for some time that Agrin was not able to interact directly with MuSK. Therefore a hypothetical molecule, myotube-associated specificity component (MASC) was proposed to serve as a binding partner for Agrin to transduce signals to MuSK (Glass et al., 1996).

In a genetic screen for genes that regulate early development in the mouse, mice carrying mutations in *Lrp4* were found to have massive defects in neuromuscular synapse formation, leading to neonatal lethality (Weatherbee et al., 2006). LDL-receptor-related protein 4 (*Lrp4*) is a member of the LDLR family and contains a large extracellular N-terminal region that possesses multiple EGF and LDLR repeats, a transmembrane domain, and a short C-terminal region without an identifiable catalytic motif (Zhang et al., 2008). Members of this family are well known for their roles in lipid metabolism, cholesterol homeostasis and Wnt signaling (He et al., 2004; Schneider and Nimpf, 2003) and some also have specific roles in the development and function of the mammalian nervous system (May and Herz, 2003).

The defects in presynaptic and postsynaptic differentiation are strikingly similar to those found in *MuSK* mutant mice, namely the absence of postsynaptic AChR clusters, extensive aberrant presynaptic branching, and reduced formation of presynaptic terminals. In addition to that, *Lrp4* is expressed preferentially by muscle synaptic nuclei (Weatherbee et al., 2006), binds to neuronal, but not muscle Agrin and is required for Agrin to stimulate MuSK phosphorylation (Zhang et al., 2008). Moreover, *Lrp4* self-associates, forms a complex with MuSK independent of Agrin and can reconstitute Agrin-stimulated MuSK phosphorylation in nonmuscle cells that express *Lrp4* and MuSK (Kim et al., 2008). Because of its self-association, *Lrp4* may promote trans-phosphorylation and activation of MuSK, which can lead to the low level of Agrin-independent MuSK activation, which is needed for prepatternning (Figure 8).

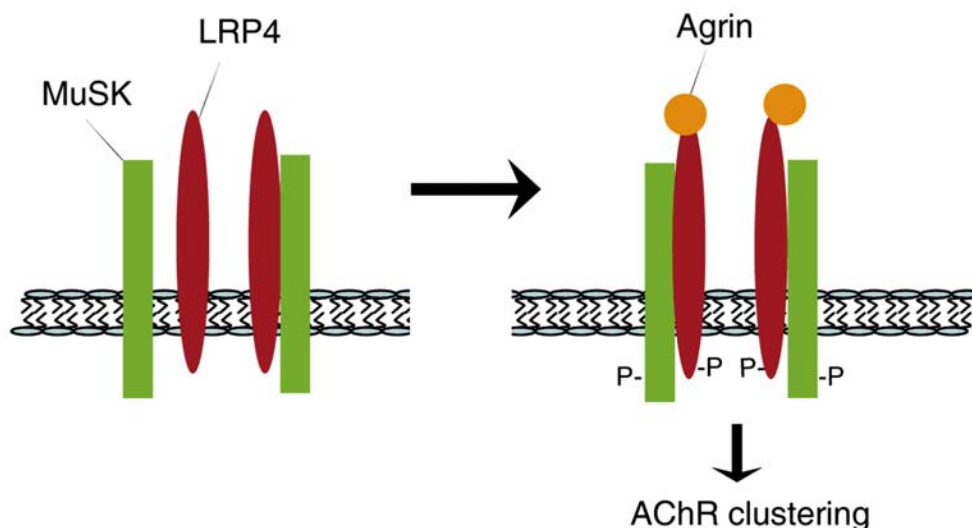


Figure 8. Working Model of the Interaction of MuSK-Lrp4 and Agrin. In the absence of neuronal Agrin, Lrp4 could interact with MuSK and this interaction is increased by Agrin stimulation. Such interaction is necessary for MuSK activation and the downstream signaling that leads to AChR clustering. P, phosphorylation (Zhang et al., 2008).

1.2.4. Downstream Signaling

The signaling downstream of MuSK is not fully understood yet. Several proteins have been shown to be necessary, but how the signal is transmitted remains unclear. Figure 9 shows a schematic drawing of the signaling complex downstream of MuSK.

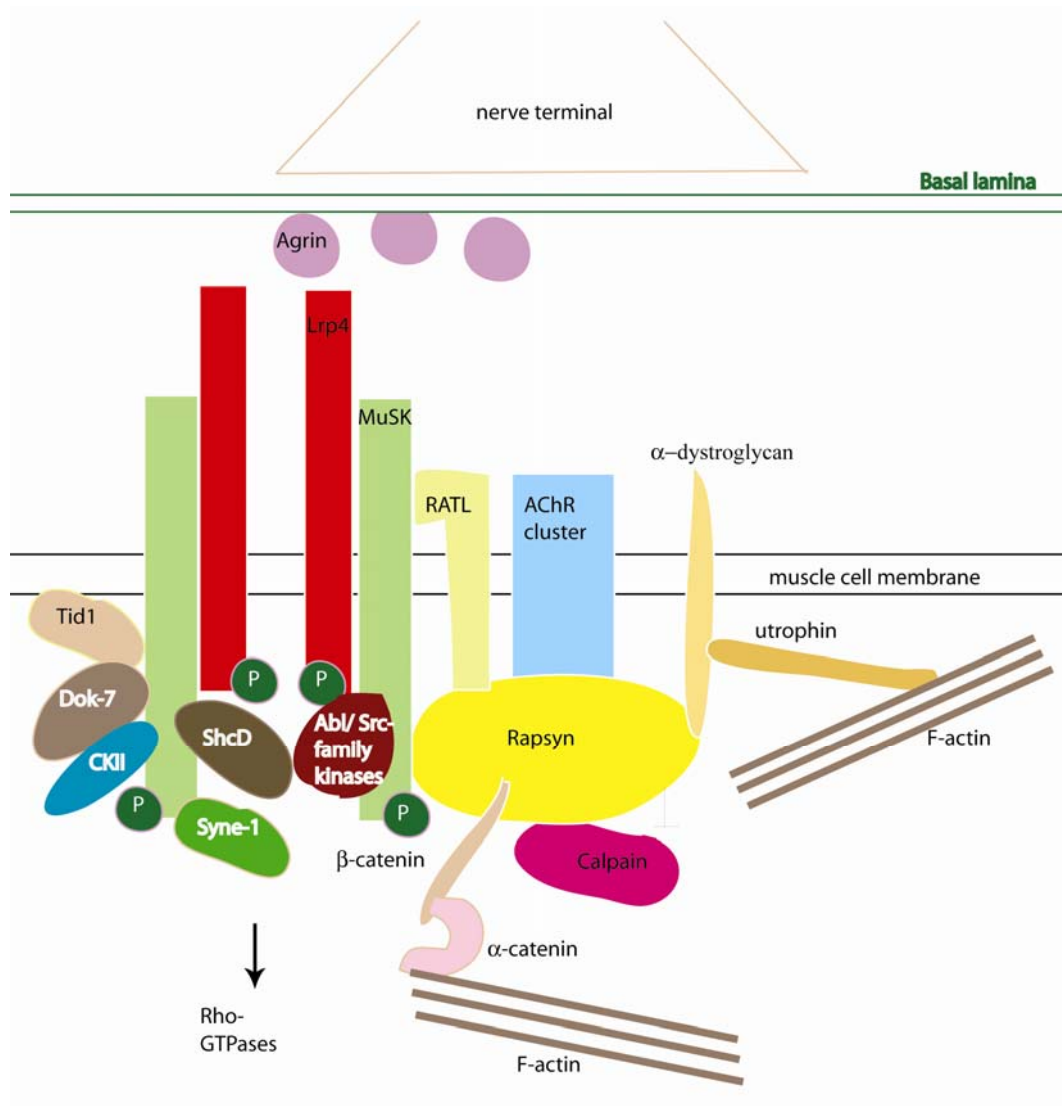


Figure 9. Cartoon of the NMS with Associated Proteins. Activation of MuSK upon Agrin binding induces clustering and tyrosine phosphorylation of AChRs required for synaptic transmission. The AChR-associated protein Rapsyn interacts with Calpain in an Agrin-dependent manner. Upon Agrin stimulation, the Agrin receptor Lrp4 induces MuSK phosphorylation and subsequent activation of several intracellular proteins. For further information see text.

Rapsyn is an entirely intracellular protein that binds to AChRs in a 1:1 stoichiometry at the NMS (LaRoche and Froehner, 1986). It interacts with the intra- and extracellular domains of MuSK in distinct ways, with the latter probably indirectly via a hypothetical protein termed RATL (for Rapsyn Associated Transmembrane Linker) (Apel et al., 1997). It also links the

AChRs to the dystroglycan/utrophin glycoprotein complex (Apel et al., 1995; Fuhrer et al., 1999) and indirectly to the α -catenin-associated cytoskeleton, through its interaction with β -catenin (Zhang et al., 2007). In mice lacking Rapsyn, NMSs fail to form, demonstrating that Rapsyn is required for the aggregation of AChRs and also critical for organization of the postsynaptic cytoskeleton (Gautam et al., 1995). Rapsyn has also been shown to stabilize AChR clusters at the NMS by its interaction with Calpain, which inhibits the protease activity of Calpain and therefore the dispersion of AChR clusters in an Agrin-dependent manner (Chen et al., 2007). Rapsyn is also able to link AChR clusters to F-actin via its interaction with α -Actinin, which has been shown to play a role in Agrin-mediated AChR clustering (Dobbins et al., 2008).

Other important proteins are Dok-7 and ShcD which both bind to the juxtamembrane NPXY motif of MuSK in a phosphorylation-dependent manner via their phosphotyrosine-binding domains (PTB domain) and therefore may compete for this binding site (Jones et al., 2007; Okada et al., 2006) and Tid1 which binds to the cytoplasmic domain of MuSK (Linnoila et al., 2008). Dok-7 is a cytoplasmic protein that is essential for MuSK activation in cultured myotubes. Mice lacking Dok-7 fail to form NMSs and die shortly after birth. Dok-7 appears to be involved in the propagation of signals from MuSK to its downstream effectors (Okada et al., 2006). Tid1 (for tumorous imaginal discs 1) is a heat shock protein (hsp) 40 homolog and has been implicated in a variety of signaling pathways, including those involving tyrosine phosphorylation. It binds to MuSK independently of Agrin and is required for both spontaneous and Agrin-induced AChR clustering in myotubes and for the maintenance of clusters at synapses in adult muscle fibers. Tid1 has been shown to be necessary for the interaction between Dok-7 and MuSK following Agrin stimulation, which is thought to lead to the activation of Tid1. Therefore, Tid1 appears to act downstream of Dok-7 in the signaling pathway (Linnoila et al., 2008). Shc family proteins serve as phosphotyrosine adaptor molecules in various receptor-mediated signaling pathways. ShcD appears to be primarily expressed in brain and skeletal muscle of adult mice. It is co-expressed with MuSK in the postsynaptic region of the NMS and seems to play a role in the early tyrosine phosphorylation of the AChRs (Jones et al., 2007).

The β -subunit of the protein kinase Casein kinase II (CKII) was identified as a protein that interacts with the phosphorylated intracellular domain of MuSK at the NMS. Additionally, the CKII-mediated serine phosphorylation of MuSK is important for normal AChR aggregation and maintenance (Cheusova et al., 2006). CKII is a highly conserved, ubiquitously expressed serine/threonine kinase present in all eukaryotes. It consists of a tetramer of two catalytic (α/α') and two regulatory (β) subunits. In mice, the knock-out of the gene encoding the CKII β subunit is lethal at a very early embryonic stage (Buchou et al., 2003).

Other important molecules that have been found to play a role in signal transduction downstream of MuSK and Agrin include tyrosine kinases like Abl kinases (Finn et al., 2003) and Src-family kinases (Mohamed et al., 2001; Sadasivam et al., 2005), Dishevelled (Dvl)/p21-activated kinase (PAK1) (Luo et al., 2002), geranylgeranyltransferase I (GGT1), which is a zinc metalloenzyme and tethers proteins to plasma membrane by prenylation (Luo et al., 2003), Rac and Cdc42, small GTPases of the Rho family (Weston et al., 2000) and Syne-1 (Apel et al., 2000).

1.3. *Cl-6 a Novel MuSK-Interaction Partner and a Potential Guanine Nucleotide Exchange Factor for Rab5*

The newly identified protein, currently termed Cl-6, was isolated in a yeast-two-hybrid (Y2H) screen with a mouse muscle cDNA library, using the MuSK cytoplasmic region as bait protein. Figure 10 shows a scheme of this originally identified protein and its domains.



Figure 10. Scheme of the Cl-6 Protein (Y2H) and Its Functional Domains.
Vps9, guanine nucleotide exchange factor domain; RH, RIN homology domain.

This new protein contains a Vps9 domain in its C-terminal part, which is homologous to the catalytic domain of the guanine nucleotide exchange factor (GEF) Vps9p. This Vps9 domain is highly conserved and present in a number of multidomain proteins and is well known for its function as a GEF for Rab5 (Hama et al., 1999). In addition to this, Cl-6 contains a RH (RIN homology) domain, which is shared by the members of the RIN family. Database search using a sequence alignment tool identified striking sequence homology of Cl-6 and the three described RIN proteins, which have been described only recently (see chapter 1.4). All of them contain a functional Vps9 domain, serve as GEF for Rab5 and seem to be involved in endocytosis and vesicular transport (Kajiho et al., 2003; Saito et al., 2002; Tall et al., 2001). Sequence alignment with the RIN proteins, resulted in high sequence homology of RIN Src homology 2 (SH2) domains and the N-terminal part of Cl-6, although a SH2 domain was not assigned to Cl-6 by database predictions. The SH2 domain represents a protein-binding module that recognizes phosphorylated tyrosines (pTyr). Therefore, proteins containing this domain play different roles in cellular tyrosine kinase signaling pathways.

The interaction of Cl-6 with MuSK was confirmed in a heterologous cell system and it turned out that the kinase activity of MuSK is not required for this interaction (Michaela Mutzl, Diploma thesis). In Y2H experiments, the region containing the RH domain turned out to be essential for the interaction of Cl-6 with MuSK (Ruth Herbst, unpublished data) and expression of Cl-6 in fibroblasts gives a punctuated staining pattern, which indicates a localization of this protein in vesicles or endosomes (Barbara Woller, Diploma thesis). The same intracellular localization was described for the RIN proteins (Kajiho et al., 2003). Taking into account the functional properties of the described RIN proteins, Cl-6 might play a role in MuSK trafficking, most likely in endocytosis. Furthermore, Cl-6 has been shown to be highly concentrated at the NMS and further co-localizes with AChRs, which is a prerequisite for a potential function during Agrin/MuSK signaling (Barbara Woller, Diploma thesis).

Analysis of the expression pattern of Cl-6 revealed that the highest expression level is found in spleen, lung and thymus, which suggests that Cl-6 might also play a role in the immune system. Additionally, lower levels of Cl-6 are found in kidney, liver, heart and brain and the lowest levels were found in muscle tissue (Gloria Ikonge, Diploma thesis).

1.4. The Family of RIN Proteins

RIN stands for Ras interaction/interference and results from the fact that RIN1 was originally identified as an inhibitor of activated Ras (Colicelli et al., 1991). Ras is a membrane-associated small G-Protein that is directly coupled to both receptor and non-receptor tyrosine kinases. RIN proteins contain a functional Ras association (RA) domain (Han and Colicelli, 1995; Han et al., 1997). It has been shown that RIN1 directly competes with RAF1 for binding to H-Ras (Han and Colicelli, 1995; Wang et al., 2002) and that each RIN interacts with certain types of Ras members (Kajiho et al., 2003). So far this family consist of three members namely RIN1, RIN2 and RIN3 (Figure 11). Within the central region of these three proteins a conserved 100 amino acid sequence has been found and subsequently termed RIN homology (RH) domain (Saito et al., 2002).

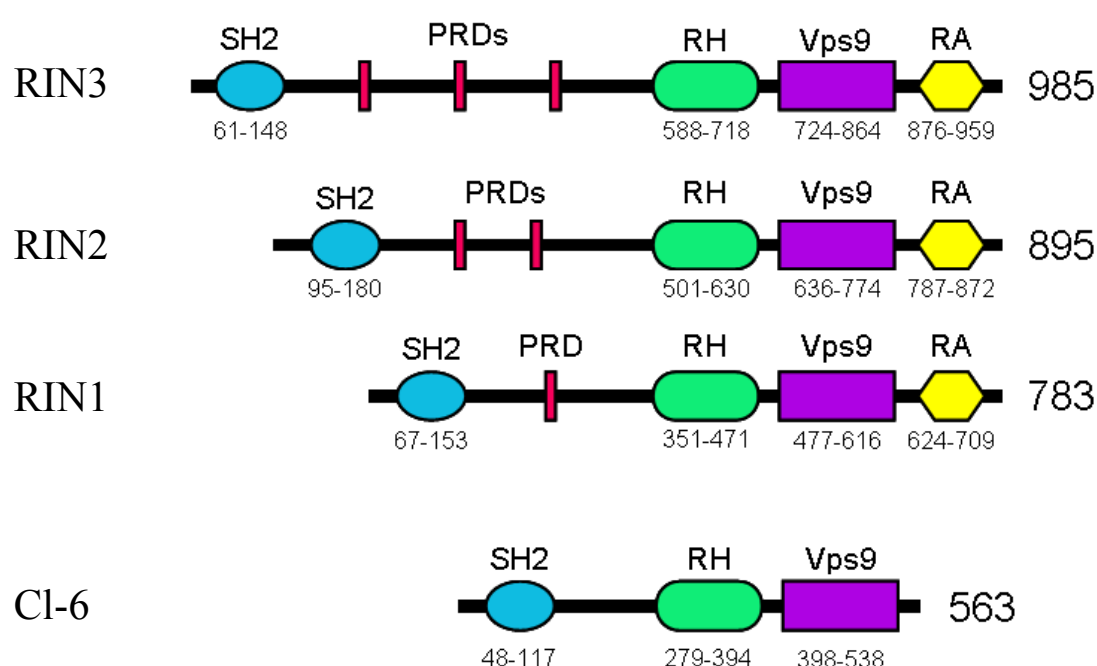


Figure 11. Scheme of the RIN Proteins and Cl-6 with the Functional Domains. Numbers below the schemes represent the amino acid residue.

SH2, Src homology domain 2; PRDs, proline-rich domains; RH, RIN homology domain; Vps9, guanine nucleotide exchange factor domain; RA, Ras association domain (Picture modified from Kajiho et al., 2003).

Further studies revealed that RIN1 also contains a region homologous to the catalytic domain of Vps9p-like Rab guanine nucleotide exchange factors (GEFs) (Tall et al., 2001). Rab GTPases are members of the Ras superfamily and represent key regulators of membrane trafficking and receptor localization in eukaryotic cells (see chapter 1.5). It has been shown, that Rab5 can be activated by the action of Vps9p (Hama et al., 1999). It turned out, that this Vps9p catalytic domain is necessary and sufficient for the interaction of RIN1 with GDP-bound Rab5. RIN1 was also shown to stimulate Rab5 guanine nucleotide exchange, Rab5a-dependent endosome fusion, and epidermal growth factor (EGF) receptor-mediated

endocytosis. Furthermore, the stimulatory effect of RIN1 on all three processes is potentiated by activated Ras (Tall et al., 2001). Additionally, RIN1 interacts with 14-3-3 proteins through the Ras binding domain (RBD), with the SH3 domain of signal-transducing adaptor molecule 2 (STAM2) and Abl tyrosine kinase through its Proline-rich domain, the latter leading to tyrosine phosphorylation of RIN1 by c-Abl (Han et al., 1997), and with the EGFR through its SH2 domain (Kong et al., 2007).

RIN2 is an additional Rab5-binding protein and has been identified due to its sequence similarities to RIN1 (Saito et al., 2002). This protein functions as a tetramer, composed of anti-parallel linkage of two parallel dimers. It has the same structure as RIN1 except for an additional Proline-rich domain. In contrast to RIN1, it preferentially binds to the GTP-bound form of Rab5, although it enhances the guanine exchange reaction of Rab5. Therefore, it may not only function as an upstream GEF but also as a downstream effector for Rab5 in the endocytic pathway. Additionally, it turned out that the RH domain, together with the Vps9 domain, constitutes a region necessary for Rab5 binding (Saito et al., 2002).

The same group also identified RIN3, which shares many functional domains with the other RIN proteins and contains three Proline-rich domains. They found that RIN3 has the same biochemical properties as RIN2, the stimulator and stabilizer of GTP-Rab5. In addition RIN3 exhibits its unique intracellular localization and localizes to Rab5-positive, but not EEA1 (early endosomal antigen 1)-positive vesicles, which is an early endosomal marker. With its Proline-rich domain, RIN3 interacts with amphiphysin II, which is involved in receptor-mediated endocytosis and cytosolic amphiphysin II is translocated into RIN3- and Rab5-positive vesicles. Altogether, this indicates that RIN3 plays an important role in the transport pathway from plasma membrane to early endosomes (Kajiho et al., 2003).

1.5. Rab Proteins – a Family of Small GTPases

Rab GTPases resemble the largest family of small GTPases. They function as molecular switches that change between two conformational states, an active, GTP-bound and an inactive, GDP-bound state. More than 60 members of the Rab family have been found and they act as coordinators of vesicle trafficking. They are localized to distinct intracellular membranes. Therefore, the specificity of vesicle trafficking is encoded in the machinery itself (Bock et al., 2001).

In Figure 12 the cycle of Rab proteins is depicted. (1) The newly synthesized Rab protein, in the GDP-bound ‘inactive’ form, is recognized by a Rab escorting protein (REP) that presents the Rab protein to (2) a geranylgeranyl transferase (GGT), which geranylgeranylates the Rab on one or two carboxy-terminal cysteine residues. (3) This geranylgeranylated, GDP-bound Rab protein is recognized by Rab GDP dissociation inhibitor (GDI), which is involved in Rab GTPase delivery to and removal from membranes. Targeting of the Rab-GDI complex to specific membranes is mediated by interaction with a membrane-bound GDI-displacement factor (GDF), which catalyses both the dissociation of prenylated, GDP-bound Rab proteins from GDI, and the subsequent transfer of Rab proteins onto the membrane. (4) At the membrane, a guanine nucleotide exchange factor (GEF) catalyzes the exchange of GDP for GTP, which causes a conformational change of the Rab protein. (5) The GTP-bound ‘active’ form recruits or activates multiple effector proteins, including sorting adaptors, tethering factors, kinases, phosphatases and motors. These Rab effector proteins are not randomly distributed on the organelle membrane, but are clustered in distinct functional domains.

Therefore, they are primary determinants of compartmental specificity in the organelles of eukaryotic cells. (6) The GTP-bound Rab gets inactivated by GTP hydrolysis, which is stimulated by a GTPase-activating protein (GAP). These GAPs are necessary, since Rab proteins only have a low intrinsic rate of GTP-hydrolysis (Reviewed by Pfeffer and Aivazian, 2004; Stenmark, 2009; Zerial and McBride, 2001).

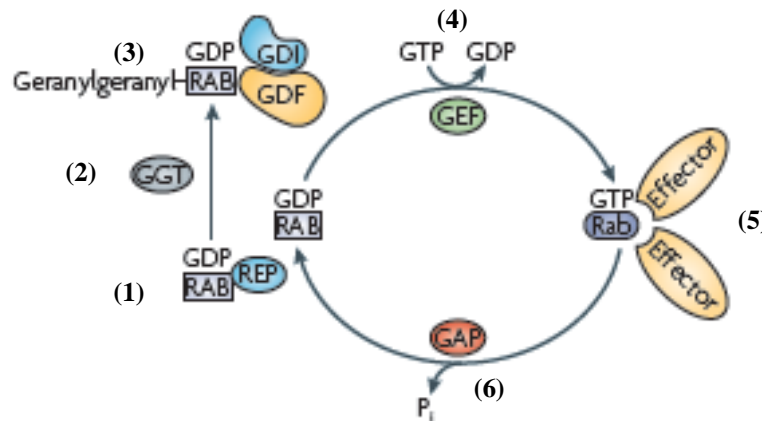


Figure 12. Schematic Drawing of Rab5 Activation and Inactivation. For information see text. REP, Rab escorting protein; GGT, geranylgeranyltransferase; GDI, GDP dissociation inhibitor; GDF, GDI displacement factor; GEF, guanine nucleotide exchange factor; GAP, GTPase-activating protein (Picture modified from Stenmark, 2009).

1.5.1. Rab5

Rab5 is among the best studied Rab proteins to date and is known as the key regulator of endocytosis, where it is involved in clathrin-coated vesicle formation, fusion between early endosomes, endosomal cargo recruitment and endosomal motility (Zerial and McBride, 2001). In mammalian systems, three Rab5 isoforms (a, b, c) have been documented, and several specific functions have been attributed to the distinct isoforms. For example, Rab5a can potentiate endocytosis of the epidermal growth factor receptor (EGFR), but Rab5b and Rab5c cannot. Phylogenetic analysis of the human Rab family suggests that Rab21, Rab22a and Rab22b might also belong to the Rab5 family. They co-localize with Rab5 and its effectors on early endocytic compartments (Carney et al., 2006). It has been shown, that Rab5 generates microdomains on endosome surfaces, which comprise specific proteins and their lipid- and protein-binding partners (Zerial and McBride, 2001). Additionally, Rab5 has been shown to be necessary for Ras stimulated pinocytosis (Li et al., 1997), and also has a function in macropinocytosis (Pfeffer, 2005). Furthermore, Rab5 is rate-limiting for endocytosis and is regulated by PKB/Akt, a kinase coupled to signal transduction (Barbieri et al., 1998).

The first direct effector of Rab5 that has been identified was Rabaptin-5, which is a mainly cytosolic protein, that is recruited to the membrane of early endosomes by GTP-bound Rab5 and is required for membrane docking and fusion (Stenmark et al., 1995). In case of Rab5, it could be shown, that nucleotide exchange is essential for endosome fusion, since active Rab5 is very unstable and rapidly hydrolysed by GAPs (Rybin et al., 1996). Therefore, only a small fraction of Rab5 is active on the membrane, which can recruit Rabaptin-5 to engage the

membrane docking and fusion machinery. Hence, the finding that Rabex-5, a Rab5 GEF, forms a tight complex with Rabaptin-5 at the membrane implies that this complex couples nucleotide exchange to effector recruitment. In addition to the activation of Rab5 by Rabex-5, this interaction with Rabaptin-5 stabilizes the activated Rab5 on the membrane by delaying GTP-hydrolysis (Horiuchi et al., 1997). Hama et al. showed that Vps9p in yeast serves as a GEF for Vps21p and the mammalian sequence homolog, Rab5. They concluded, that Vps9p and Rabex-5 may represent a novel class of GEFs that modulate the activity of Rab5 (Hama et al., 1999). It turned out that this consensus Vps9p catalytic domain is present in a variety of multi-domain proteins and that Alsln (ALS2) and a new family of proteins, the RIN proteins, which also contain these Vps9 domain, function as GEFs for Rab5. RIN1 was shown to bind to the GDP-bound form of Rab5, while the other two RIN proteins preferentially interact with the GTP-bound form (Kajiho et al., 2003; Saito et al., 2002; Tall et al., 2001). ALS2 showed a slight preference for the Rab5a isoform, but the significance of this observation remains unclear. Furthermore, ALS2 serves additionally as an exchange factor for Rac1 via its Dbl homology (DH) and Pleckstrin (PH) domains (Topp et al., 2004). The crystal structure of the Vps9 domain of Rabex-5 was determined, revealing that the catalytic core of Rabex-5 exhibits high specificity for the Rab5 subfamily, and has a tandem helical architecture in which the Vps9 domain is stabilized by an essential helical bundle (Delprato et al., 2004). It has been shown that the Vps9 domain of Rabex-5 possesses GEF activity on only Rab5, Rab21 and more weakly Rab22 (Carney et al., 2006). It turned out, that GTP-bound Rab22 serves as a binding site on early endosomes for direct recruitment of Rabex-5 and activation of Rab5. Therefore, Rabex-5 is an effector for Rab22 (Zhu et al., 2009). The presence of other signaling domains within Vps9 domain-containing proteins suggests that these GEFs might serve to integrate Rab5 activation with signal transduction cascades (Carney et al., 2006). Structural analysis of Rabex-5 further revealed the presence of an autoinhibitory element, that overlaps with the binding site for Rabaptin-5 and potentially suppresses the exchange activity of Rabex-5 (Delprato and Lambright, 2007).

Additionally, it turned out that some Rab5 effectors, namely Rabaptin-5 and Rabenosyn-5, actually act as bifunctional effectors of the GTPases Rab5 and Rab4. A cooperatively interaction of these two proteins seems to be required, to ensure a strong physical link between the Rab4 and Rab5 domains. Therefore, they may provide spatial control integrating Rab5-dependent fusion and Rab4-dependent fission events (De Renzis et al., 2002; Deneka and van der Sluijs, 2002).

Other Rab5 interacting proteins are Rabankyrin, Vps34 and EEA1 (van der Blik, 2005).

1.6. RNA Interference

1.6.1. Overview

With the RNA interference (RNAi) technique genes can be silenced at the mRNA level, due to the inhibition of the expression of specific genes by double stranded RNAs (dsRNAs). With this method the function of a gene both *in vivo* and *in vitro* can be quickly and easily determined. When exposed to foreign genetic material (RNA or DNA), many organisms mount highly specific counter attacks to silence the invading nucleic-acid sequences, before these sequences can integrate into the host genome or subvert cellular processes. Interestingly, dsRNA does more than help to defend cells against foreign nucleic acids – it also guides

endogenous developmental gene regulation, and can even control the modification of cellular DNA and associated chromatin (Mello and Conte, 2004). RNAi is a multi-step process involving the generation of small interfering RNAs (siRNAs) *in vivo* through the action of the RNase III endonuclease Dicer (Shi, 2002). The resulting 21- to 23-nucleotide (nt) siRNAs guide distinct protein complexes to their targets. These silencing complexes include the RNA-inducing silencing complex (RISC), which pairs with the complementary mRNA sequences and leads to their degradation (Mello and Conte, 2004).

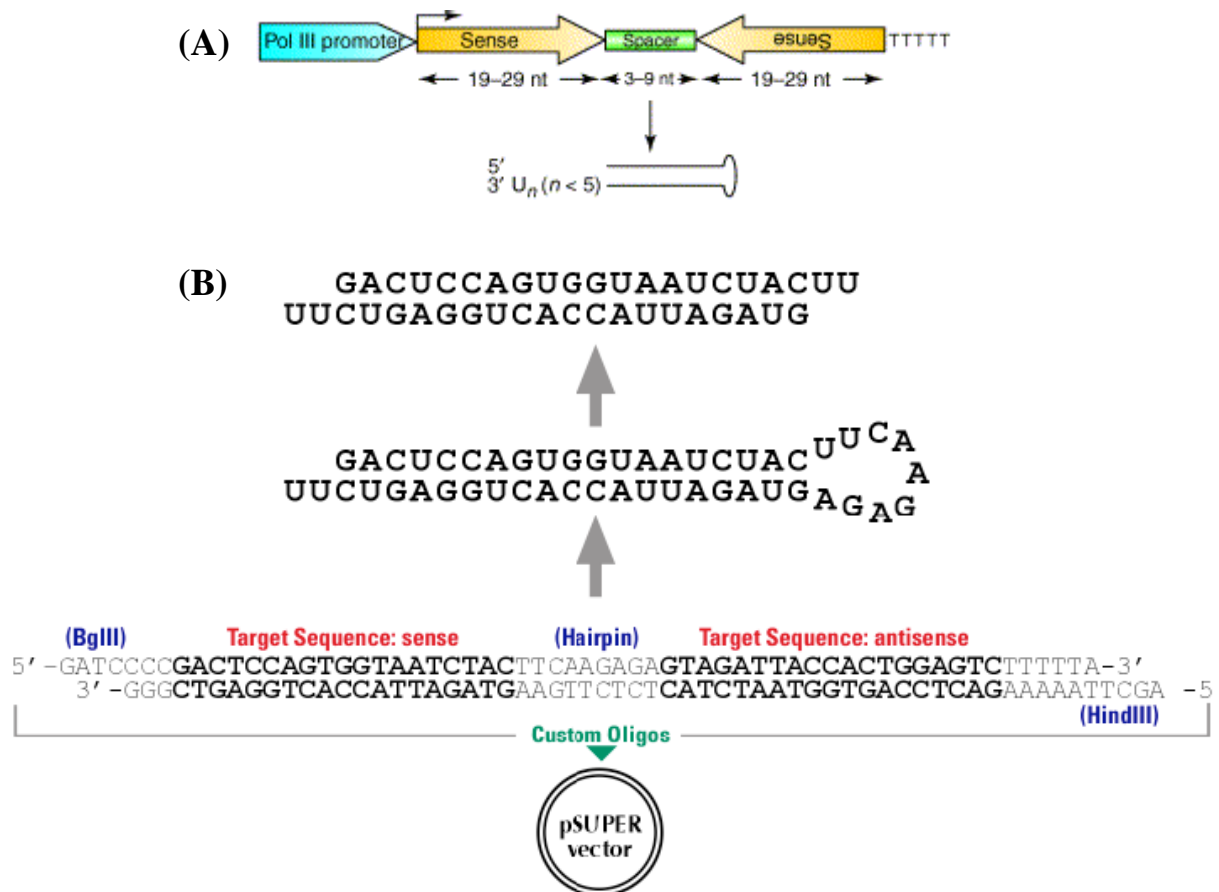


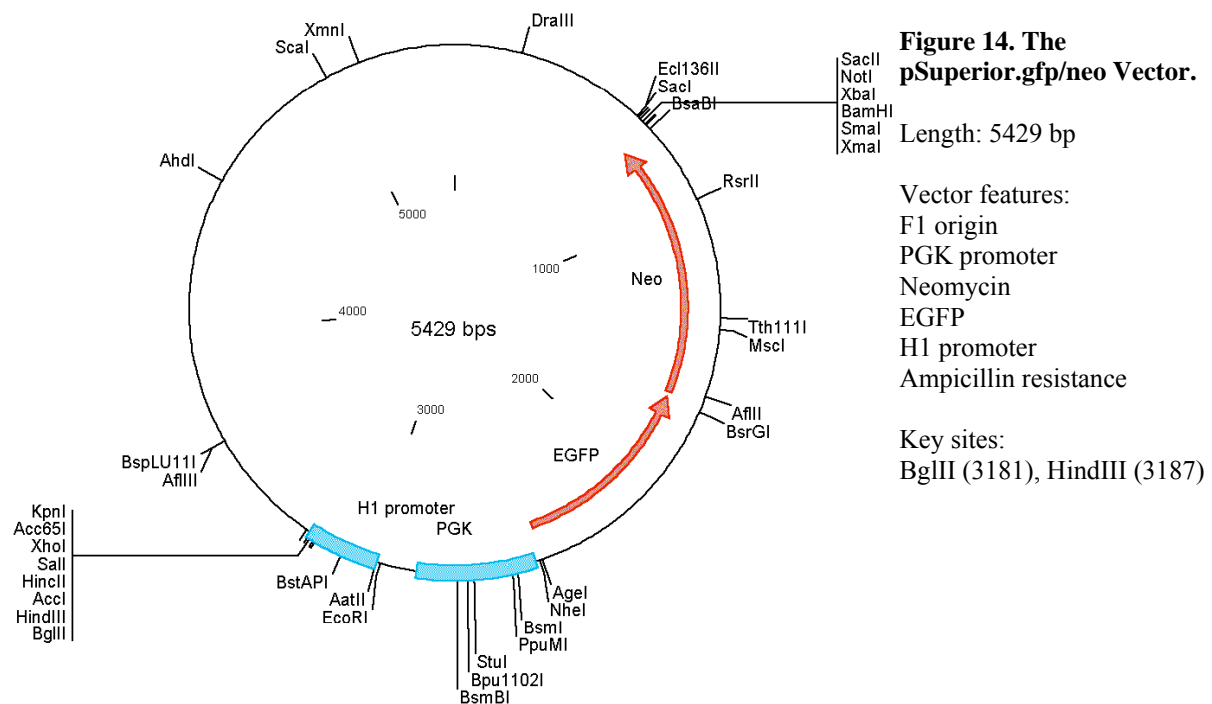
Figure 13. The DNA Vector-Based RNAi Technology. (A) Generation of a small hairpin RNA (shRNA) directed by a Pol III promoter. The two motifs that form the inverted repeat are separated by a spacer and the resulting RNA is predicted to fold back forming a shRNA as shown (Shi, 2003). (B) The pSuper RNAi System; overview of the insert design. Custom oligos of 60 nt are cloned into the pSuper vector between the unique BglIII and HindIII enzyme sites. The resulting transcript of the recombinant vector forms a 19 base pair stem-loop structure that is quickly cleaved in the cell to form the functional siRNA (pSuper Manual, Oligo Engine, 2003).

In vitro synthesized, 21 to 23 nt long dsRNAs can act as siRNAs to elicit gene-specific inhibition in mammalian cells. Lately, DNA-based plasmid vectors for the delivery of siRNA into mammalian cells have been described (Figure 13). With these vectors small hairpin RNAs (shRNAs) are transcribed, which can be processed into functional siRNAs by Dicer. In addition to providing a low-cost alternative to the chemically synthesized siRNAs, this DNA-vector based strategy is capable of mediating stable target gene inhibition, thus allowing gene function analysis over an extended period of time. This approach appears to function in a

wide variety of cells, including established cell lines, such as NIH3T3, HeLa, HEK-293T and COS cells. However, in mammalian cells, siRNA-mediated gene inhibition, leads to a maximal reduction of 80-90% and never completely eliminates the target gene product. Therefore, it must be considered a 'knock-down' rather than a 'knock-out' approach (Shi, 2002).

1.6.2. pSuper and pSuperior RNAi System

The pSuper RNAi system provides a mammalian expression vector (Figure 13) that directs the synthesis of siRNA-like transcripts. This vector uses the polymerase-III Histone1 (H1)-RNA gene promoter, as it produces a small RNA transcript lacking a polyadenosine tail. Additionally, the H1 promoter has a well-defined start of transcription and a termination signal consisting of five thymidines in a row (T5). Most importantly, the cleavage of the transcript at the termination site occurs after the second uridine, yielding a transcript resembling the ends of synthetic siRNAs, which also contain two 3' overhanging T or U nucleotides (Brummelkamp et al., 2002). The pSuperior.gfp/neo vector carries EGFP and a neomycin resistance gene (Figure 14).



2. Aim of the Project

The aim of my diploma project was to characterize the role of Cl-6, a novel MuSK binding protein, *in vitro*. My work focused on four distinct goals: (1) to narrow down the binding site of Cl-6 with MuSK, (2) to investigate the interaction of Cl-6 with Rab5 and the region required on Cl-6 for this interaction, (3) to investigate the interaction between Cl-6 and endogenous MuSK expressed in muscle cells and (4) to determine the function of Cl-6 during Agrin-induced AChR clustering.

The results of this study are described in the next chapter.

3. Results

3.1. Interaction Studies of Cl-6

3.1.1. Cloning of GST-Fusion Proteins

Different Cl-6 constructs were cloned into glutathione S-transferase (GST) containing vectors to gain constructs with a N-terminal GST-Tag (see Materials and Methods). The cloning strategies are shown in Figure 15.

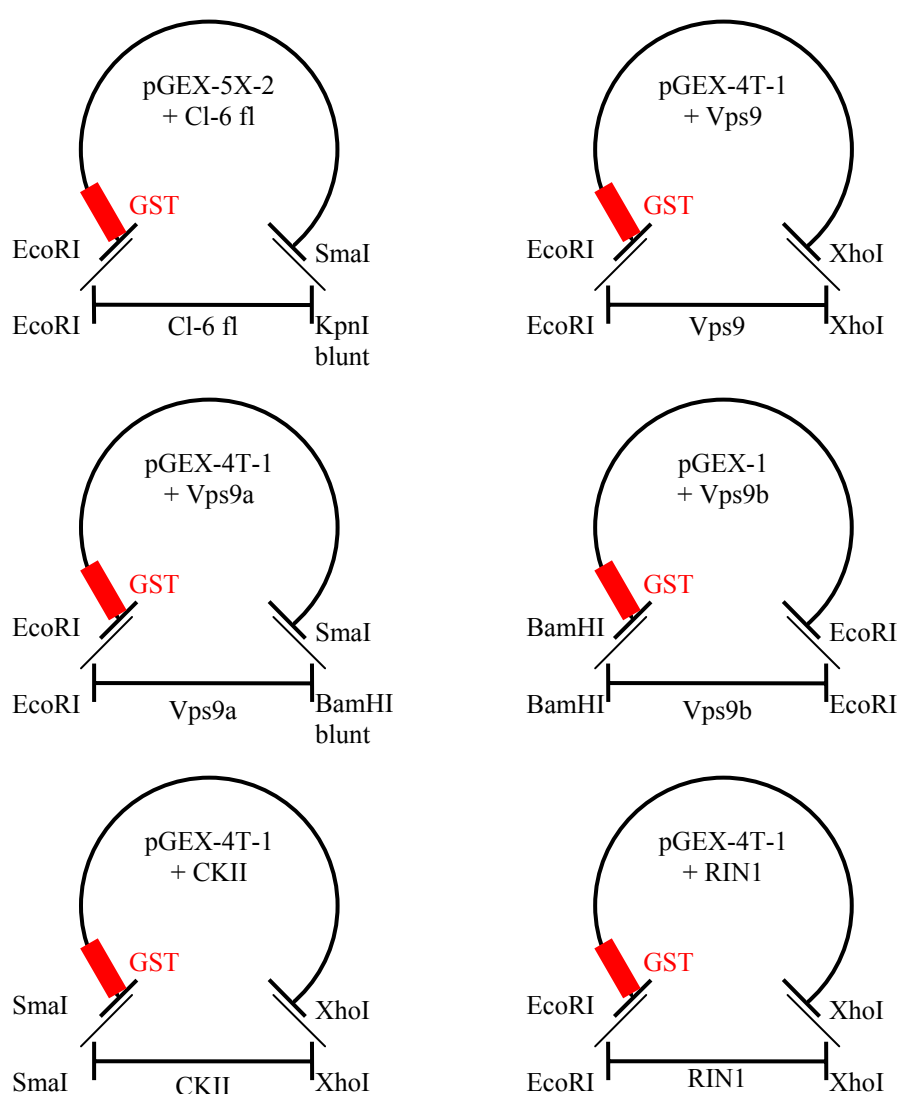


Figure 15. Scheme of the Different GST-Fusion Protein Containing Plasmids. For further information about the cloning procedure see Materials and Methods.

Figure 16 shows an overview of the Cl-6 truncation proteins, which were used for the pull-down experiments.

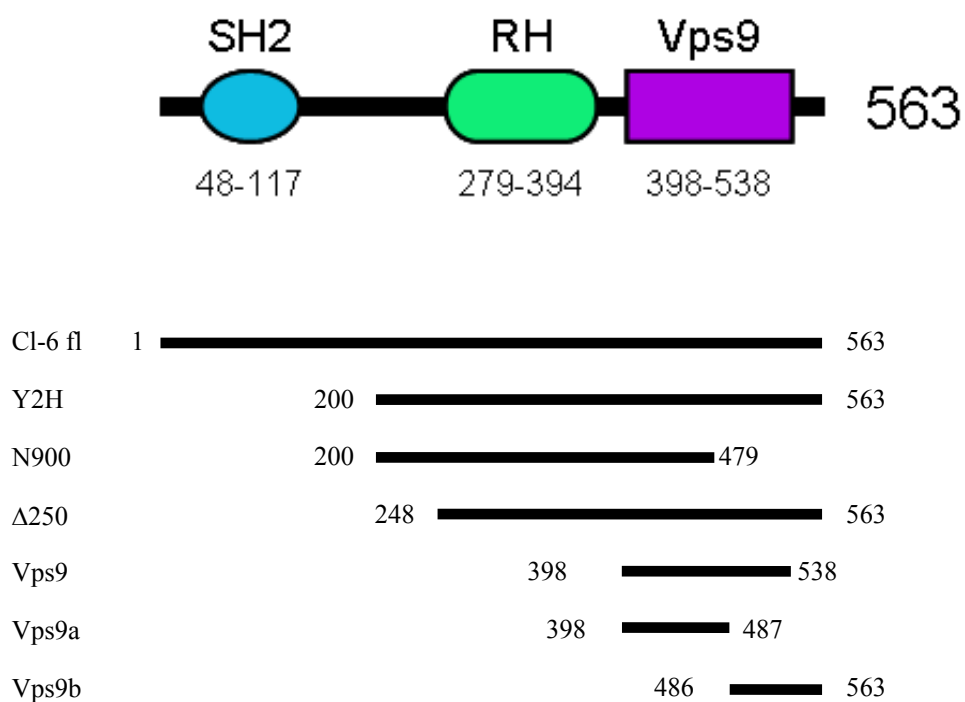


Figure 16. Schematic Diagram of the Different Cl-6 Constructs Used for GST Pull-Downs. Numbers next to the schemes represent the amino acid residues. SH2, Src homology domain; RH, RIN homology domain; Vps9, guanine nucleotide exchange factor domain.

3.1.2. Expression and Purification of GST-Fusion Proteins

3.1.2.1. Expression of GST-Fusion Proteins

The different GST-fusion protein containing plasmids were transformed into the *E. coli* strain Rosetta and expression was induced by the addition of IPTG (see Materials and Methods).

3.1.2.2. “Test Purifications” of GST-Fusion Proteins

For the purification of the GST-fusion proteins glutathione-agarose beads were used. At first the protocol was adjusted to achieve good yields for all proteins. Therefore “test purifications” were performed with and without sarkosyl. This determination was important, because on one hand sarkosyl helps to keep the proteins soluble, but on the other hand it interferes with the binding of the GST-fusion proteins to the beads. After the purification aliquots were subjected to SDS-PAGE. The bands were then visualized by Coomassie Brilliant Blue staining. In Figure 17 examples of such purifications of two proteins are shown. In the case of the GST-Tag alone the purification works better without sarkosyl, but in the case of RIN1 sarkosyl is necessary.

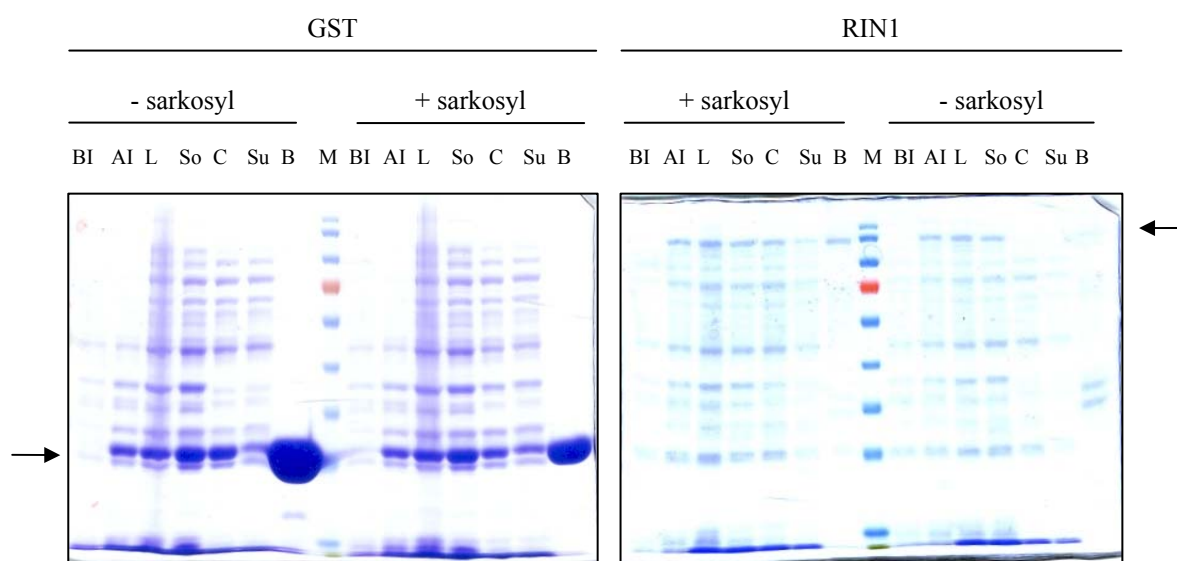


Figure 17. „Test Purifications“ of GST-Fusion Proteins. The GST-fusion proteins were expressed in Rosetta bacteria by induction with 0.5mM IPTG. Aliquots were taken before (BI) and after induction (AI). Comparing these two lanes successful induction of protein expression is observed. The bacteria were lysed and to some samples 0.25% sarkosyl was added before sonication. After a centrifugation step the clear lysates were diluted with lysis buffer to reduce the amount of sarkosyl and incubated with glutathione-agarose beads. Aliquots taken during the purification procedure were run on a 10% SDS polyacrylamide gel and proteins were visualized by staining with Coomassie Brilliant Blue. M, marker; BI, before induction; AI, after induction; L, lysate; So, sonicated; C, centrifuged; Su, supernatant; B, beads.

3.1.2.3. Large Scale Purifications of GST-Fusion Proteins

With the “test purifications” the method was specified and also the amount of how much protein can be gained was determined. Therefore large bacterial cultures were used and treated in the way that had been proven to be the best. Again aliquots were collected and subjected to SDS-PAGE followed by Coomassie Brilliant Blue staining to determine the purity and the concentration of the proteins (Figure 18).

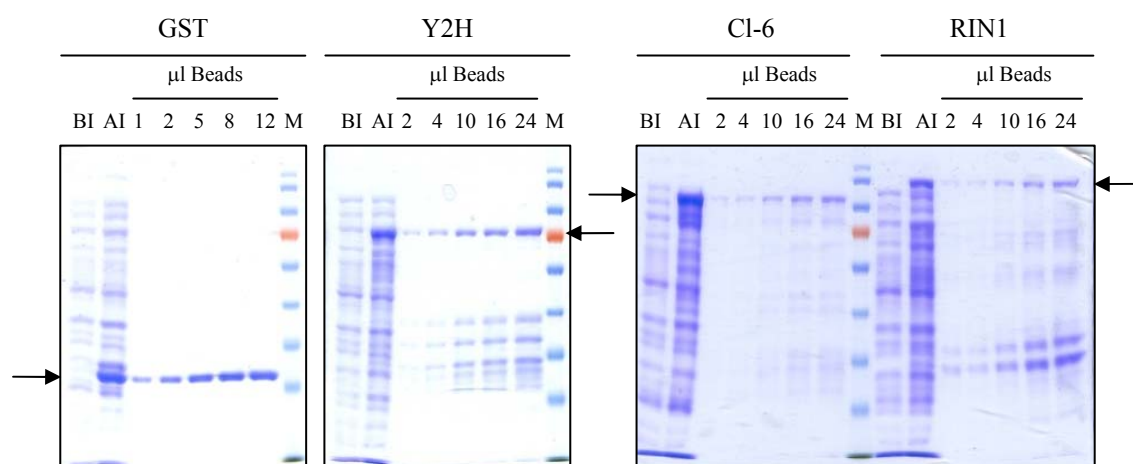


Figure 18. Purifications of GST-Fusion Proteins. The GST-fusion proteins were expressed in Rosetta bacteria by induction with 0.5mM IPTG. Aliquots are shown before (BI) and after induction (AI). Aliquots of the purified proteins were separated by SDS-PAGE and proteins were visualized by staining with Coomassie Brilliant Blue.

M, marker; BI, before induction; AI, after induction.

3.1.3. Interaction of CI-6 with Rab5

COS-7 cells were transfected with different GFP-Rab5 constructs, namely wild type (wt), the dominant negative mutant (S34N) and the constitutively active mutant (Q79L). In addition GFP-Rab7 (wt) was used to test for specificity. Aliquots of pooled lysates expressing the same GFP-Rab protein were separated by SDS-PAGE and subjected to Western blot analysis to determine the expression of the different constructs within the cells (Figure 19). This experiment was necessary to include similar amounts of GFP-Rab within one GST pull-down experiment.

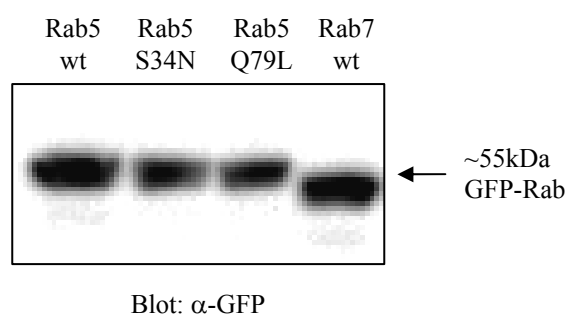


Figure 19. Expression of Different Rab Constructs. Lysates of C-7 cells transfected with GFP-Rab5 wt, S34N, Q79L or Rab7 were subjected to SDS-PAGE and Western blot analysis, using α -GFP as antibody. The expression of Rab7 and Rab5 wt was stronger compared to Rab5 S34N and Q79L.

For the pull-down experiments approximately equal amounts of GFP-Rab proteins were mixed with purified GST-fusion proteins coupled to the beads. Pull-downs were performed as described in Material and Methods. The samples were subjected to SDS-PAGE followed by Western blot analysis. Figure 20A shows that Cl-6 interacts specifically and robustly with the dominant negative mutant of Rab5 (S34N) but not with the other Rab proteins that were tested. Several control reactions were performed. Rab7 was used to check the specificity of the binding between Cl-6 and Rab5. RIN1 served as a positive control, since it has already been published, that RIN1 preferentially interacts with Rab5 S34N (Tall et al., 2001), and the GST alone was used to show that the interaction is specific and not due to cross-reaction via the GST-Tag or unspecific binding to the glutathione-agarose beads (Figure 20B).

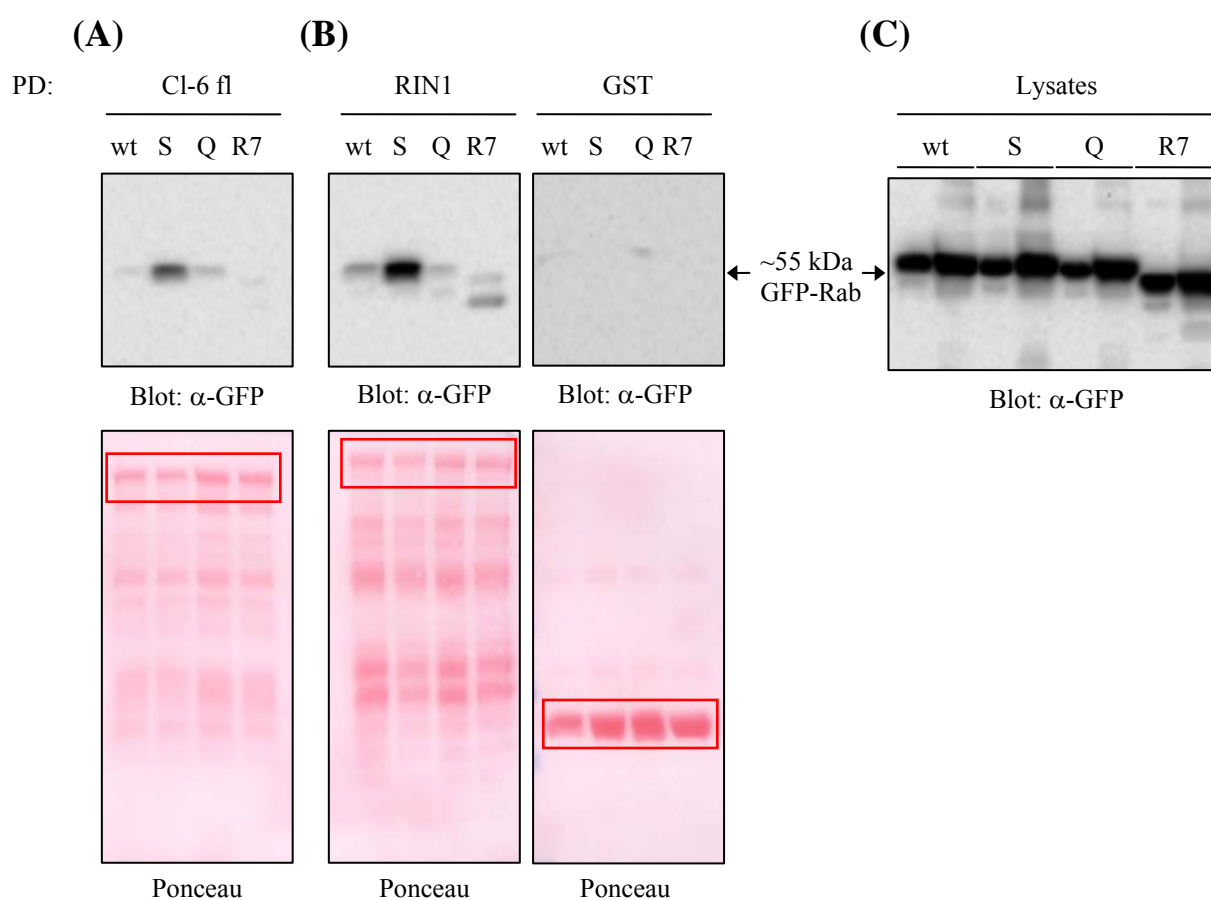


Figure 20. Interaction of Cl-6 with Rab5. COS-7 cells were transfected with different GFP-Rab proteins. The cells were lysed and a GST pull-down was performed with the indicated GST-fusion proteins followed by immunoblotting with α -GFP (1:1000) and Ponceau staining. **(A)** The pull-down with Cl-6 fl is shown. **(B)** The pull-downs with RIN1 and GST are shown. **(C)** Two different volumes of lysates are shown to confirm that the amounts of GFP-Rab proteins that have been used are comparable. Red rectangles mark the used GST-fusion proteins. PD, pull-down; wt, Rab5 wt; S, Rab5 S34N; Q, Rab5 Q79L; R7, Rab7.

To determine which parts of the Cl-6 protein are needed for its interaction with Rab5 S34N, different truncation mutants, namely Y2H, N900, Δ 250 and Vps9 (see Figure 15), were used. Our predictions were that all but the N900 should be able to interact with Rab5, since all but the N900 contain the Vps9 domain. Again COS-7 cells were transfected with GFP-Rab5 (wt, S34N, Q79L) and after cell lysis subjected to a pull-down using the above described GST-fusion proteins. Then the samples were separated by SDS-PAGE and Western blotting was performed using α -GFP antibodies (Figure 21). As expected, all Cl-6 constructs with the exception of N900, which lacks the Vps9 domain, did interact with Rab5 and all of them interacted specifically with the dominant negative mutant of Rab5 (S34N). In case of the Vps9 truncation only a very weak interaction was detectable. Crystallization structure previously revealed that a helical bundle N-terminal to the Vps9 domain, is needed for its function (Delprato et al., 2004). Therefore, this Vps9 truncation might not be fully functional and this might explain the weak interaction.

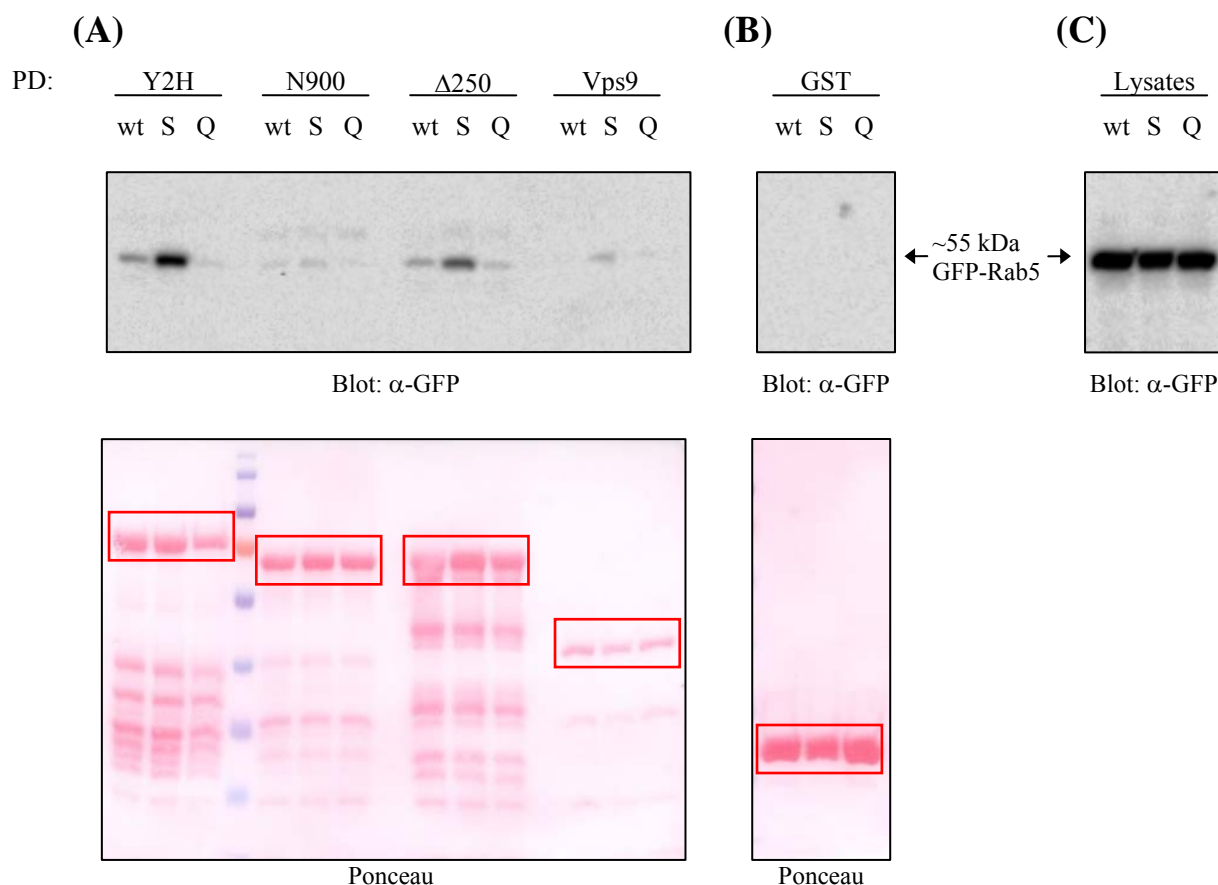


Figure 21. Interaction of Deletion Constructs of Cl-6 with Rab5. COS-7 cells were transfected with different GFP-Rab5 constructs as mentioned. The cells were lysed and a GST pull-down was performed with the indicated GST-fusion proteins followed by immunoblotting with α -GFP (1:1000) and Ponceau staining. (A) Interactions of the Cl-6 constructs with the different Rab5 proteins are shown. (B) Results of the control experiments where the pull-downs were only performed with the GST-Tag are shown. (C) An aliquot of each lysate is shown to confirm that the amounts of GFP-Rab5 proteins that have been used are comparable. Red rectangles mark the used GST-fusion proteins. PD, pull-down; wt, Rab5 wt; S, Rab5 S34N; Q, Rab5 Q79L.

3.1.4. Interaction of Cl-6 with MuSK

Cl-6 was found as an interaction partner of the cytoplasmic region of MuSK in a Y2H screen and this interaction has been confirmed by co-immunoprecipitation and co-localization studies (unpublished data by Barbara Woller). Therefore, the next step was to determine the region within Cl-6 that is necessary for this interaction. For that purpose, HEK-293T cells were transfected with MuSK-myc and after lysis, the cell lysates were subjected to a pull-down using the different purified GST-fusion proteins described in Figure 15. As shown in Figure 22, Cl-6, Y2H, N900 and $\Delta 250$ interacted with MuSK, whereas the Vps9 constructs did not. CKII was used as a positive control, since it has been shown previously, that CKII interacts with MuSK (Cheusova et al., 2006). As expected, a strong interaction of CKII with MuSK can be observed on the blot. Surprisingly, RIN1 also interacted with MuSK, similarly to Cl-6.

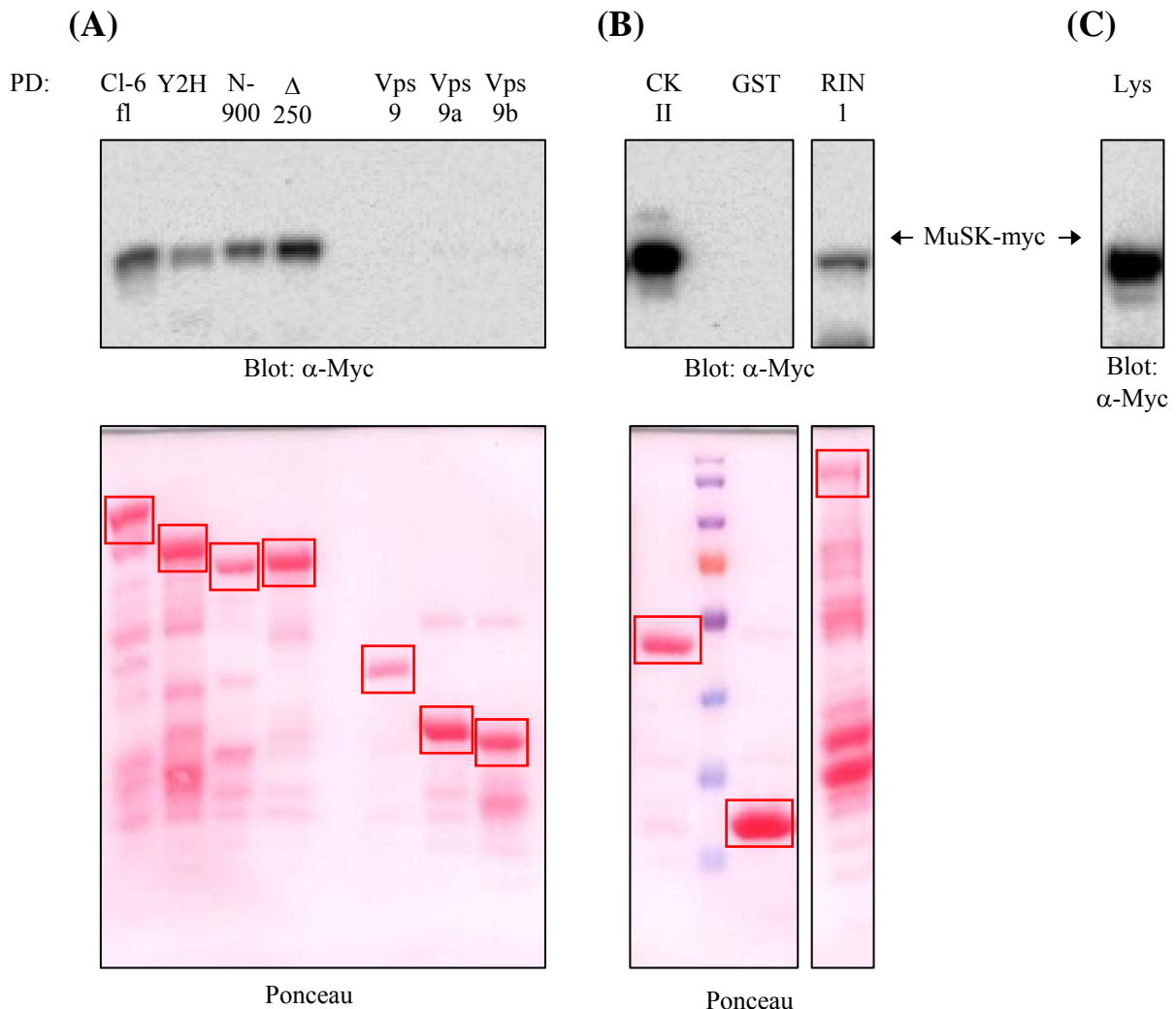


Figure 22. Interaction of Cl-6 with MuSK. HEK-293T cells were transfected with MuSK-myc. The cells were lysed and a GST pull-down was performed with the indicated GST-fusion proteins followed by immunoblotting with α -Myc (1:5000) and Ponceau staining. (A) The pull-down experiments with the different Cl-6 constructs are shown. (B) The pull-down experiments of the two controls, CKII and GST, and of RIN1 are shown. (C) An aliquot of the lysate is shown.

Red rectangles mark the used GST-fusion proteins. PD, pull-down; Lys, lysate.

3.1.5. Interaction of Cl-6 with Endogenous MuSK of C2C12 Cells

Since the GST pull-down experiments showed a specific and direct interaction between MuSK-myc and Cl-6, we next asked, whether this interaction can also be detected with the endogenous MuSK expressed in C2C12 muscle cells. Therefore, differentiated C2C12 cells were starved for 2.5 to 3 hours, treated with or without Agrin for 30min and then lysed. The lysates were subjected to a pull-down using GST fusion proteins. In this experiment Cl-6 was used as well as GST and CKII, which served as negative and positive control, respectively. As an additional control, cell lysates were immunoprecipitated with α -MuSK antibodies. As shown in Figure 23, no interaction between MuSK and Cl-6 can be detected. Although Agrin induces a strong phosphorylation of MuSK as detected with α -pY antibodies, no interaction of MuSK with CKII is observed. This is surprising since CKII has been found to interact preferentially with phosphorylated MuSK (Cheusova et al., 2006). All of the observed signals appear to be unspecific, since they are either above or below the MuSK signal which was gained by immunoprecipitation or signals are the same for GST as for CKII and Cl-6. This suggests that the pull-down is not sensitive enough to detect interactions between endogenous MuSK and GST-fusion proteins.

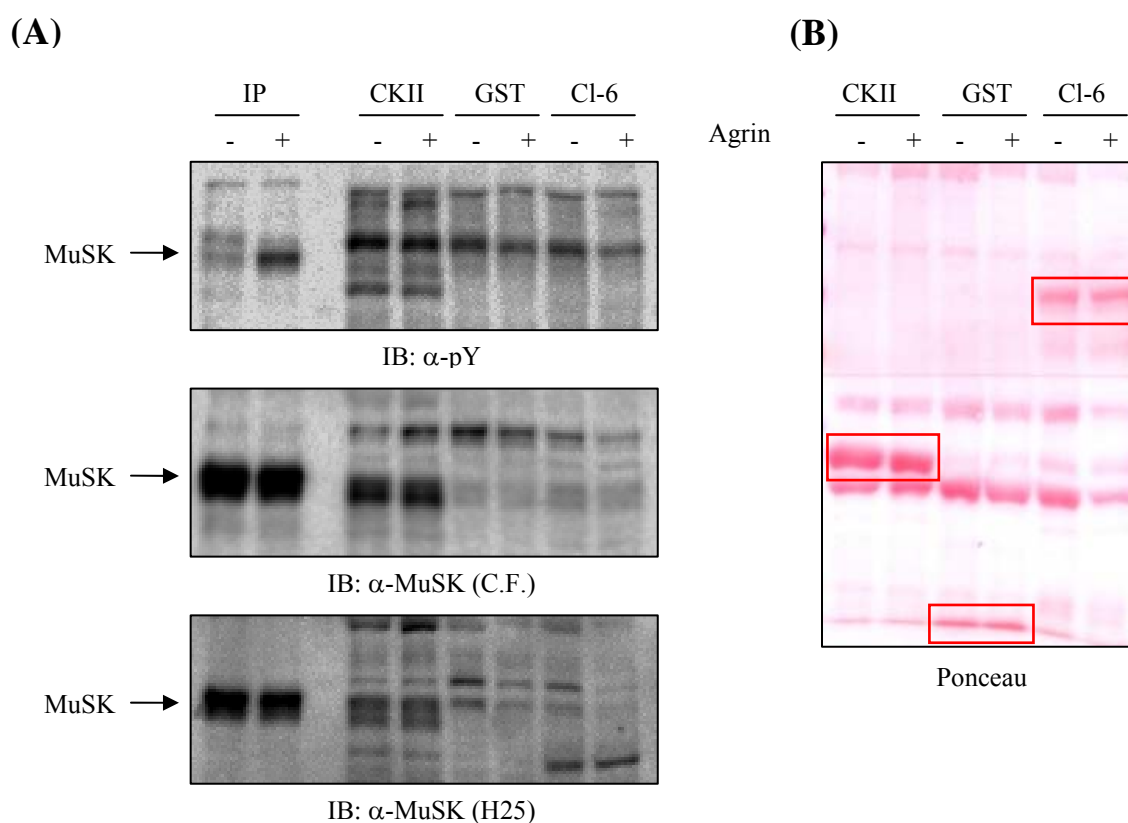


Figure 23. Interaction of Cl-6 with Endogenous MuSK of C2C12 Cells. Differentiated C2C12 cells were treated with or without agrin and lysed. The lysates were then subjected to GST pull-downs with the indicated constructs or immunoprecipitation (IP) with α -MuSK (1:125) antibodies and followed by **(A)** immunoblotting (IB) with α -pTyr (Cell Signaling, p100 1:2000; Santa Cruz, p99 1:1000), α -MuSK (C.F.) and α -MuSK (H25, 1:250) antibodies and **(B)** Ponceau staining. Red rectangles mark the used GST-fusion proteins.

3.2. RNAi Experiments to Knock-Down Cl-6 Expression

To study the function of Cl-6 an already established RNAi approach (Barbara Woller, Diploma thesis) was used, to knock-down the expression of Cl-6 and determine its influence on the clustering of AChRs.

3.2.1. RNAi Oligos Targeting Cl-6

To suppress Cl-6 expression, siRNA sequences were selected using different RNAi design tools.

Three different sequences, located at position 348, 1529 and 2034 of the full-length *Cl-6* cDNA were chosen (Figure 24).

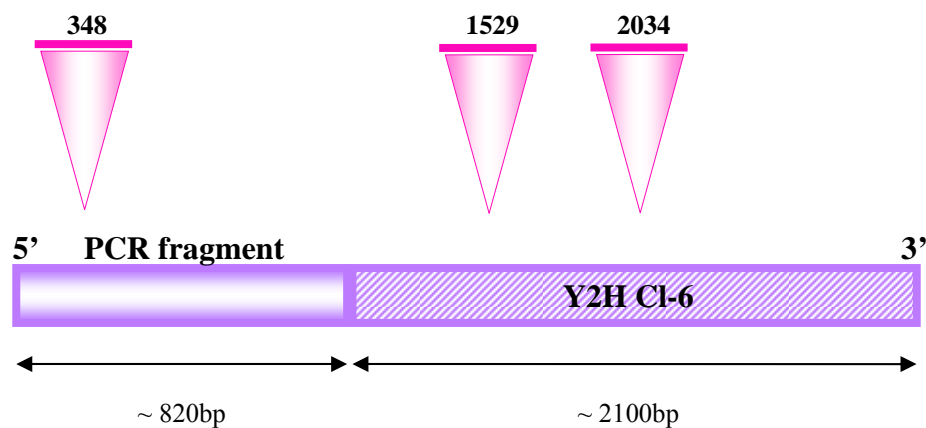


Figure 24. Scheme of the Cl-6 cDNA with RNAi Oligos Targeting the Gene at Different Positions. Oligos 1529 and 2034 are located in the original Y2H cDNA sequence, 348 targets the Cl-6 5'-end (Barbara Woller, Diploma thesis).

Additionally, two constructs with the scrambled siRNA base pair composition located at position 348 and 2034 of the full-length Cl-6 cDNA were made, in order to test the specificity of suppression of Cl-6 expression and to produce a negative control for siRNA-mediated knock-down (Michaela Mutzl, Diploma thesis).

3.2.2. Selection of Clones with Stable siRNA Expression

For analysis of Cl-6 function, several cell lines stably carrying the pSuperior.gfp/neo vector with different siRNA oligos were generated. The pSuperior.gfp/neo RNAi system expresses EGFP and has a neomycin resistance in addition to its function of siRNA mediated downregulation of gene translation. Cell lines expressing the different pSuperior constructs were generated (Michaela Mutzl, Diploma Thesis).

The clones were expanded and tested for proper myotube formation as well as for the presence of GFP in myotube cell lysates (Figure 25). Positive clones showing normal myotube formation and expression of GFP are listed in Table 1. Only for two siRNA

constructs (348 and 2034) and two control constructs (empty and 2034 scrambled) GFP expression was detectable.

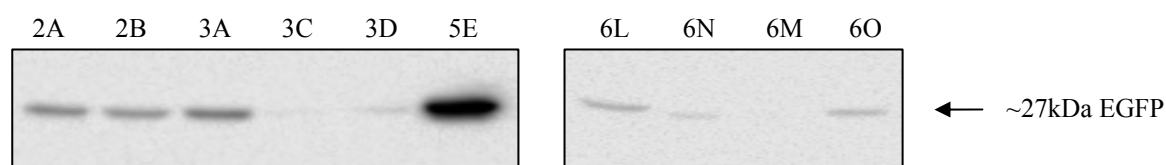


Figure 25. Stable Cell Lines Carrying the pSuperior.gfp/neo Vector. C2 cells were transfected with Lipofectamine 2000 and selected using 700µg/ml G418 in the culture medium. Single clone colonies were picked, cells were again expanded and myotube cell lysates were subjected to immunoblotting (IB) with α -GFP (Santa Cruz, 1:1000) antibody.

2, pSuperior-empty; 3, pSuperior-348; 5, pSuperior-2034; 6, pSuperio-2034 scrambled; the letters just denote the different cell lines that have been tested.

Cell line	Myotube formation	GFP positive clones
pSuperior-empty	Normal	2A, 2B
pSuperior-348	Normal	3A
pSuperior-2034	Normal	5E
pSuperior-2034 scrambled	Normal	6L, 6O

Table 1. Stable Cell Lines Containing pSuperior/RNAi Constructs.

3.2.3. Role of Cl-6 in AChR Clustering

To investigate the influence of endogenous Cl-6 on AChR clustering, one positive cell line of each construct (2B, 3A, 5E, 6L) was used. For this purpose differentiation of the cells into myotubes was induced by applying differentiation medium containing G418. After differentiation into myotubes, the clustering of AChRs was induced by stimulation with Agrin and AChRs were visualized by staining with Alexa594-conjugated α -bungarotoxin (BTX). Myotubes were then fixed with 2% PFA and mounted using VectaShield (Vector Laboratories Inc.) or Mowiol. As shown in Figure 26A, the cell lines stably expressing the constructs containing the two siRNAs (348 and 2034) show a reduced AChR cluster length compared to the controls (empty and 2034 scrambled).

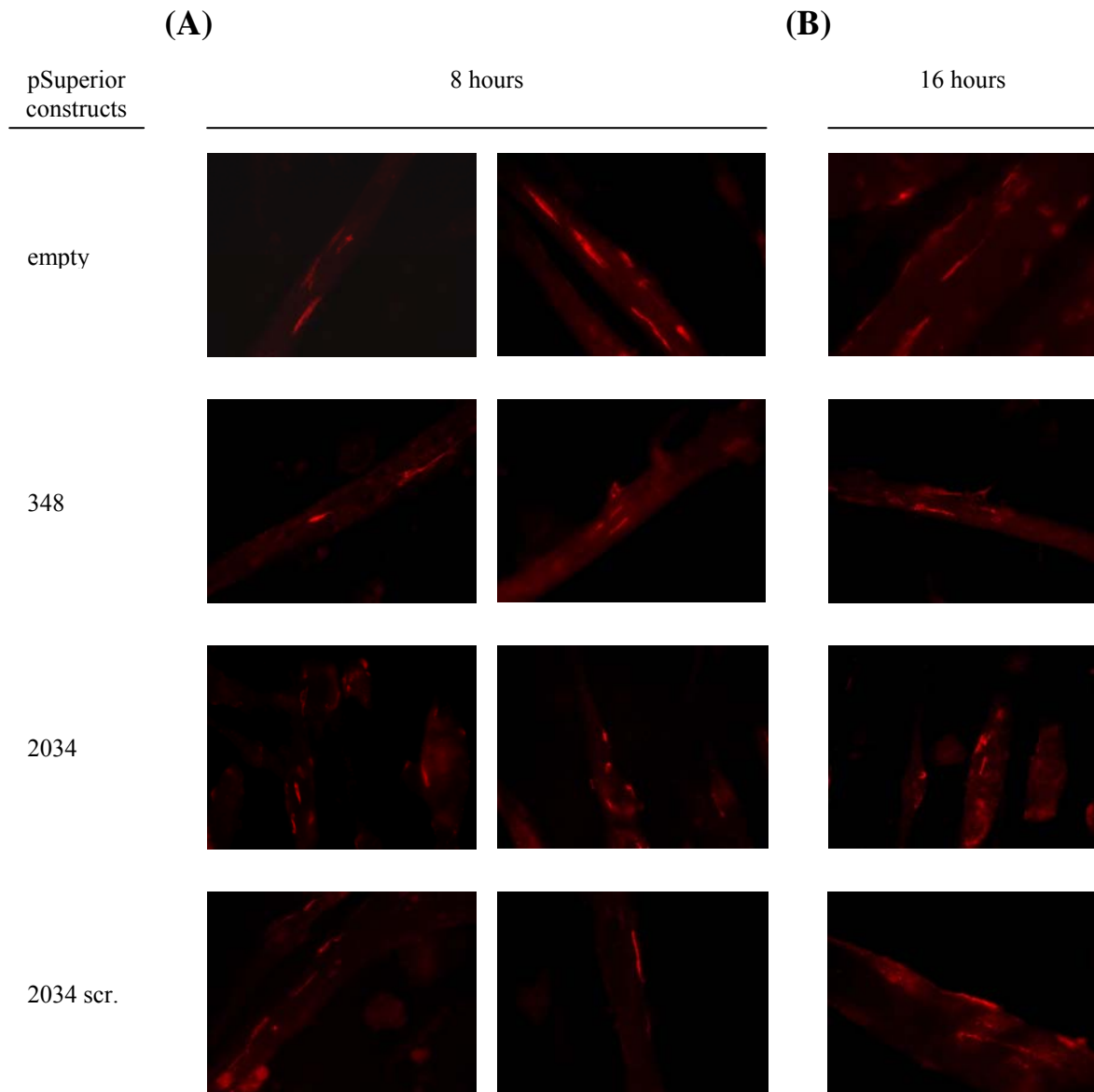


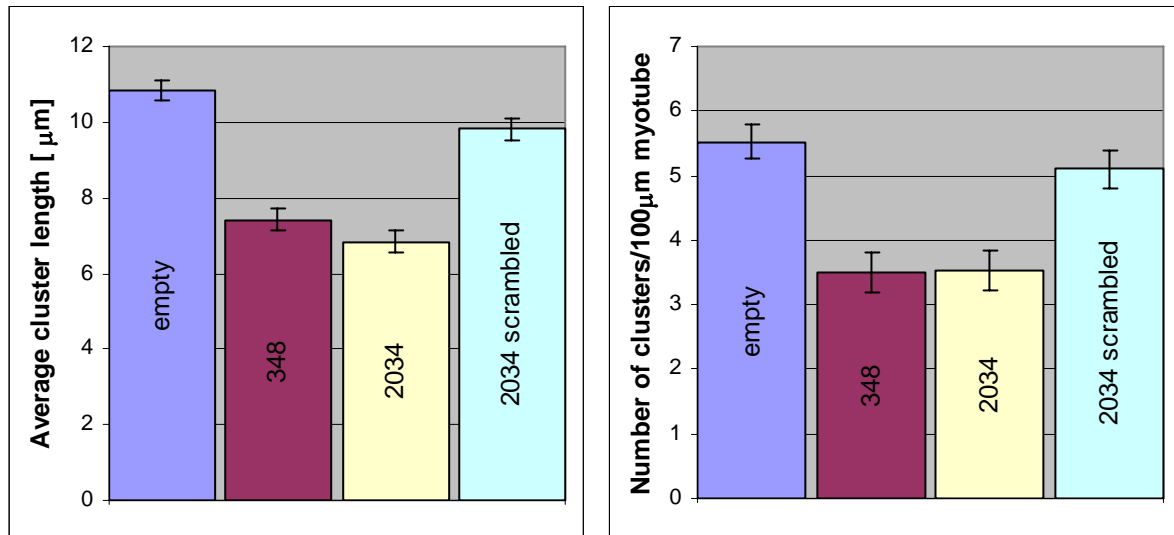
Figure 26. Downregulation of Cl-6 Interferes with AChR Clustering. Representative images from different BTX-labeled C2 stable cell lines are shown. Clustering of AChRs was induced by incubation in nerve-derived Agrin-containing medium for (A) 8 or (B) 16 hours as indicated.

A detailed analysis was performed by measuring the AChR cluster length and counting the AChR cluster numbers. The number and the average length of AChR clusters were significantly decreased in the stable cell lines, which express the siRNA 348 or 2034 compared to myotubes expressing pSuperior empty or 2034 scrambled (Figure 27A). In addition, the size distribution of AChR clusters was altered (Figure 27B). The number of microclusters was increased at the expense of large-size clusters ($>10\mu\text{m}$). The fraction of the medium sized clusters increased also a little bit but not as dramatically as the fraction of the microclusters.

Many studies published by other researchers use 16 hours of Agrin stimulation. To test whether this treatment has the same effect, I stimulated myotubes for 16 hours (Figure 26B). I observed that a longer Agrin treatment leads to a reduction in the average cluster length and the number of cluster per myotube (compare Figure 27A and 28A) and also changes the size

distribution of the AChR clusters (compare Figure 27B and 28B). In general, this longer treatment with Agrin reduced the difference seen between the RNAi treated and the control cell lines.

(A)



(B)

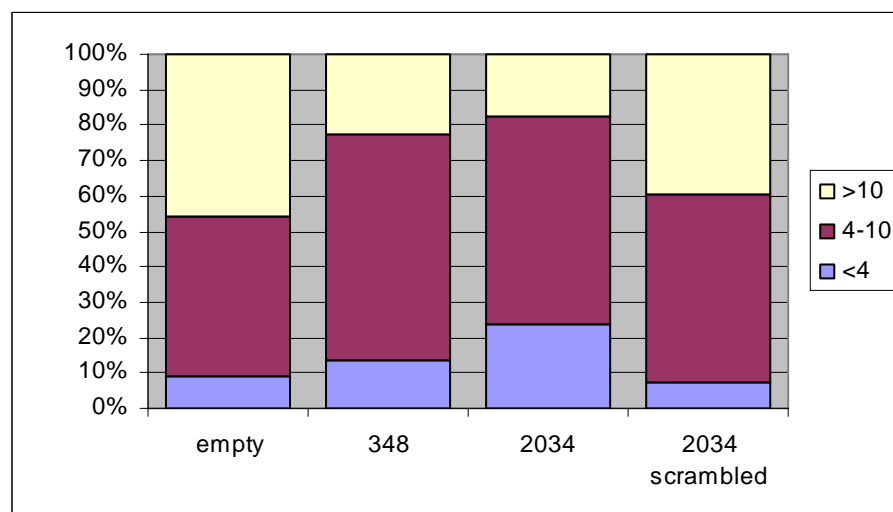
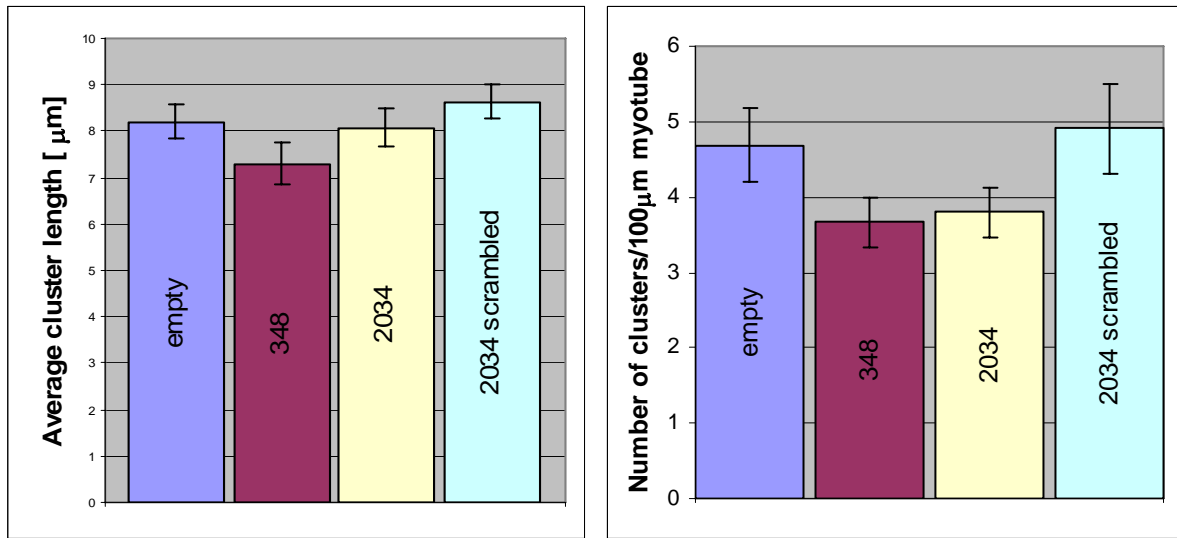


Figure 27. Downregulation of Cl-6 Leads to a Reduction of AChR Cluster Length and Number in Stable Cell Lines and Alters the Distribution of the Size of the AChR Clusters. Quantification of the effect after 8 hours of Agrin stimulation. (A) The average cluster length (on the right) and the number of clusters per 100μm myotube are shown. (B) All visible clusters were assigned to the three groups according to their size, and percentage of each group is shown. Error bars \pm 1.0 SEM (Standard error of mean).

(A)



(B)

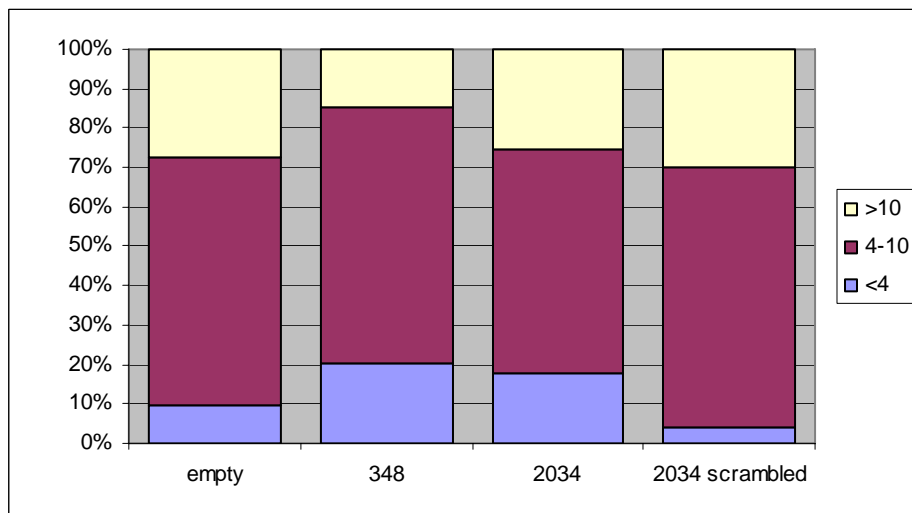


Figure 28. Downregulation of Cl-6 Leads to a Reduction of AChR Cluster Length and Number in Stable Cell Lines and Alters the Distribution of the Size of the AChR Clusters. Quantification of the effect after 16 hours of Agrin stimulation. **(A)** The average cluster length (on the right) and the number of clusters per 100 μm myotube are shown. **(B)** All visible clusters were assigned to the three groups according to their size, and percentage of each group is shown. Error bars ± 1.0 SEM (Standard error of mean).

3.2.4. Agrin-Induced MuSK Phosphorylation in Muscle Cells Expressing Cl-6 RNAi

To check whether Cl-6 downregulation also has an influence on MuSK expression and/or activation, differentiated myotubes were stimulated with or without Agrin and cells were lysed. The cell lysates were immunoprecipitated with α -MuSK followed by SDS PAGE and immunoblotting with α -pTyr and α -MuSK. An aliquot of each lysate was immunoblotted with α -Actin to check for equal loading (Figure 29).

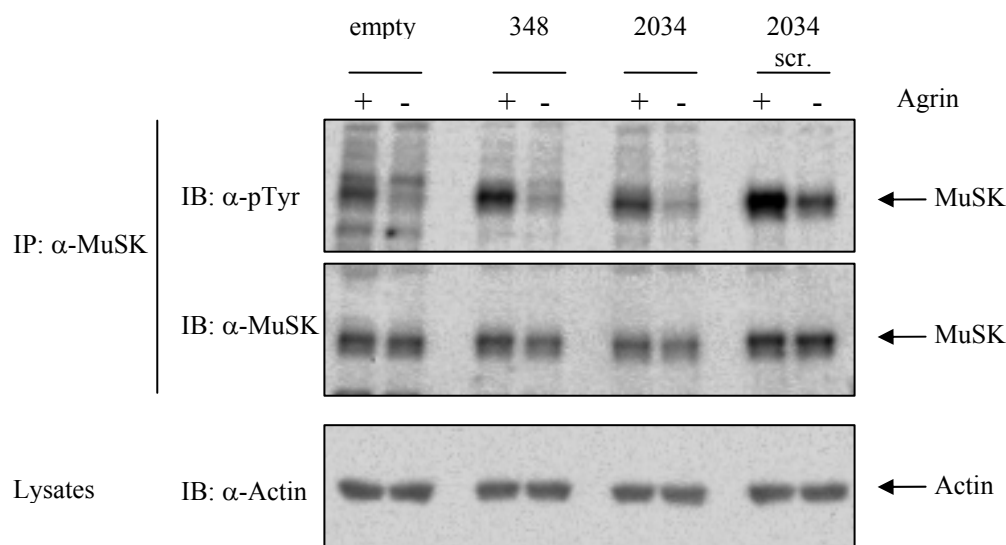
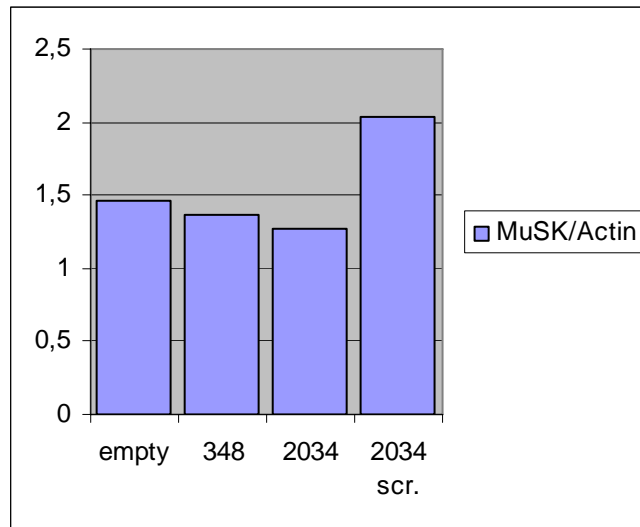


Figure 29. Expression and Phosphorylation of MuSK in C2 Stable Muscle Cell Lines. Lysates of stable cells lines were immuoprecipitated (IP) with α -MuSK (1:125) and immunoblotted (IB) with α -pTyr (Cell Signaling, p100 1:2000; Santa Cruz, p99 1:1000) and α -MuSK (1:250). To ensure that the same amount of lysate was used, an aliquot of the lysate was immunoblotted with α -actin.

A quantification of the results from Figure 29 revealed that no strong effect on MuSK expression and phosphorylation can be observed when Cl-6 is downregulated by siRNAs (Figure 30). These data were only gained from one experiment and therefore need to be confirmed by further studies.

(A)



(B)

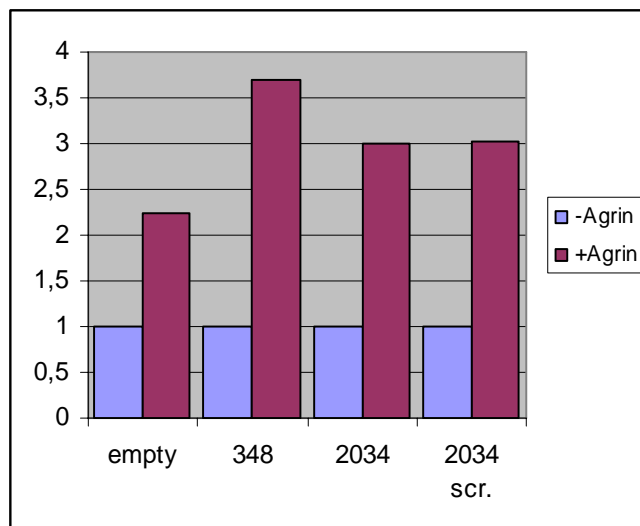


Figure 30. MuSK Activation and Expression in C2 Cell Lines Expressing pSuperior/RNAi. Quantification of the data shown in Figure 29. (A) The expression of MuSK is shown as the ratio between the MuSK and the actin signals. (B) MuSK phosphorylation is shown as the fold induction after Agrin treatment, with values of unstimulated cells set to 1.

4. Discussion

MuSK represents the central key player during the development of the NMS. However, the signaling cascade regulated by MuSK is not yet fully understood. Likewise, few direct interaction partner of MuSK have been identified, especially in comparison to RTKs, like EGFR (epidermal growth factor receptor). Binding partners include Scr, Dishevelled, Syne-1, Dok-7, Tid1, Abl and CKII (Apel et al., 2000; Cheusova et al., 2006; Finn et al., 2003; Linnoila et al., 2008; Luo et al., 2002; Mohamed et al., 2001; Okada et al., 2006; Sadasivam et al., 2005).

This study focuses on a protein termed Cl-6, which was found as an interaction partner of MuSK in a Y2H screen (Ruth Herbst, unpublished). The interacting regions of Cl-6 with MuSK and Rab5 were narrowed down using a GST pull-down assay supporting the notion that this newly identified protein may play a role during MuSK and Rab5 functions. Another important finding of this study was the observation that the targeting of Cl-6 with siRNAs attenuates Agrin-induced AChR clustering. This suggests that Cl-6 is an important component of the Agrin/MuSK signaling pathway and plays an important role in AChR clustering at the NMS.

4.1. Cl-6 – a Novel Member of the RIN Family

Database searches with Cl-6 revealed striking homologies to the three members of the RIN protein family, RIN1, RIN2 and RIN3 (Han and Colicelli, 1995; Kajiho et al., 2003; Saito et al., 2002). In particular, the domain structure between the proteins appears highly conserved (Barbara Woller, Diploma thesis). Cl-6 shares the SH2, the Vps9 and most importantly also the RH (RIN homology) domain with the RIN proteins, but lacks proline rich domains and the RA (Ras association) domain. Consequently, we propose that Cl-6 shares functional properties with the members of the RIN family.

4.2. Interaction of Cl-6 with MuSK and Rab5

In this study the interactions of Cl-6 with MuSK and Rab5 have been investigated. Figure 31 shows a scheme summarizing these interactions.

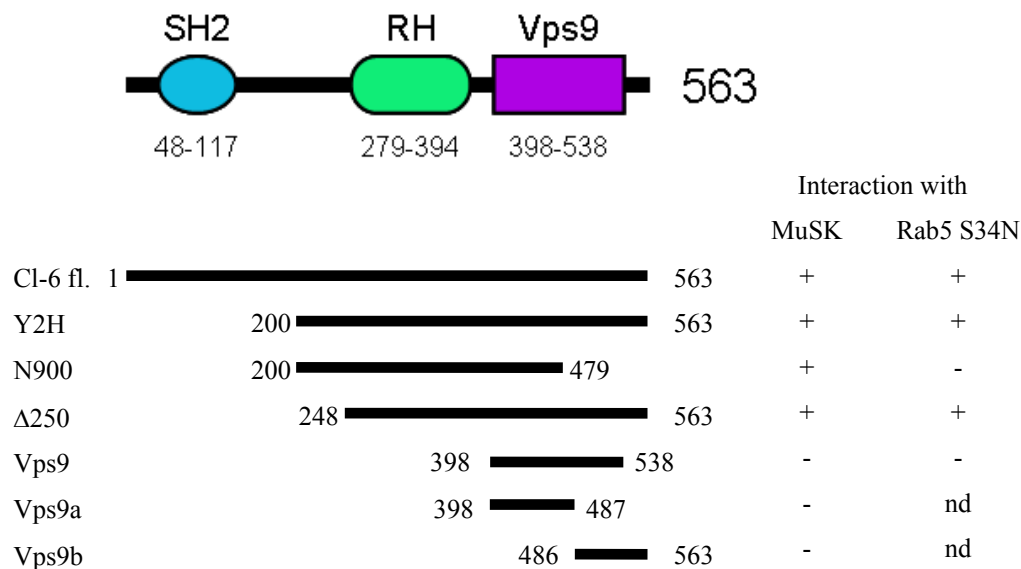


Figure 31. Scheme of the Interactions of Cl-6 with MuSK and Rab5.
+, interaction; -, no interaction; nd, not determined.

4.2.1. Interaction of Cl-6 with MuSK-Myc and Endogenous MuSK of C2C12 Cells

As shown in Figure 31, the Cl-6 truncations, which contain the RH domain, were able to interact with MuSK, namely Y2H, N900, Δ250. These results were consistent with a previous Y2H screen, in which the RH domain has also been identified as the interacting domain of Cl-6 with MuSK (Ruth Herbst, unpublished data). Since the RH domain, which is shared by all proteins of the RIN family, mediates this interaction, it is not surprising that RIN1 also interacts with MuSK. Although it has not been tested whether the interaction between MuSK and RIN1 depends on the RH domain of RIN1, it seems likely that the RH domain mediates this interaction. It would be interesting to determine, whether this interaction of RIN1 with MuSK has any significance *in vivo* and if RIN2 and/or RIN3 are also able to interact with MuSK through this domain.

Additionally, we wanted to show the interaction between Cl-6 and the endogenous MuSK expressed in C2C12 cells. We reasoned that this assay would be more specific since it does not depend on the overexpression of MuSK-myc in a heterologous cell system. However, I was unable to detect any interaction of Cl-6 with MuSK. There are several possible explanations. First of all, the GST pull-down assay has a limited sensitivity. A lot of MuSK protein was required to gain an interaction, which lead to the conclusion, that the amount of MuSK present in the C2C12 cells might have been too low. Secondly, we had to use a MuSK antibody for detection, which showed unspecific binding activity. In particular it recognized some other proteins of similar size as MuSK, making it difficult to check for the presence of a weak band eventually corresponding to MuSK. Furthermore, it was not possible to detect an interaction between CKII and endogenous MuSK. It has been reported that CKII interacts preferentially with the phosphorylated MuSK (Cheusova et al., 2006). I however, was never

able to detect an interaction using either phosphotyrosine or MuSK antibodies. This further supports the notion that the GST pull-down is not suitable to detect interactions between Cl-6 and the endogenous MuSK of C2C12 cells.

4.2.2. Interaction of Cl-6 with Rab5

Rab5 has already been shown to interact with the Vps9 domain of all three RIN proteins (Kajiho et al., 2003; Saito et al., 2002; Tall et al., 2001). Therefore, we hypothesized an interaction between the Vps9 domain of Cl-6 and Rab5. Using GST pull-down, only Cl-6, Y2H and Δ 250 interacted with Rab5, while N900 and Vps9 did not (Figure 31). This result suggests that the classical Vps9 domain is required but not sufficient and that additional amino acids N-terminal of the Vps9 domain are essential. Indeed, it turned out that the predicted Vps9 domain, does not represent the whole Vps9 domain, but lacks the helical bundle. This has been shown for Rabex-5 by crystallization studies (Delprato et al., 2004) and also for RIN1 by Y2H screens (Tall et al., 2001). Therefore, the Δ 250 construct represents the whole functional Vps9 domain, with the helical bundle and the classical Vps9 domain.

Cl-6 interacted preferentially with the dominant negative mutant of Rab5, S34N, which has also been demonstrated for RIN1 (Tall et al., 2001), although the interaction between Cl-6 and Rab5 appeared weaker than between RIN1 and Rab5. In contrast, the other two members of the RIN family, RIN2 and RIN3, have been shown to preferentially interact with the constitutively active mutant of Rab5, Q79L, thereby stabilizing GTP-Rab5 (Kajiho et al., 2003; Saito et al., 2002). It has been shown that all three RIN proteins have GEF (guanine nucleotide exchange factor) activity towards Rab5 (Kajiho et al., 2003; Saito et al., 2002; Tall et al., 2001).

Rab5 is known as the key regulator of endocytosis, where it is involved in clathrin-coated vesicle formation, fusion between early endosomes, endosomal cargo recruitment and endosomal motility (Zerial and McBride, 2001). This GTPase is rate-limiting for endocytosis and is regulated by PKB/Akt, which is a kinase coupled to signal transduction (Barbieri et al., 1998). GEFs of Rab5 can catalyze the exchange of GDP with GTP and thereby activate Rab5, which in turn can then activate or recruit effector molecules (Stenmark, 2009). For RIN1 it has been shown that it can interact with Rab5a, and that this interaction is necessary for EGFR endocytosis (Chen et al., 2009). Therefore, evidence that Cl-6 also possesses GEF activity for Rab5, would strongly indicate that Cl-6 plays a role in the endocytic processing of surface molecules, maybe of MuSK.

4.2.3. RIN1 and Cl-6

The observation that Cl-6 and RIN1 show the same interaction pattern regarding Rab5 and MuSK, suggests that Cl-6 might have a similar function in the cell as RIN1. RIN1 has been shown to interact with a variety of different proteins through its different functional domains. The SH2 domain is responsible for the interaction with activated receptors via phosphotyrosine residues and the proline rich domain is able to interact with SH3 domains. SH2 and SH3 domains are involved in the assembly of signal transducing protein complexes and in the regulation of non-receptor tyrosine kinases, such as Src and Abl (Han et al., 1997). Additionally, there are a Vps9 domain, which has been shown to serve as GEF for Rab5 (Tall et al., 2001) and the RA domain, which directly interacts with Ras (Colicelli et al., 1991).

The EGFR, the interleukin-3 (IL3) receptor, the insulin receptor and EphA4 receptor have been found as interaction partners of the SH2 domain of RIN1. Additionally, RIN1 has been shown to play an important role in the endocytosis and signaling of these receptor (Barbieri et al., 2003; Deininger et al., 2008; Hunker et al., 2006a; Hunker et al., 2006b). The interaction with activated EGFR may direct RIN1 to the correct membrane localization (Galvis et al., 2009). Overexpression of RIN1 accelerated EGFR degradation in EGF-stimulated cells. Consistently, depletion of endogenous RIN1 by RNAi resulted in a remarkable reduction of EGFR degradation. Additionally, signal-transducing adaptor molecule 2 (STAM2), which associates with hepatocyte growth factor-regulated substrate and plays a key role in the endosomal sorting machinery, was found as interaction partner of the proline rich domain of RIN1. This suggests that RIN1 regulates EGFR degradation in cooperation with STAM2, which defines a novel role for RIN1 in regulating endosomal trafficking (Kong et al., 2007). Other interaction partners of this proline rich domain are the Abl kinases, which bind via their SH3 domains (Han et al., 1997). However, Abl has been shown to phosphorylate a tyrosine (Y36) on RIN1 after interaction with the proline rich domain and this phosphorylated tyrosine residues interacts then with the SH2 domain of Abl (Hu et al., 2005). This interaction seems to promote a more accessible Abl catalytic site through relief of autoinhibition (Cao et al., 2008). Recently, it has been shown, that RIN1 preferentially interacts with Rab5a and that Rab5a is needed for EGFR degradation (Chen et al., 2009). It would be interesting to determine, if Cl-6 also possesses a preference for any Rab5 isoform.

RIN1 and Raf1 compete directly for binding to activated Ras. The normal localization and function of RIN1, but also its ability to compete with Raf1, are regulated in part by 14-3-3 proteins, which act as negative regulators of RIN1 membrane localization and Ras association (Wang et al., 2002). Activated Ras potentiates the stimulatory role of RIN1 on Rab5 guanine nucleotide exchange, Rab5a-dependent endosome fusion and EGFR-mediated endocytosis (Tall et al., 2001).

Ras signaling following its interaction with Raf is also known to mediate long-term potentiation (LTP) and long-term memory formation in postmitotic neurons. RIN1 seems to function through the competitive inhibition of Ras-Raf binding and leads to Ras signaling through alternate pathways. Additionally, it could be shown, that RIN1 is preferentially expressed in postnatal forebrain neurons in which it is localized in dendrites and physically associated with Ras. This suggests a role of RIN1 in Ras-mediated postsynaptic neuronal plasticity. RIN1 knock-out mice are viable and appear to develop normally, but show enhancement of aversive learning and memory. This suggests a negative modulating role for the Ras effector RIN1 in normal learning and memory (Dhaka et al., 2003).

RIN1 has been shown to be highly expressed in the brain, although the expression of RIN1 was not uniform in the whole brain. In addition, lower levels of RIN1 expression were found also in pancreas, kidney, lung, liver and placenta (Han and Colicelli, 1995). Cl-6 on the other hand is most strongly expressed in spleen, thymus and lung and additionally, lower levels are found in kidney, liver, heart, brain and skeletal muscle (Gloria Ikonge, Diploma thesis). Giving the different expression pattern of Cl-6 and RIN1 they might have some complementary roles in different tissues. Therefore, it will be interesting to determine how Cl-6 behaves towards the other interacting proteins of RIN1 and how its function is regulated, since it lacks the RA domain, which has been shown to have a regulatory function on the GEF activity of RIN1 (Tall et al., 2001).

4.3. Cl-6 Plays a Role in AChR Clustering

It has been shown that MuSK is internalized after Agrin stimulation (Zhu et al., 2008), just like other ligand-induced receptor tyrosine kinases, including EGFR (Schlessinger, 2004) and Trk Receptors (Zhang et al., 2000). The internalization of activated receptors is promoted via ligand binding and leads either to the attenuation of signaling via degradation of the ligand-receptor complex or signaling from endosomes (Ceresa and Schmid, 2000; Di Fiore and De Camilli, 2001). Agrin-mediated endocytosis of MuSK seems to be required for AChR clustering (Zhu et al., 2008). Endocytosis, which is characterized by the internalization of molecules from cell surface into internal membrane compartments, can be divided into two main pathways: the classic, clathrin-mediated endocytic pathway and the nonclassic, clathrin-independent but lipid-raft-dependent route (Le Roy and Wrana, 2005). Which, of these two pathways is used by MuSK is not yet clear, but it has been shown, that MuSK endocytosis and AChR clustering is attenuated by the dominant-negative K44A dynamin mutant, which is deficient in GTP hydrolysis (Zhu et al., 2008). Additionally, it has been shown, that Agrin induces rapid translocation of MuSK into lipid rafts, which is required for downstream signaling and AChR clustering (Zhu et al., 2006). Dynamin has been shown to be essential for raft-dependent endocytosis and the GTPase activity of dynamin is required for the budding of lipid rafts from purified endothelial plasma membranes (Oh et al., 1998). How endocytosis of MuSK is actually linked to AChR clustering remains unclear. AChR phosphorylation and clustering are relatively slow, suggesting that multiple steps are likely to lie between Agrin activation of MuSK and AChR phosphorylation and clustering (Burden, 1998). Nevertheless, endocytosis of MuSK seems to be involved in Agrin signaling (Zhu et al., 2008). In muscle cells, endocytosis of AChRs is barely detectable, but a significant number of AChRs recycle back into the postsynaptic membrane *in vivo* (Akaaboune et al., 1999; Bruneau et al., 2005; Bruneau and Akaaboune, 2006). This suggests, that AChR endocytosis might be mediated by a different mechanism (Zhu et al., 2008). Indeed, it turned out, that the endocytosis of AChRs is independent of dynamin, but depends on the activity of the Rho-GTPase Rac1 (Kumari et al., 2008). The identification of the different internalization pathways used by cells is of tremendous relevance, because cells use different internalization mechanisms to control signaling, the magnitude, duration and nature of signaling events and receptor turnover.

In this study, it has been shown, that Cl-6 downregulation interferes with AChR clustering at the NMS. Therefore, Cl-6 seems to be an important molecule in downstream signaling of the Agrin/Lrp4/MuSK pathway. In spite of the necessary role of Cl-6 in AChR cluster formation, the way how this is achieved, remains to be determined. Burke et al. could show for the EGFR, that the receptor remained active in early endosomes, after internalization, and also interacted with different signaling molecules. Additionally, they observed a difference in the proteins, which interacted with the EGFR at the surface and in early endosomes. This suggests, that internalization is needed for the modulation of receptor signaling. Although receptor internalization is the first step in receptor degradation, internalization is not necessarily an attenuation process itself (Burke et al., 2001). Although ligand-induced endocytosis was originally thought to be a mechanism of receptor inactivation, many studies suggest that receptors remain active within endosomes (Wiley and Burke, 2001).

From these observations, the conclusion can be drawn that Cl-6 might function in the endocytosis of MuSK. For RIN1 it has been shown, that it stimulates the degradation of EGFR by its interaction with Rab5 (Chen et al., 2009). Since Cl-6 has been shown to interact with Rab5 in the same way as RIN1, it could be possible that Cl-6 stimulates the degradation of MuSK via Rab5. This would mean that the downregulation, of Cl-6 results in decreased

endocytosis of surface MuSK and therefore, prolonged MuSK surface signaling and a reduction in the internalization-dependent signaling events. Since downregulation of Cl-6 leads to smaller AChR cluster, I propose that the signals, necessary for AChR clustering come from within the cell, rather than from the cell surface or that both ways of signaling contribute equally to the formation of cluster and that a reduction of one mechanism leads to smaller clusters. Consistent with these data, blocking endocytosis using a dominant negative mutant of Dynamin 2 leads to the generation of smaller and fragmented AChR clusters upon Agrin-stimulation (Zhu et al., 2008). Another possibility would be that Cl-6 has no influence on MuSK endocytosis, but might just stabilize MuSK at the NMS. This hypothesis is somehow supported by the observation, that the interaction of Cl-6 with MuSK is independent of its activation status (Michaela Mutzl, Diploma thesis). Further studies will be needed to really clarify the role of Cl-6 at the NMS. An important step in this direction would be the identification of tyrosine phosphorylated proteins that interact with the SH2 domain of Cl-6. The interaction of such proteins would depend on the phosphorylation of these proteins, most probably by the Agrin-MuSK signaling pathway. It has been shown that binding of EGF to the EGFR activates the receptor, which leads to tyrosine phosphorylation of the EGFR. These activated EGFR can then bind to the SH2 domain of RIN1, which couples it to the endocytic machinery via its interaction with Rab5 through the Vps9 domain. My third hypothesis, about the functional importance of the interaction between Cl-6 and MuSK rests on these data. Thereby, interaction of Cl-6 with MuSK is required to target Cl-6 to the NMS, where it can interact with other proteins and regulate the endocytosis of proteins via its interaction with the SH2 domain.

So far the trafficking of MuSK and the responses it generates are poorly understood. Therefore, further research will be needed, to clarify the function and influence of Cl-6 on MuSK at the NMS. Recent studies provide strong evidence that receptor trafficking is an important mechanism to regulate the specificity of receptor tyrosine kinase (RTK)-generated responses (Vieira et al., 1996; Zhang et al., 2000). Therefore, Cl-6 might represent an important link between MuSK signaling and endocytosis.

4.4. Future Outlook

In the future the consequences of Cl-6 interactions with Rab5 and MuSK have to be determined. In particular, it will be important to show that Cl-6 acts as GEF for Rab5. Consequently, it has to be tested whether this mechanism plays a role during MuSK endocytosis. Additionally, it will be necessary to determine whether the downregulation of Cl-6 has any influence on MuSK expression or the amount of MuSK present at the surface. Stable muscle cell lines carrying different siRNA constructs will assist in answering this question as well as the recent finding that Lrp4 is the Agrin receptor, which allows for the determination of MuSK function in heterologous cell systems. This will make it easier to determine whether Cl-6 has any influence on MuSK endocytosis or stability. Furthermore, Cl-6 knock-out mice and a conditional knock-out in skeletal muscle are being generated to study the role of Cl-6 during NMS formation, maintenance and function *in vivo*.

5. Materials and Methods

5.1. Chemicals and Reagents

10xPBS	PAA
40% Acrylamide	Roth
Acetic acid	Roth
Agar-Agar	Roth
Alexa594- α -bungarotoxin	Molecular Probes
Ampicillin	Roth
Aprotinin	Roth
APS	Roth
BSA	PAA
Bromphenol blue	Roth
Calcium chloride	Roth
DABCO	Roth
DMEM	Sigma
DMSO	Roth
EDTA	Roth
Ethanol	Roth
Ethidium bromide	Roth
FBS	Gibco
Gelatine	Sigma
Gel extraction kit	Omega
Geneticidin disulfate (G418)	Roth
Glutathione-agarose beads	Sigma
Glycerol	Roth
Glycine	Promega
HCl	Roth
IPTG	Roth
Isopropanol	Roth
Kanamycin	Roth
LB-broth	Roth
Leupeptin	Roche
Lumi-Light	Roche
Lysozyme	Sigma
Maxi prep kit	Omega
Methanol	Roth
MgCl ₂	Roth
MgSO ₄	Roth
Midi prep kit	Omega
Mini prep kit	Fermentas
Modifying enzymes	Fermentas
Mowiol 4-88	Roth
NaOH	Roth
N-Lauroylsarcosine	Sigma
NP-40	Sigma
Paraformaldehyde	FLUKA

PCR buffer 10x	Sigma
PEG	Roth
Penicillin/Streptomycin	Sigma
Pepstatin A	US biological
Phenylmethylsulfonylfluorid (PMSF)	Roth
Potassium chloride	Roth
Prestained protein ladder (Gene ruler)	Fermentas
Protein A-agarose beads	Roche
Restriction enzymes	Fermentas
Sodium acetate	Roth
Sodium chloride	Roth
Sodium dodecyl sulfate	SERVA
Sodiumdihydrogen phosphate	Roth
Sodiumfluoride (NaF)	Fluka Chemika
Sodiumhydrogen phosphate	Roth
Sodium orthovanadate	Sigma
Taq buffer 10x	Fermentas
Taq DNA polymerase	BioLabs
TEA	Sigma
Tris	Roth
Tris-HCl	Roth
Triton X-100	Sigma
Trypsin-EDTA	Gibco
Tween 20	Promega
Vectashield	VECTOR
α -Cl-6 antibody	Barbara Woller (unpublished)
α -GFP antibody	Santa Cruz
α -MuSK antibody	Ruth Herbst (Herbst & Burden, 2000)
α -MuSK antibody	Christian Fuhrer
α -myc antibody	Sigma
α -phosphotyrosine antibody	Cell Signaling p100; Santa Cruz p99
β -Mercaptoethanol	Roth
1kb DNA base-pair ladder	Fermentas

5.2. Oligonucleotides

Primer name	Sequence (5'→3')
Cl-6 primers	
Vps9 EcoRI for	CGAATTCGCGGGGCTGAGGGACAGAGC
Vps9 XhoI rev	GCGCTCGAGCCGTCTTCGATGCCACTGGCG
RNAi oligos	
348s	<u>GATCCCC</u> GCCAGGTCCTGATGTT <u>GAATTCA</u> AGAGATTCAACATCAGGACCTGGCTTTTTA
348as	AGCTTAAAAAGCCAGGTCCTGATGTTGAATCTCTT <u>GAATTCA</u> ACATCAGGACCTGGCGGG
2034s	<u>GATCCCC</u> ACCCTCTCGCCTGAATCTCTTCAAGAGAGAGATTCAAGGCGAGAGGGTTTTTA
2034as	AGCTTAAAAAACCTCTCGCCTGAATCTCTCTCTTGAAGAGATTCAAGGCGAGAGGGTGGG
scrambled 348s	GATCCCCGGGTACCAGCGACTATGTTTCAAGAGAACATAGTCGCTGGTAACCTTTTTTA
scrambled 348as	AGCTTAAAAAGGGTTACCAGCGACTATGTTCTCTTGAACATAGTCGCTGGTAACCCGGG
scrambled 2034s	GATCCCCGCCCTCCACGACTTTCATTTCAAGAGAATGAAAGTCGTGGGAGGGCTTTTTTA
scrambled 2034as	AGCTTAAAAAGCCCTCCACGACTTTCATTCTCTTGAATGAAAGTCGTGGGAGGGCGGG

Underlined bases represent restriction sites.

5.3. Plasmids

Plasmid name	Description/Cloning	Reference
Mammalian expression vectors		
pEGFP-Rab5 wt	Rab5 wt sequence with a N-terminal GFP-tag.	M. Zerial
pEGFP-Rab5 S34N	Dominant negative form of Rab5 with a N-terminal GFP-tag	M. Zerial
pEGFP-Rab5 Q79L	Constitutively active form of Rab5 with a N-terminal GFP-tag	M. Zerial
pEGFP Rab7	Rab7 sequence with a N-terminal GFP-tag.	C. Bucci
MuSK-myc	A mammalian expression vector backbone pRc/RSVr. Promoter: RSC LTR; bacteria resistance: ampicillin. Insert MuSK with a myc tag at 10 aa site.	Backbone from Invitrogen
pCMX.PL1 CKII β	The CKII β -subunit	S. Hashemolhosseini
Plasmids for protein expression in <i>E. coli</i>		
pGEX-4T-1	Control plasmid.	GE Healthcare
GST-Y2H	CI-6 DNA that was isolated in a Y2H screen was subcloned into the pGEX-3 Vector. It contains aa 200 to 563 of the full-length construct	R. Herbst
GST-N900	A CI-6 DNA fragment containing the aa 200 to 479 was subcloned into the pGEX-2 vector	R. Herbst
GST- Δ 250	A CI-6 DNA fragment containing the aa 248 to 563 was subcloned into the pGEX-2 vector	R. Herbst
RNAi plasmid		
pSuperior.gfp/neo	See plasmid map in Introduction; the siRNA expression cassette in all pSUPER and pSUPERIOR vectors are completely identical, except for one key feature: a sequence modification of the H1 promoter between the TATA box and the RNA hairpin transcription start site;	OligoEngine, Brummelkamp et al., 2002

5.4. Bacterial Strain

E. coli strain: Rosetta(DE3)pLysS (F^- ompT hsdS_B(R_B⁻ m_B⁻) gal dcm λ (DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5) pLysSRARE (Cam^R))

5.5. Cloning of GST-Fusion Proteins

5.5.1. GST-CI-6

For the CI-6 insert the vector pEGFP/N3-CI6 fl was cut with KpnI and the ends were removed using T4 DNA polymerase. Then a second digestion with EcoRI was performed. The vector pGEX-5X-2 was cut using SmaI and EcoRI. CI-6|EcoRI, KpnI blunt was ligated into pGEX|EcoRI, SmaI.

5.5.2. GST-Vps9

For this construct the Vps9 part of the CI-6 sequence was amplified by PCR using the following primers: Vps9 EcoRI for and Vps9 XhoI rev. The resulting PCR fragment was then cut with EcoRI and XhoI and ligated into a pGEX-4T-1 vector that had been cut the same way. Crystallization studies revealed that this construct lacks the helical bundle and is therefore not the functional Vps9 domain of CI-6 (Delprato et al., 2004).

5.5.3. GST-Vps9a

For this construct the GST-Vps9 construct was cut with BamHI and the ends were filled in using Klenow fragment. Then a second digestion with EcoRI was performed. The vector pGEX-4T-1 was cut using SmaI and EcoRI and ligation of these two fragments led to the destruction of the BamHI/SmaI restriction site. This construct contains the Vps9 domain lacking the last helix.

5.5.4. GST-Vps9b

For this construct the GST-Y2H construct was cut with EcoRI and BamHI and ligated into the pGEX-1 vector that had been cut the same way. This construct just consists of the last helix of the Vps9 domain and should therefore be not functional at all.

5.5.5. GST-CKII

For this construct the Casein Kinase II (CKII) containing vector pCMX.PL1_CKII_β was cut with SmaI and XhoI and ligated into the pGEX-4T-1 vector that had been cut the same way. This construct was used as a control for the GST pull-downs with MuSK, because it has been shown, that CKII strongly interacts with MuSK (Cheusova et al., 2006).

5.5.6. GST-RIN1

For this construct BS-RIN1 was cut with EcoRI and XhoI and ligated into the pGEX-4T-1 vector that had been cut the same way. This construct was used as a control for the GST pull-downs with Rab5, because it has been published that RIN1 interacts with Rab5 and preferentially with the dominant-negative mutant of Rab5, S34N (Tall et al., 2001).

5.6. Plasmid Preparation from *E. Coli*

5.6.1. Small Scale Plasmid Preparation

1.5-3ml of an overnight culture (in LB medium containing 50µg/ml Ampicillin or 30µg/ml Kanamycin) were centrifuged at maximum speed in a table-top centrifuge and plasmids were isolated using the Fermentas Gene JetTM Plasmid Miniprep kit. All steps were carried out as suggested by the manufacturer.

5.6.2. Middle Scale Plasmid Preparation

100-200ml of an overnight culture (in LB medium containing 50µg/ml Ampicillin or 30µg/ml Kanamycin) were centrifuged at 5000g (SLA-1500 rotor) and plasmids were isolated using the Omega biotek Plasmid Midiprep kit. All steps were carried out as suggested by the manufacturer. The DNA concentration was determined by measuring an aqueous dilution at 260nm.

5.6.3. Large Scale Plasmid Preparation

200-400ml of an overnight culture (in LB medium containing 50µg/ml Ampicillin or 30µg/ml Kanamycin) were centrifuged at 5000g in a Sorvall centrifuge using the SLA-1500 rotor. Plasmids were isolated using the Omega Biotek Plasmid Maxiprep kit. All steps were carried out as suggested by the manufacturer. The DNA concentration was determined by measuring an aqueous dilution at 260nm.

5.7. Manipulation of DNA with Enzymes

5.7.1. Restriction Enzyme Digestion

Enzymes were supplied by Fermentas or New England Biolabs and used as suggested by the manufacturer. Digestions were mostly carried out in a 20µl volume using 0.5-1µl restriction enzyme solution and approximately 0.5 to 1µg DNA.

5.7.2. Dephosphorylation of DNA

For dephosphorylation of linearized plasmids 1µl SAP (shrimp alkaline phosphatase, 1U/µl) was added right after digestion, followed by a 1h incubation period at 37°C.

5.7.3. Filling in Ends with Klenow Polymerase

Klenow polymerase fills in sticky ends and makes them blunt. 0.5µl Klenow polymerase (5U/µl) were added directly to the restriction mix together with 1.5µl dNTPs (2mM). The vial was incubated at 37°C for 15min, afterwards the enzyme was heat-inactivated by putting the vial to 75°C for 10min.

5.7.4. Isolation of DNA Restriction Fragments

After digestion the DNA was run on a 0.8-2% agarose gel/1xTAE containing 2µl EtBr per 50ml of gel. The bands were visualized by subjecting the gel onto a UV source. The right bands were excised from the gel and purified using a Gel Extraction kit. All steps were carried out as suggested by the manufacturer. Concentrations of isolated DNA fragments were estimated by loading 1µl aliquots onto an agarose gel and comparing the resulting bands to the 1kb DNA base pair ladder, that was loaded at known concentration.

50x TAE:	242g Tris base
	57.1ml acetic acid
	100ml 0.5M EDTA
	volume adjusted to 1l with dH ₂ O

5.7.5. Ligation of DNA

Ligation reactions were always carried out in a 20µl volume with a molar ration of vector to insert of approximately 1:3, except for very small inserts, which were used in higher excess. Prior to ligation, vector and insert were digested with restriction enzymes that produced the same or compatible cohesive ends. The ligation was performed by combining vector and insert DNA in an Eppendorf tube and adding 1µl T4 DNA ligase (5u/ml), 2µl 10x T4 buffer and dH₂O up to 20µl. After an incubation for at least 4h or over night at RT, the whole ligation mix was transformed into competent bacteria.

5.8. Generation of Competent Bacteria

3ml LB without antibiotics were inoculated with bacteria and left on a shaker at 37°C over night. 1ml of this overnight culture was added to 100ml of LB-Medium, the resulting culture was incubated on a shaker at 37°C until the OD₆₀₀ reached a value between 0.3 and 0.6. Then the bacteria were centrifuged at 5000rpm (SLA-1500 rotor) for 5min and the pellet was immediately resuspended in 10ml ice-cold TBS buffer. Aliquots of competent bacteria were frozen and stored at -80°C. The quality of the competent bacteria was assayed by transforming them with a plasmid carrying an antibiotic resistance gene. High fidelity bacteria should yield at least 10⁶ colonies per 1µg DNA. The bacteria alone were also put on LB-plates, which contained antibiotics, to test that there is no contamination in the preparation.

TBS buffer: 10% PEG
5% DMSO
10mM MgCl₂
10mM MgSO₄

5.9. Transformation of Competent Bacteria

In an Eppendorf reaction tube 20µl ligation mix, 20µl 5xKCM buffer, 60µl dH₂O and 100µl competent bacteria were mixed. Then the tube was placed on ice for 30min, afterwards on 42°C for a 2min heat shock and then put back on ice again. After the addition of 900µl LB medium without antibiotics the tube was incubated at 37°C in a water bath for 30min. Then the cells were pelleted and ~1ml of the supernatant was discarded. The rest was plated on selective media.

5xKCM buffer: 0.5M KCl
0.15M CaCl₂
0.25M MgCl₂

5.10. Protein Gel Electrophoresis and Western Blot Analysis

Different percentage polyacrylamide gels were prepared, according to the size of proteins to be analyzed. Samples were mixed with 4xSDS loading dye and loaded into the gel slots. Then gels were run at 100-130V in 1xRunning Buffer. After separation of the proteins, the gels were taken out of the electrophoresis apparatus and immediately put into transfer buffer for Western blot analysis or into Coomassie staining solution.

For the Coomassie staining procedure the gels were approximately 30min incubated in the Coomassie staining solution. Afterwards the gels were destained in Coomassie destain until the protein-bands became visible at RT and preferably the destaining was continued at 4°C over night.

For Western blot analysis the gel was placed on top of a PVDF-membrane (which was incubated in methanol and washed in transfer buffer right before use) between two transfer buffer-drained papers in a blotting apparatus and a current of 50mA was applied per gel. The proteins were allowed to migrate onto the membrane for 80-90min. Afterwards, the membrane was immediately put into TBS-Tween for washing. For further analysis the membrane was blocked. This was achieved by incubating the membrane on a shaker at room temperature in a 5% BSA/TBS-Tween solution for 2h or in a 5% instant milk powder in TBS-Tween solution for 1h. The primary antibody, stored at 4°C in 5% BSA + 0.1% NaN₃ in TBS-Tween, was then added to the membrane. The membrane was then incubated with the antibody over night at 4°C. The next day, the membrane was washed 3 times for 10min with TBS-Tween and then the secondary antibody, a horse reddish peroxidase conjugated antibody was added, diluted in 5% instant milk powder in TBS-Tween. After 1h incubation, the membrane was washed another 3 times for 10min with TBS-Tween and incubated with Roche

Lumi-Light, Western blot, horse reddish peroxidase substrate. The bands were visualized by subjecting the membranes to BIO-RAD Fluor-STM MultiImager. Bands were analyzed using BIO-RAD Quantity One – 4.1.1 Software. To loosen the antibody binding, blots were stripped either by incubating the membranes with 10% acetic acid for 2h on a shaker and afterwards washing the blots with TBS-Tween or by incubating the membranes for 5min in 0.2M NaOH and washing with dH₂O before and afterwards. Then the blots were reused for another antibody reaction.

To visualize the protein bands on the membranes, the blots can be stained with Ponceau solution for a few minutes at RT and then destained with dH₂O. Afterwards, the blots can be completely destained with TBS-Tween and can then be used for antibody reactions again.

5.10.1. Solutions and Gels

4xSDS Loading Buffer:	240mM Tris.Cl pH6.8 8% β-mercaptoethanol 8% SDS 40% glycerol bromphenol blue
5xRunning Buffer:	15.05g Tris base 72g glycine dissolved in almost 1l dH ₂ O 5g SDS were added and the volume adjusted to 1l
10xTransfer Buffer:	58g Tris 29g Glycine 5g SDS filled with dH ₂ O to 1l
1xTransfer Buffer:	50ml 10xTransfer Buffer 50ml methanol 400ml dH ₂ O
10x TBS-Tween:	100mM Tris.Cl pH8 1.5M NaCl 0.5% Tween 20
Coomassie Staining:	225ml methanol 50ml acetic acid 225ml dH ₂ O 1,25g Coomassie Brilliant Blue was added to the solution
Coomassie Destaining:	90ml methanol 20ml acetic acid 90ml dH ₂ O
Ponceau Solution:	0.5g Ponceau 500ml 1% acetic acid

PAA-Gels	Running Gel								
	7.5%			10%			12.5%		
total volume [ml]	5	10	20	5	10	20	5	10	20
40% AA mix [ml]	0.94	1.88	3.76	1.25	2.5	5	1.55	3.1	6.2
1.5M Tris/0.5M HCl [ml]	1.25	2.5	5	1.25	2.5	5	1.25	2.5	5
dH ₂ O [ml]	2.7	5.4	10.8	2.4	4.8	9.6	2.1	4.2	8.4
10% SDS [μl]	50	100	200	50	100	200	50	100	200
APS [μl]	50	50	100	50	50	100	50	50	100
TEMED [μl]	5	10	20	5	10	20	5	10	20

PAA-Gels	Stacking Gel	
	5%	
total volume [ml]	5	10
40% AA mix [ml]	0.625	1.25
0.02M Tris/0.48M Tris.Cl [ml]	1.25	2.5
dH ₂ O [ml]	3.025	6.05
10% SDS [μl]	50	100
APS [μl]	50	100
TEMED [μl]	5	10

5.11. Polymerase Chain Reaction (PCR)

DNA was amplified from a DNA template (plasmid or cDNA) using two specific primers (0.625μl of each primer with a concentration of 10μM), 1.25μl PCR-buffer, 0.5μl 10mM dNTPs, 0.1μl Taq polymerase. The mixture was filled up with water to a final volume of 12.5μl. The reaction mix was incubated for 120sec at 95°C to denature the DNA, and then there were 30 cycles which contained 3 steps: 30sec at 95°C, 30sec at 60±7.5°C (gradient) and 30sec at 72°C. After that there was a final elongation of the DNA which was 10min at 72°C.

5.12. Expression and Purification of GST-Fusion Proteins in *E. Coli*

The *E. coli* strain Rosetta was transformed with the expression plasmids that encode for a GST-fusion protein. One single colony was grown up in LB medium supplemented with 50μg/ml Ampicillin overnight. This pre-culture was diluted 1:25, incubated shaking at 220rpm at 37°C and left growing until OD₆₀₀ reached a value of approximately 0.8-0.9. Protein production was induced by the addition of 0.5mM IPTG and the culture was incubated for another 2h. Afterwards, the bacteria were chilled on ice and centrifuged for 15min at 5000g (SLA-1500 rotor). The pellet was resuspended with ice-cold 1xPBS+1%

Triton. Per ml resuspension 20µl lysozyme (50mg/ml) and 2µl PMSF solution were added. The pellets were lysed on ice for 1h. After that 0.25% sarkosyl was added to some of the samples. All the samples were then sonicated twice for 10sec with 20sec on ice inbetween. Afterwards, the samples were centrifuged for 15min at 15000g either in an Eppendorf 5415 D centrifuge or with a SS-34 rotor. The cleared supernatant was then added to glutathione-agarose beads, which had been washed with 1xPBS+1% Triton for three times before use. In case of the addition of sarkosyl to the lysate, the volume of the supernatant was doubled with 1xPBS+1% Triton to decrease the sarkosyl amount to 0.125%. The samples were then incubated on a rotating device at 4°C for 1h. Then the samples were centrifuged for 5min. The glutathione agarose beads were then washed 3 times with 1xPBS+1% Triton for 15min. Then aliquots were made. One aliquot was used for SDS-PAGE to determine the concentration and purity of purified protein by performing Coomassie staining, while the other aliquots were put in liquid nitrogen and were stored at -80°C for later use.

10xPBS:	1.37M NaCl
	27mM KCl
	43mM Na ₂ HPO ₄
	14mM KH ₂ PO ₄

5.13. Cell Culture

5.13.1. HEK-293T and COS-7 Cells

HEK-293T cells and COS-7 cells were grown in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin (=general medium, GM). The incubator was adjusted to 37°C and 6% CO₂. Cells were split every two days when they were about 80-90% confluent.

5.13.2. C2 Muscle Cells

C2HG cells stably expressing different RNAi (empty, 348, 348 scrambled, 2034 and 2034 scrambled) constructs were generated by Michaela Mutzl during her diploma thesis. Therefore C2HG cells were transfected with this constructs using Lipofectamin. Afterwards, single clone colonies were picked and frozen in liquid nitrogen.

C2HG cells stably expressing different siRNAi constructs and C2C12 cells were grown in DMEM supplemented with 15% FBS, 0.25% chicken embryonic extract and 1% penicillin/streptomycin (= general medium, GM). Additionally, 700µg/ml G418 were added to the GM medium of stable cell lines. The incubator was adjusted to 37°C and 6% CO₂. Cells were split every two days when they were about 80-90% confluent. For differentiation of C2 myoblasts into myotubes cells were plated on gelatine coated dishes (C2HG: ~1.3*10⁵ cells/3.5cm dish; C2C12: ~3*10⁵ cells/10cm dish) and differentiation was started the next day by switching to DMEM supplemented with 2% HS and 1% penicillin/streptomycin (= differentiation medium, DM) at ~90% confluency. DM was changed every day for 3-4 days when the differentiation into myotubes was completed.

5.13.3. Freezing of Cells

A 10cm plate of happily growing cells that were about 60-70% confluent was trypsinized. The cells were washed off the plate with 5ml GM, then centrifuged for 5min at 1500rpm. The cell pellet was well resuspended in 1ml freeze medium (GM + 10% DMSO). Cells were frozen at -80°C and can be later put into a liquid nitrogen tank for long-time storage.

5.13.4. Transfection of HEK-293T Cells and COS-7 Cells by Ca²⁺ DNA Precipitation

2xBBS solution and the 0.25M CaCl₂ solution were thawed quickly and placed at room temperature. For every 10cm plate 20µg DNA was used in total and pipetted into a round bottom polystyrene tube. Then, 875µl 0.25M CaCl₂ were added and the solution was mixed. Afterwards, 875µl 2xBBS were added and the tube was vortexed, followed by an incubation at room temperature for 11min. After this incubation step, the DNA mix was added drop-wise on top of the cells. ~15-17h after transfection the growth medium was changed to remove the precipitate and let the cells recover.

2xBBS:	80ml sterile ddH ₂ O
	5.6ml 5M NaCl
	5.0ml 1M BES
	150µl 1M Na ₂ HPO ₄
	pH adjusted to 6.95
	volume raised to 100ml

5.13.5. Preparation of Cell Lysates

C2 cells were starved for 2.5-3h and treated with Agrin 4.8 or 0.0 for 30min before cell lysis. The lysis procedure itself was then the same for all different cell types.

After incubation at 37°C the plates were put on ice and the growth medium was aspirated. The cells were washed twice with ice-cold PBS. Afterwards, NP-40 lysis buffer containing protease inhibitors was added (1ml per 10cm plate, 400µl per 6cm plate, 100µl per 3.5cm plate). The protease inhibitors Leupeptin, Aprotinin, Orthovanadate, Pepstatin and PMSF were added to the lysis buffer right before usage. For lysis to occur, plates were left on a shaker at 4°C for 10-15min. Then the cells were scraped off each plate and transferred into an Eppendorf tube. Lysates were centrifuged at 4°C for 10min at maximum speed in a table-top centrifuge. The supernatants were transferred into new centrifugen tubes. Samples were either used immediately for further analysis or frozen in liquid nitrogen and stored at -80°C.

NP-40 lysis buffer:	1% NP40
	5mM EGTA
	50mM NaCl
	30mM TEA, pH 7.5
	50mM NaF
	in dH ₂ O

5.14. GST Pull-Down

Cell lysate aliquots of 350-1000µl were incubated with the respective GST-fusion proteins which were already bound to glutathione-agarose beads on a rotating device. After 4-18h incubation the lysates were centrifuged for 1min at maximum speed in an Eppendorf 5415 D centrifuge. The glutathione-agarose beads were washed 3-4 times with 1ml lysis buffer for 2-15min. Bound proteins were eluted with SDS sample buffer and subjected for further analysis to SDS-PAGE and Western blotting. All steps were carried out at 4°C.

5.15. Immunoprecipitation

Cell lysate aliquots of 500-1000µl were incubated with the respective antibody on a rotating device. After 4-16h incubation 20-30µl protein A agarose was added and incubated for 1-1.5h on a rotating device. Lysates were centrifuged for 1min at maximum speed in an Eppendorf 5415 D centrifuge. The protein A agarose beads were washed 3 times using 1ml lysis buffer. Bound proteins were eluted with SDS sample buffer and subjected for further analysis to SDS-PAGE and Western blotting. All steps were carried out at 4°C.

5.16. Staining of AChRs

Stable transfected C2 cells cultured on gelatine-coated 3.5cm dishes were treated with Agrin 4.8 for 8 or 16h. As a control cell were treated with Agrin 0.0 for the same time. After that the cells were incubated with Alexa-594 conjugated α -bungarotoxin for 60min. Then, the cells were washed twice with PBS for about 2min before fixation with 2% PFA in PBS for 10min at RT. After another two washing steps for 2min, plates were mounted onto glass slides in Vectashield mounting medium or Mowiol supplemented with DABCO. After that the plates were viewed on a fluorescence microscope using an excitation wavelength of 546nm.

4% PFA/PBS: 0.8g PFA were dissolved in 20ml 1xPBS
100µl 0.5M NaOH to reach pH7
The solution was gently heated and stirred until clear.

5.17. Quantification of AChR Clusters

Quantification of the AChR cluster length and number was done using the Metamorph Imaging Software (Molecular Devices). Pictures of the myotubes were taken using the 63x objective and the Cy3 filter to visualize AChR clusters on the surface of myotubes. The AChR cluster and myotube length were determined by applying a line across the cluster using the free hand tool of the software. The quantification was done in a double-blind manner. The length of AChR clusters was presented in micrometers.

6. Abbreviations

APS	ammoniumperoxidisulfate
ATP	adenosine triphosphate
bp	base pair
BBS	BES buffered saline
BES	N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid
BLAST	basic local alignment research tool
BSA	bovine serum albumin
Da	Dalton
DMEM	Dulbeco's Modified Eagles Medium
DMSO	dimethylsulfoxide
dNTPs	deoxyribonucleoside triphosphates
E. coli	Escherichia coli
EDTA	ethylenediaminetetraacetate
EtBr	ethidium bromide
FBS	fetal bovine serum
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
GST	glutathione S-transferase
GTP	guanosine triphosphate
h	hour
HEPES	2-[4-(2-Hydroxyethyl)-1-piperazinyl]-ethanesulphonic acid
IPTG	isopropylthio- β -D-galactoside
kb	kilobase
kDa	kiloDalton
LB	Luria-Bertani Medium
MCS	multiple cloning site
min	minute
NP-40	Nonidet P-40
nt	nucleotide
OD	optical density
oligo	oligonucleotide
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PFA	paraformaldehyde
PMSF	phenylmethylsulfonylfluoride
pTyr	phosphorylated tyrosine
PVDF	polyvinylidene difluoride
RH	RIN homology
RT	room temperature
SDS	sodium dodecyl sulfate
sec	second
SH2	Src homology 2
TAE	tris-acetate-EDTA
TBS	tris-buffered saline

TBE	tris-borate-EDTA
TE	tris-EDTA
TEA	triethanolamine acetate
TEMED	N,N,N,N-tetramethylethylene diamine
Tris	tris (hydroxymethyl) aminomethane
Vps9	vacuolar protein sorting
Y2H	Yeast-2-hybrid

7. References

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