Pro gradu -tutkielma

Biochemical and functional characterization of the interaction between two receptor tyrosine kinases, ROR1 and MuSK

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ABSTRACT

Receptor tyrosine kinase-like orphan receptor 1 (ROR1) is a closely related homolog to MuSK, Muscle-specific kinase. MuSK functions mainly in the muscle where it is active in the formation of neuromuscular junction (NMJ) by gathering acetylcholine receptors at the synaptic sites, although MuSK has been detected in other tissues as well. ROR1 is present in the brain during embryonic development, where it functions in neurite elongation, differentiation of neural cells and synapse formation. Even though ROR1 is mostly absent in adult tissues except in several types of cancer, it has also been detected in muscle, spleen and intermediate B-cell population called hematogones.

We hypothesized that ROR1 and MuSK interact during the neuromuscular junction formation. The aim of this study was to characterize the properties of ROR1-MuSK interaction and show their endogenous expression in mouse skeletal muscle cell line C2C12 myotubes and neurons. Our results show that ROR1 can interact with MuSK and ROR1 is phosphorylated by MuSK when these receptors are overexpressed in 293T and Cos-7 cells, via co-immunoprecipitation and Western blot. We detected the endogenous expression of ROR1 in mouse skeletal muscle C2C12 cells with qPCR and co-immunoprecipitation.

Since ROR1 was previously believed to only be expressed during embryonic development and in cancer, it became a target for cancer drug development. Our results show that ROR1 has a function in muscle cells, in the formation of the NMJ by interacting with MuSK. We also show that ROR1 is present in muscle tissue, which has clinical importance in the development of cancer treatments. Taken together, our studies provide novel information about ROR1 expression and function.

ABSTRAKTI

Receptor tyrosine kinase-like orphan receptor 1 (ROR1) sekä Muscle-specific kinase (MuSK) ovat reseptori tyrosiinikinaasi –perheen keskenään läheisimmät sukulaiset. MuSKin tunnetaan parhaiten tehtävästään lihassoluissa, joissa se säätelee lihas-hermosynapsien muodostumista edeltävää asetyylikoliinireseptorien kokoamista lihassolun solukalvolle kohtaan, johon synapsin muodostuu. Nimestään huolimatta MuSK toimii kuitenkin myös muissa kudoksissa. ROR1 on vahvimmin ekspressoituneena alkionkehityksen aikana, jossa sillä on monta tärkeää tehtävää erityisesti aivoissa. Alkion kehittyvissä aivoissa ROR1:n toiminta on yhdistetty mm. hermosolujen erilaistumineen, hermosynapsien muodostumiseen, sekä neuriittien pidentymiseen. Pitkään luultiin, että ROR1 –proteiinia löytyykin kudoksista ainoastaan alkionkehityksen aikana, minkä lisäksi aikuisilla ainoastaan syöpäsoluissa. Viime aikaisissa tutkimuksissa ROR1-proteiinia on kuitenkin havaittu ekspressoituvan pienissä määrin myös useissa normaaleissa aikuisen kudoksissa, kuten haimassa, lihaksissa, b-soluissa sekä keuhkoissa.

Hypoteesimme oli, että ROR1 ja MuSK vuorovaikuttavat lihashermosynapsin muodostumisprosessissa. Työn tarkoituksena oli tutkia ja todistaa ROR1:n MuSK:n välisen vuorovaikutuksen olemassaolo. Lisäksi tarkoituksena oli osoittaa, että ROR1 ekspressoituu endogeenisesti hiiren jo erilaistuneissa C2C12 luustolihassoluissa. Tuloksemme todistavat ROR1:n ja MuSK:n todella vuorovaikuttavan C2C12-soluissa, sekä sen, että tämän vuorovaikutuksen aikana MuSK:n kinaasiaktiivisuus saa aikaan ROR1:n fosforyloitumisen. Menetelminä käytimme immunopresipitaatiota sekä Western blotia. Endogeenisen ekspression osoitimme qPCR:n, sekä niin ikään immunopresipitaation avulla.

Tuloksillamme on merkitystä erityisesti lääkesuunnittelun kannalta. Koska vielä jonkin aikaa sitten ROR1 –proteiinia pidettiin aikuisen kudoksissa syöpämarkkerina sen puuttuessa terveistä kudoksista kokonaan, siitä tulikin mielenkiintoinen kohde syöpälääkkeiden suunnittelulle. Tämä tutkimus osoittaa, että ROR1 -proteiinilla on oma roolinsa lihas-hermosynapsien syntymisessä ja MuSK:n signaloinnissa. Tutkimustuloksemme paljastivat uutta tietoa ROR1-proteiinista, sen ekspressiosta, sekä roolista.

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ABBREVIATIONS

AChR	Acetylcholine receptor
CRD	Cysteine rich domain
Dok-7	Down-stream of tyrosine kinase 7
IP	Immunoprecipitation
Lrp4	Lipoprotein-related protein 4
MuSK	Muscle-specific kinase
MB	Myoblast
MT	Myotube
NMJ	Neuromuscular junction
NPC	Neural progenitor cell
PR	Proline rich domain
ROR	Receptor tyrosine kinase-like orphan receptor
RTK	Receptor tyrosine kinase
S/T	Serine/Threonine rich domain
WB	Western blot

1 INTRODUCTION

Receptor tyrosine kinases (RTK) are a family of type 1 transmembrane receptors involved in several vital processes in the cell. The structure and domain architecture of the extracellular domains vary between RTK subfamilies, but the catalytically active intracellular tyrosine kinase domain is highly conserved between the subfamilies and also between species from *Caernohabditis elegans* to humans. This kind of interspecies evolutionary conservation underlines the importance of RTKs. Not surprisingly, defective RTKs are the cause of many developmental disorders, diabetes and many cancers (Robinson et al., 2000). The binding of an extracellular ligand molecule initiates a signaling cascade inside the cell. RTKs function in cell communication, signaling in processes including adhesion, growth, proliferation and cell death.

The receptor tyrosine kinase family consists of 20 different subfamilies with the total of 58 proteins (Robinson et al., 2000). This thesis will focus on two different receptor tyrosine kinases from two different subfamilies; the receptor tyrosine kinase-like orphan receptor 1 (ROR1) and the muscle-specific kinase (MuSK). ROR1 was previously believed to be expressed only during embryonic development and in cancer. The believed absence of ROR1 in normal adult tissues has made it an interesting target for cancer therapy development. Here, we intend to show that ROR1 is in fact found in skeletal muscle as well, and that it has an important role in the process where MuSK functions, in the formation of the neuromuscular synapse.

1.1 Receptor Tyrosine Kinase-like Orphan Receptor 1

Receptor tyrosine kinase-like orphan receptors 1 and 2 make up the ROR family of receptor tyrosine kinases. ROR1 was discovered for the first time in 1992 (Masiakowski & Carroll, 1992). The sequence of the RTK kinase domain is highly conserved, but these catalytic domains of ROR1 and ROR2 lack several of the

conserved amino acids. RORs thus have been considered pseudokinases with no ability to phosphorylate substrates (Green et al., 2008). The extracellular part of a ROR receptor consists of an IgG-domain, a cysteine rich domain (CRD) also known as Frizzled domain, and a Kringle domain (Figure1.). The intracellular part of the protein consists of the tyrosine kinase domain, two serine/threonine rich domains, and a proline rich domain (Endo et al., 2015). The Frizzled domain is known to bind Wnt molecules, but not much is known about the functions of the other domains (Endo et al., 2015).

RORs are known to function in embryonic development especially in the brain, where ROR1 is considered to participate in several essential processes including neural progenitor cell (NPC) proliferation, neurite elongation, and synapse formation (Endo et al., 2011). During embryonic development and also in cancer, the ROR receptors function as Wnt molecule receptors in Wnt signaling pathways (Endo et al., 2015, Green et al., 2008). One study suggested ROR1 and ROR2 might bind to form a heterodimer that functions in Wnt-5a signaling network in the brain (Paganoni et al., 2010). The same study with cultured hippocampal neurons showed that downregulation of ROR1 and ROR2 expression resulted in formation of fewer synaptic connections, suggesting a role for RORs in synapse formation in the central nervous system. Generally, ROR2 and its signaling have been more widely studied, while ROR1 and its possible activity especially in adult tissues have remained some of a mystery until recently. It was believed that ROR1 is not expressed in adult tissues at all, except in cancer cells (Balakrishnan et al, 2017).

A recent study demonstrated a role for ROR1 in muscle regeneration after injury (Kamizaki et al, 2017). Depletion of ROR1 led to inhibition of muscle cell progenitor satellite cell proliferation, indicating an important role for ROR1 in muscle regeneration. During muscle injury, the production of inflammatory cytokines tumor necrosis factor TNF- α and interleukin IL-1β enhances both the expression of ROR1 and the proliferation of satellite cells (Kamizaki et al., 2017, Chaweewannakorn et al., 2018, Yang and Hu, 2018). In addition to this, a recent review discussed that ROR1 may have a role in suppression of satellite cell differentiation in aged mice (unpublished data, Kamizaki et al., 2020). The

proposition was based on the increased expression of Frizzled1, which results in suppressed myogenic differentiation of satellite cells in aged mice (Doi et al., 2014) and on the finding of ROR1 expression being reduced at the same time. Another study found that in contradiction to what has been previously thought, ROR1 is indeed expressed in several normal adult cells found in pancreatic islets, esophagus, duodenum, stomach and parathyroid (Balakrishnan et al, 2017).

Our studies also revealed that ROR1 is found in adult muscle tissue. Since ROR1 was previously thought to be absent in adult tissues other than tumors, it has been an interesting target for cancer therapy development. Investigating the expression of ROR1 is essential for the development of cancer treatments and drugs to avoid targeting healthy tissues.

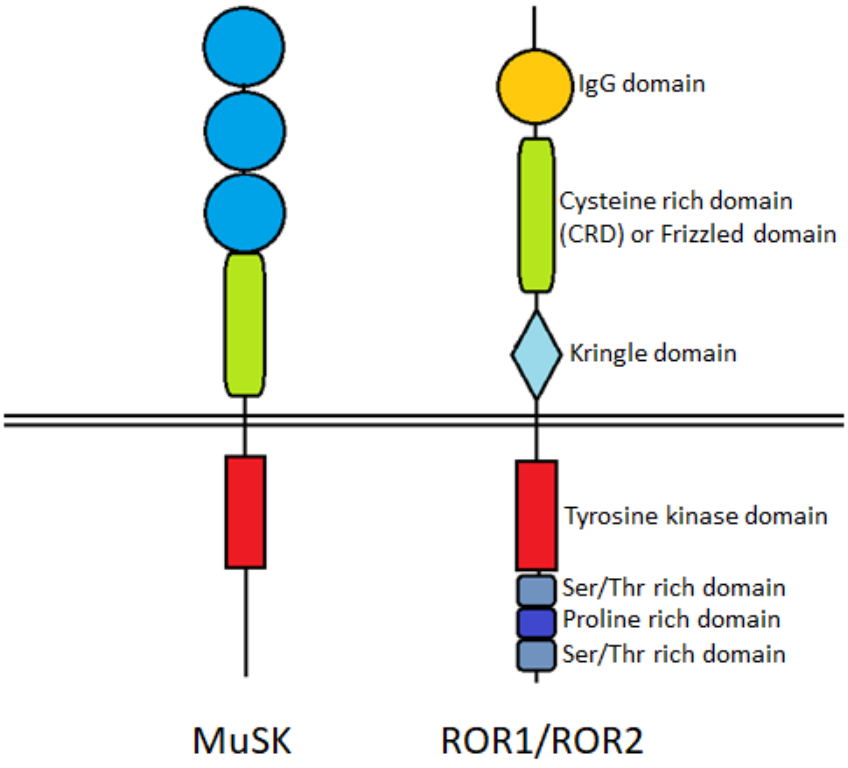

Figure 1. The structure of receptor tyrosine kinases MuSK and ROR1/ROR2

1.2 Muscle Specific Kinase

The closest relative of ROR proteins in the RTK family is the Muscle Specific Kinase MuSK. Despite its name, MuSK has been discovered in other tissues including the brain, where it functions in mediating memory consolidation and synaptic plasticity (Garcia-Osta et al., 2006). MuSK receptor consists of three extracellular Ig-like domains crucial for MuSK activation, an extracellular Frizzled-like CRD domain, and an intracellular region containing a juxtamembrane region and the conserved tyrosine kinase domain (Figure 1.) (Burden et al., 2015). MuSK expressed in fish, avians and amphibians contains a kringle domain like RORs, but the mammalian MuSK lacks the kringle domain. RORs and MuSK share tyrosine kinase domain amino acid similarity of 80% (Green et al., 2008). MuSK plays an essential role in the formation and maintenance of neuromuscular synapses (or neuromuscular junction NMJ). The formation of NMJ is a complicated process involving the signaling of several crucial proteins. MuSK is activated in response to signal protein Agrin, which is secreted by the motor neurons (Burden et al., 2015). Upon activation at the neuromuscular junction, MuSK is responsible for preparing the muscle postsynaptic membrane for differentiation by redistributing the acetylcholine receptors (AChRs) in the postsynaptic membrane (Garcia-Osta et al., 2006). Then in turn, MuSK activation and signaling at the NMJ also affects the motor nerve terminal differentiation (Burden at al., 2015).

MuSK has been found to be expressed at least in the central nervous system, lung liver, heart and spleen (Garcia-Osta et al., 2006, Burden et al., 2015). In the brain MuSK functions in the process of memory formation by maintaining and remodeling the synaptic connections. MuSK is also suggested to have a role in several neuropsychic activities including learning, sleep and movement (Garcia-Osta et al., 2006).

1.3 MuSK activation and neuromuscular synaptogenesis

MuSK activation is known to require the binding of at least three proteins, a signal protein Agrin, a low-density lipoprotein-related protein 4 (Lrp4) and downstream

of tyrosine kinase 7 (Dok-7). Dok-7 is an adaptor protein located in the cytoplasm of myotubes. Dok-7 doesn't have any catalytic activity, but its necessary for the phosphorylation of MuSK (Inoue et al., 2009). Its role in MuSK activation leading to clustering of the acetylcholine receptors is crucial for the neuromuscular synaptogenesis. Mutations in Dok-7 lead to deficient synaptogenesis which causes diseases like myasthenia (Beeson et al., 2006). Dok-7 binds to one of MuSK tyrosine residues, Y553, of the intracellular juxtamembrane region of the tyrosine kinase domain (Burden et al., 2015). In addition to being vital for MuSK phosphorylation since MuSK is not tyrosine phosphorylated in the absence of Dok-7, Dok-7 also becomes phosphorylated itself after binding MuSK. This leads to recruitment of more proteins on the site of NMJ formation (Burden et al., 2015).

A neural signal protein Agrin, secreted by motor neurons, is an extracellular activator required in MuSK phosphorylation. However, Agrin does not directly bind to MuSK, but to low-density lipoprotein receptor-like protein 4 (Lrp4), the receptor of Agrin and another protein required in MuSK activation (Burden et al., 2015)). Agrin also stimulates the phosphorylation of Dok-7. The activation of MuSK triggers a signaling cascade which results in clustering of Acetylcholine receptors (AChR) on a cell membrane, priming postsynaptic membrane of the NMJ for the synapse formation.

In this study, we show that in addition of Dok-7 being necessary for MuSK phosphorylation, it also directly interacts with ROR1. The requirement for ROR1 becoming phosphorylated is that it needs to be bound to both MuSK and Dok-7. ROR1 also interacts with Lrp4 (Karvonen et al., 2018) suggesting that ROR1 and MuSK could be involved in a signaling complex, where the two interact regulating down-stream signaling with other proteins.

1.4 Aims of the study

We intend to show here, that ROR1 is endogenously expressed in normal adult tissues, more specifically in myogenic cells and neurons, and that it interacts with MuSK in the formation of neuromuscular junction. We also show, that ROR1 binds

MuSK adaptor protein Dok-7. In our studies, only ROR1 and not ROR2 bound MuSK and Dok-7. We hypothesize, that ROR1 is a substrate for MuSK phosphorylation and has a crucial regulatory role in MuSK activation in the formation of NMJ.

Since ROR1 expression has previously been believed to occur only during embryonic development and in cancer, ROR1 became a target for cancer drug development. Here in this study, we wanted to show that ROR1 is found in normal adult tissues and functions in the formation of neuromuscular junction, interacting with its closest relative, MuSK.

The aims of the study listed:

1. To show the endogenous expression of ROR1 in mouse myogenic cells
2. To examine the biochemical and functional interaction of ROR1 and MuSK both *in vitro* in C2C12 cells and while endogenously expressed in myogenic cells
3. To provide novel information about ROR1 and its function

2 MATERIALS AND METHODS

2.1 Cell culture and transfections

Human embryonic kidney cells (293T cells) and monkey fibroblast-like kidney cells (Cos-7 cells) were grown in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Life Technologies), 2mM L-glutamine and 1% penicillin-streptomycin (Pen-Strep, Lonza). Cells were incubated at 37 °C and 5% CO_2. Cells were transfected with expression plasmids using TurboFect Transfection Reagent (Thermo Scientific). Wilhelmiina Niininen, the lab technician, also performed many of the transfections for these experiments.

2.1.2 C2C12 culture and differentiation

C2C12 mouse myoblasts were cultured as above. Differentiation into myotubes was induced by changing the growth media to differentiation media, DMEM containing 2% horse serum after the cells had reached 100% confluency. In the experiments in which C2C12 cells were also transfected, the differentiation was induced 24h after transfections. For the Western blot of endogenous protein expression, the differentiated C2C12 myotubes were collected and lysed as below 6 and 8 days into differentiation.

2.1.3 Cell lysis

Cells were lysed and collected in NP-40 or Triton X lysis buffer with 1:100 Protease Inhibitor Cocktails (Biomake, Houston, TX, USA), protease (Biotool) and 1:1000 NEM added in 24-48h after transfection. After 15min incubation on ice, cell lysates were centrifuged at 16.1 x rpm and at 4°C for 15min.

Table 1. Reagents used for cell lysis

Buffer	**Content**	**Proportion**
TritonX lysis buffer	TritonX-100 (T8787-50ML, Sigma)	1 %
	Glycerol	10 %
	NaCl	150 mM
	EDTA	1 mM
	NaF	50 mM
	Tris-HCl pH 7.5	50 mM
	Phosphatase Inhibitor Cocktail A (Bimake)	1 %
	Phosphatase Inhibitor Cocktail B (Bimake)	1 %
	Protease Inhibitor Cocktail (B15001, Bimake)	1 %
Laemlli Sample Buffer	Laemlli Sample Buffer 4x (1610747, Bio-Rad), Laemlli Sample Buffer 2x (1610737, Bio-Rad)	
	β-mercaptoethanol	

2.2 SDS-PAGE, co-immunoprecipitations and Western Blot

2.2.1 SDS-PAGE

The samples were prepared by adding 2X Laemmli Sample Buffer to cell lysates, then the lysates were boiled on a heat block at 95°C for 5 to 10min and centrifuged for 30s at 13.1 x g before running the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), with gels from 7 to 8%. PageRuler ™Plus Prestained Protein Ladder (Thermo Scientific) was run along the samples.

Table 2. Reagents used for SDS-PAGE and Western Blot

Step	**Buffer**	**Content**
Gel run	Gel	30 % Acrylamide/Bis Solution (161-0156, Bio-Rad)
		1,5 M Tris pH 8.8 with 0,4 % SDS
		0,5 M Tris pH 6.8 with 0,4 % SDS
		10 % Ammonium persulfate
		10 % TEMED
	Running buffer	25 mM Tris
		192 mM Glycine
		0,1 % SDS
	2x Laemmli Sample Buffer	2x Laemmli Sample Buffer
		β-mercaptoethanol
	Marker	PageRuler Plus prestained protein ladder (Thermo Scientific)
Transfer	Transfer Buffer	30,8 mM Tris
		0,24 mM Glycine
		20 % methanol
Blocking	Blocking Buffer	0,05 % Tween TBS
		4 % BSA (Thermo Fisher)

Washing	TBS-Tween buffers	0,5 % Tween TBS
		0,1 % Tween TBS
		0,05 % Tween TBS
Antibody dilution	Antibody dilution buffer	0,05 % Tween TBS
		0,5 % BSA (Thermo Fisher)
		0,005 % NaN_3

2.2.2 Western Blot

In Western Blot, the protein samples from the SDS-PAGE gels were blotted onto a nitrocellulose membrane (Amersham) with Bio-Rad Trans blot SD Cell. Membranes were blocked in 4% BSA or Odyssey Blocking buffer (LI-COR) for 1h. The membranes were stained with primary antibodies (His, Flag, HA, Ror1, MuSK or pTyr) for 1h in rt or overnight at 4°C and secondary antibodies (rabbit, goat or mouse) for 1h in rt. The membranes were washed in between and after the antibody stainings for 3 x 10min with washing buffers1 x TBS + 0,5% Tween, 1 x TBS + 0,1% Tween and 1 x TBS + 0,05% Tween. The antibodies are listed below (Table1). Finally, the membranes were visualized with Odyssey CLX LI-COR imaging system and images processed with Image Studio Lite (LI-COR).

Table 3. List of antibodies used in western blotting and immunoprecipitations

Primary antibodies	Antibody	Catalog Number	Manufacturer
	Flag	#F1804	Sigma-Aldrich
	HA	#901513	BioLegend
	His	#MA1-135	Thermo Fisher Scientific
	MuSK	#PA1-1741	Thermo Fisher Scientific
	ROR1	#AF2000	R&D Systems
	ROR1 4A5	#564464	BD Biosciences
	ROR2	#565550	BD Biosciences
	ROR1 6D4		Dr. Riddell lab

	pTyr 4G10	#05-777	Merck, Darmstadt, Germany
Secondary antibodies	RDye® 680RD Donkey anti-Goat IgG		LI-COR
	IRDye®800CW Donkey anti-Mouse IgG		LI-COR
	IRDye® 680RD Donkey anti-Rabbit IgG		LI-COR

2.2.4 Co-immunoprecipitations

To reveal the protein-protein interactions, the cell lysates were first incubated with the antibodies of proteins of interest for 1h or O/N on a rotator at 4°C. Protein A-agarose or G-Plus Agarose beads washed twice by centrifuging the beads for 30s at 10 000 x g, removing the solution, and resuspending the beads in NP-40 Lysis buffer (with added 1:50 Na_3VO_4, 1:100 aprotinin, 1:250 pepstatin and 1:250 PMSF). The beads suspensions were then added into the cell lysates. The lysates were incubated for 1h on a rotator at 4°C. The lysate beads mixture was then centrifuged at 10 000 x g and 4°C for 1min, the cell lysate supernatant was removed and the beads were again washed twice as above. The wash buffer was removed and 2X Laemmli Sample Buffer was added to release the protein by breaking the bonds between the protein, antibody and bead. The samples were heated on a 90°C heat block for 5min and centrifuged at 10 000 x g for 30s. SDS-PAGE and Western Blots were performed as above.

2.3 qPCR

Ror1 and MuSK expression in C2C12 myoblasts, and 4-, 6-, and 8-day-differentiated myotubes was examined with qPCR. Ror1 expression was also studied in neurons (cell pellet received). First, mRNA was extracted for cDNA synthesis using NucleoSpin RNA Plus purification kit (Macherey-Nagel). PCR program for cDNA synthesis was run using iScript Select cDNA synthesis kit (Bio-Rad). For qPCR,

cDNAs were diluted 1:50 with H_2O. 18Sr was used as a reference gene for normalization of the results and Baf3 cells as a negative control for C2C12 cells. When examining Ror1 expression in neurons, Actin was used as a reference gene and Jeko cells as a positive control, and MEC-1 cells as a negative control. Primers (Sigma) used for qPCR listed in Table1. The reagent used was SSo Fast Evagreen Supermix (Bio-Rad), qPCR runs were performed with Bio-Rad CFX96 Real-Time System, C1000 Thermal Cycler.

Table 4. The sequences of the primers used in qPCR analysis

Primer	**Sequence**
Ror1 mouse forward	5′-GCTGCGGATTAGAAACCTTG-3′
Ror1 mouse reverse	5′-TACGGCTGACAGAATCCATC-3′
18S rRNA mouse forward	5′-ATGGCCGTTCTTAGTTGGTG-3′
18S rRNA mouse reverse	5′-CGCTGAGCCAGTCAGTGTAG-3′
MuSK mouse forward	5′-CTGCTGCATAGCCAACAATG-3′
MuSK mouse reverse	5′-CCCTTGATCCAGGACACAGA-3′
Ror1 human forward	5′-AGCGTGCGATTCAAAGGATT-3′
Ror1 human reverse	5′-GACTGGTGCCGACGATGACT-3′
b-actin forward	5′-CGACAGGATGCAGAAGGAGA-3′
b-actin reverse	5′-CGTCATACTCCTGCTTGCTG-3′

2.4 DNA plasmids and Cloning

2.4.1. DNA Plasmids

The ROR1, ROR2, and MuSK DNA plasmids used had been previously cloned into pClNeo expression vector (Promega, Madison, WI, USA) with a tag, either HA, myc, flag or His. Other MuSK and Dok-7 containing expression plasmids were gifted by Dr. Hubbard's lab at NYU.

2.4.2 Site-directed mutagenesis and digestion

MuSK K608A and Ror1 K506A mutations were induced to examine the interactions of the proteins when their site of phosphorylation was mutated. Mutations were performed using QuickChange II Site-directed mutagenesis kit (Agilent technologies, Santa Clara, CA, USA) and the following primers from Sigma:
MuSK K608A forward: 5'-ACTATGGTGGCTGTGGCGATGCTGAAGGAGGAG-3'
MuSK K608A reverse: 5'-CTCCTCCTTAGCATCGCCACAGCCACCATAGT-3'
Ror1 K506A forward: 5'- CAGCTGGTTGCTATCGCGACCTTGAAAGACTAT
Ror1 K506A reverse: 5'-ATAGTCTTTCAAGGTCGCGATAGCAACCAGCTG-3'
The buffer and enzyme used for the mutagenesis PCR were Pfu 10X Cloned Reaction Buffer and Pfu Turbo DNA Polymerase (Agilent). Mutagenesis was performed according to manufacturer's instruction manual. After the PCR, digestion enzyme DpnI (Thermo Scientific) was used to digest the plasmids, and the digestion reactions were then incubated at 37°C for 1-2h.

2.4.3 Transformations

E. Coli XL1-blue supercompetent bacterial strain was used for transformation reactions. Bacteria was thawed on ice. 1µl of digestion reaction was used per 50µl of bacteria. DNA was pipetted on top of bacteria and mixed by tapping. The cells and the DNA were incubated on ice for 30min followed by 45s and 42°C heat shock and again 2min incubation on ice. 500µl of SOC-medium was added and the tubes were placed on a shaker for 1h at 37°C. The bacteria were then plated on ampicillin plates and the plates were placed at 37°C for 16-18h. Colonies from the plates were picked into liquid culture tubes to grow on a shaker o/n at 37°C. DNA was purified for sequencing by using Gene Jet Plasmid Miniprep Kit (Thermo Scientific).

2.4.4 Sequencing

PCR for sequencing was performed using Big Dye Terminator 3,1 Cycle Sequencing RR-100 Kit (Thermo Scientific). Following the PCR reaction, samples were prepared accordingly and taken to the sequencing facility at the University of Tampere.

2.5 Immunostaining

Differentiated C2C12 were immunostained to visualize ROR1 and MuSK at acetylcholine receptors in the cells. Cells were fixed with 4% PFA by incubating in rt for 15 minutes. After two rinsing with 1xPBS, the cells were incubated in 0.1% Triton-X in PBS for 10 minutes to permeabilize cell membranes, and then rinsed again with 1xPBS. Cells were blocked in 1% BSA/PBS for 1h and then incubated in primary antibody solution for 1h, followed by 2x 5min 1xPBS washes. Secondary antibody incubation took place in the dark for 40min. Cells were visualized with Life Technologies EVOS FL Digital Microscope.

3 RESULTS

3.1 ROR1 interacts with MuSK and Dok-7

To examine whether ROR1 and ROR2 can interact with MuSK *in vitro,* we transfected Cos-7 and 293T cells with ROR1, ROR2, MuSK and Dok-7 expression plasmids. Both ROR1 and ROR2 were transfected with MuSK or Dok-7 or both to investigate the interactions. 24 to 48 hours after transfections the cells were lysed, ROR1 and ROR2 were immunoprecipitated using anti-ROR1- and anti-ROR2-specific antibodies, and then western blotted to uncover the possible interactions with MuSK and Dok-7. Here we observed ROR1 interaction with both MuSK and Dok-7 both individually and also in a ROR1-MuSK-Dok-7 complex when all three expression plasmids were co-transfected. ROR2 immunoprecipitation showed no interaction between ROR2 and MuSK or Dok-7 (Fig.2).

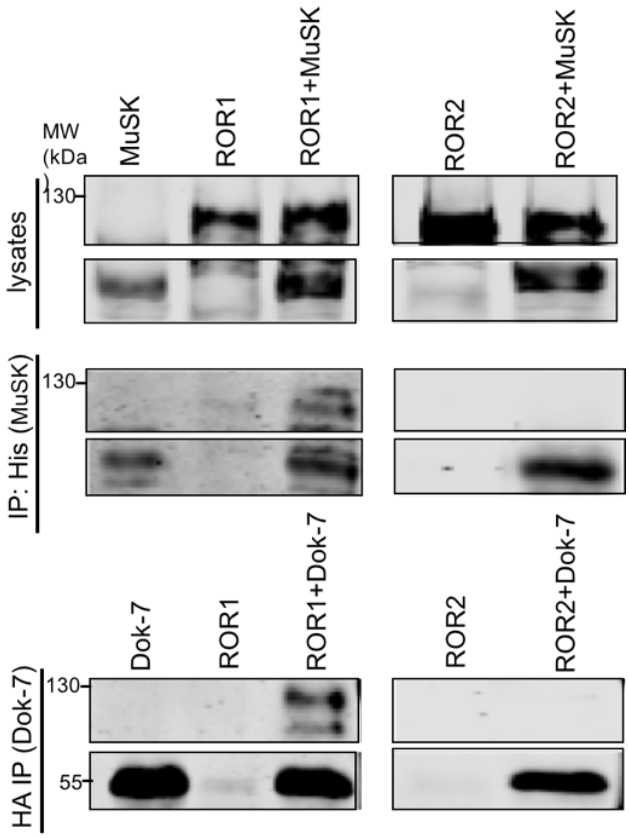

Figure 2. ROR1 immunoprecipitates with both MuSK and Dok-7. ROR2 does not immunoprecipitate with either one.

3.2 ROR1 is only phosphorylated when interacting with both MuSK and Dok-7

Dok-7 is crucial for the activation of MuSK in the neuromuscular synaptogenesis. Both proteins become phosphorylated upon Dok-7 binding MuSK. We wanted to examine whether ROR1 affects the phosphorylation. We observed ROR1 becoming strongly phosphorylated in addition to MuSK and Dok-7, when cotransfected with both of them (Fig.3). When transfected with only either MuSK or Dok-7, no ROR1 phosphorylation was detected.

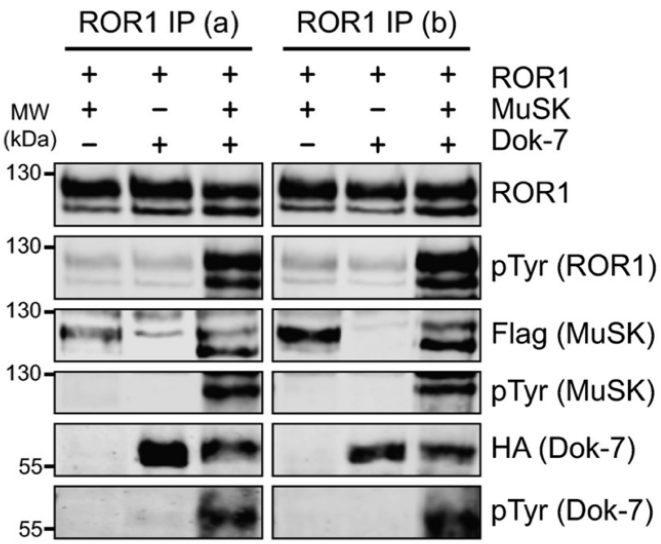

Figure 3. Cos-7 cells were transfected with ROR1 (His-tag), MuSK (flag-tag) and Dok-7 (HA-tag). ROR1 immunoprecipitation was performed and the results analyzed with Western blot. From (Karvonen, et al., 2018), Copyright Federation of European Biochemical Societies, Reproduced with Permission.

3.3 MuSK kinase activity is required for ROR1 phosphorylation, but not for interaction

To examine whether the kinase activity of MuSK is required for ROR1 phosphorylation and ROR1-MuSK interaction, we created a kinase-dead mutant of MuSK (K608A) by mutating the invariant Lys in β3 strand (Till et al., 2002). Cos-7 cells were transfected with ROR1, Dok-7 and either MuSK wild type (WT) or the kinase-dead mutant MuSK K608A. ROR1, MuSK and Dok-7 immunoprecipitations were performed followed by Western blot analysis with spesific antibodies (Fig. 4A). ROR1 was able to coimmunoprecipitate with both MuSK WT and MuSK K608A. In fact, even more with the kinase-dead mutant MuSK K608A than the wildtype MuSK. However, ROR1 phosphorylation was not present with MuSK K608A. This suggests MuSK kinase activity is required for ROR1 phosphorylation, but not for the ROR1-MuSK interaction. Dok-7 phosphorylation could not be detected either in the presence of MuSK K608A, confirming that MuSK kinase

activity is needed for Dok-7 phosphorylation, as previously demonstrated (Bergamin et al., 2010).

It was recently suggested that ROR1 would be catalytically active and able to phosphorylate Receptor tyrosine-protein kinase erbB-3 (Her3), and that the function was eliminated with K506A mutation (Li et al., 2017). Next we tested whether a K506A mutation in ROR1 kinase domain would affect ROR1 phosphorylation by MuSK. An equally strong phosphorylation was detected with both ROR1 K506A and ROR1 WT, when transfected with MuSK and Dok-7 (Fig 4B). The phosphorylation of neither MuSK nor Dok-7 was affected by ROR1 K506A mutant, indicating that the mutant ROR1 does not affect the interaction between ROR1, MuSK and Dok-7.

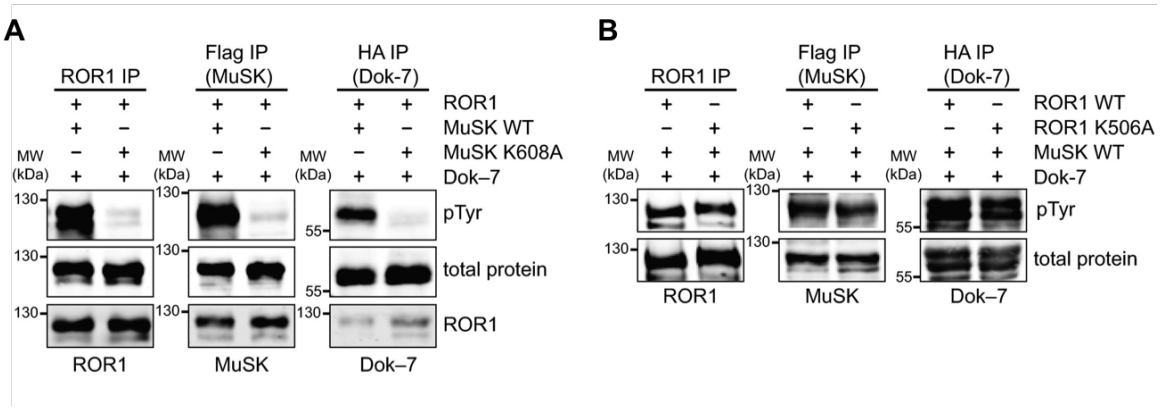

Figure 4. (A) ROR1 interacts with both MuSK wild type and the kinase-dead mutant MuSK K608A, but is only phosphorylated by the wild type MuSK. (B) Both wild type ROR1 and mutant ROR1 K506A interact and become phosphorylated when transfected with both MuSK and Dok-7. 293T cells were transfected with (A) ROR1, Dok-7 and either MuSK wild type or a kinase-dead mutant MuSK K608A and (B) MuSK, Dok-7, and either wild type ROR1 or a mutant ROR1 K506A. ROR1, MuSK (flag) and Dok-7 (HA) immunoprecipitations were done followed by a Western blot analysis with anti-ROR #AF2000, anti-Flag and anti-HA antibodies. From (Karvonen, et al., 2018), Copyright Federation of European Biochemical Societies, Reproduced with Permission.

3.4. MuSK phosphorylates ROR1 at the cytoplasmic proline-rich domain

We created ROR1 truncation plasmids (Fig.5), each missing a portion of the cytoplasmic domain of ROR, to identify the domain of ROR1 that is phosphorylated by MuSK. 293T cells were transfected with ROR1 truncation plasmids together with both MuSK and Dok-7. ROR1 immunoprecipitation with His-antibody was then

performed followed by anti-pTyr Western blot. Results show the full-length ROR1 WT, ROR1 1-876 and ROR1 1-851 were phosphorylated, while the shortest truncations, ROR1 1-782 and ROR1 1-752 were not (Fig.6). This indicates that MuSK phosphorylates ROR1 at its cytoplasmic proline-rich domain.

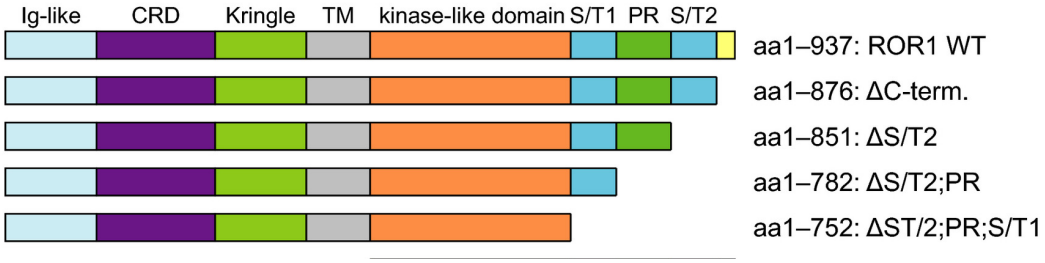

Figure 5. Truncated versions ROR1 lacking part of the cytoplasmic domain. The structure of full-length ROR1 and the domains explained in Fig.1. From (Karvonen, et al., 2018), Copyright Federation of European Biochemical Societies, Reproduced with Permission.

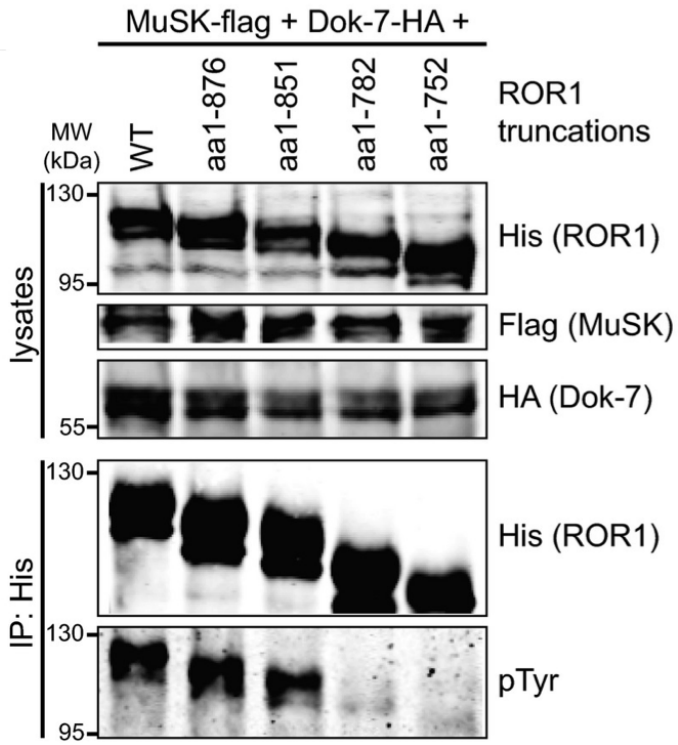

Figure 6. Identifying which cytoplasmic domain of ROR1 becomes phosphorylated. 293T cells were transfected with MuSK (flag-tag), Dok-7 (HA-tag), and the truncated versions of ROR1 (with His-tags). ROR1 immunoprecipitation was performed with His-antibody, followed by a Western blot analysis. From (Karvonen, et al., 2018), Copyright Federation of European Biochemical Societies, Reproduced with Permission.

3.5 ROR1 is endogenously expressed in differentiated C2C12 myotubes and neurons

After validating ROR1-MuSK interaction *in vitro,* we investigated the *in vivo* endogenous expression and interaction using mouse myogenic cell line C2C12. After inducing the differentiation of myoblasts into myotubes under low serum condition, ROR1 and MuSK mRNA levels were monitored with qPCR analysis 4, 6 and 8 days after the differentiation initiation. The qPCR analysis showed an increase in mRNA levels of both MuSK and ROR1 starting from day 4 (Figure 7).

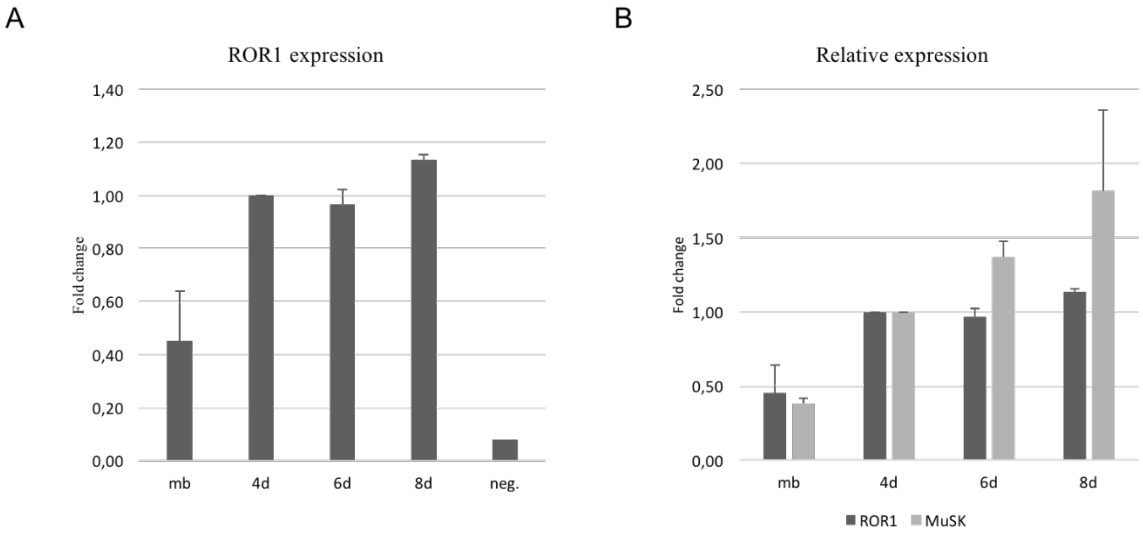

Figure 7. A. Expression of ROR1 in C2C12 myoblasts (mb) and 4-, 6-, and 8-day-differentiated myotubes. Baf3 as a negative control. B. Relative expression of ROR1 and MuSK in myoblasts and myotubes, that have been differentiating for 4, 6 and 8 days.

The results of co-immunoprecipitation experiments with C2C12 cells were in agreement with qPCR results and showed ROR1 expression (Fig8.). Western blot after immunoprecipitation also revealed ROR1 and MuSK interaction *in vivo* in differentiated myotubes.

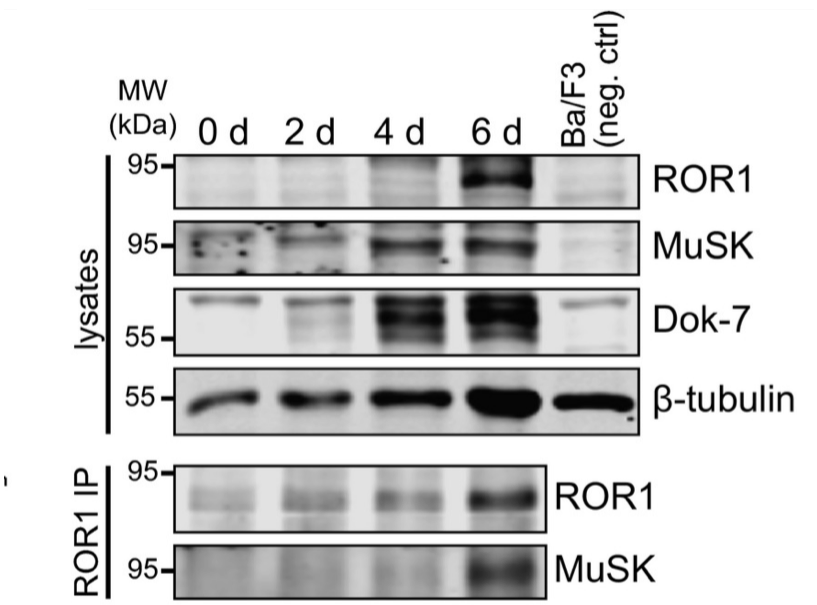

Figure 8. A Western blot of differentiated C2C12 cell lysates was performed to evaluate the protein levels of ROR1, MuSK and Dok-7. The level of ROR1 in cell lysates was quantified relative to β-tubulin levels. ROR1 immunoprecipitation and a Western blot analysis for MuSK and ROR1 were performed. From (Karvonen, et al., 2018), Copyright Federation of European Biochemical Societies, Reproduced with Permission.

3.6 ROR1 transfection affects the differentiation of C2C12 cells

After detecting the endogenous expression of ROR1 in C2C12 cells, we examined the effect of an additional ROR1 transfection on the differentiation of myoblasts into myotubes. C2C12 myoblasts were transfected with ROR1 and differentiation was induced 24h after transfection. The transfection of additional ROR1 resulted in fast and robust differentiation of myotubes (Fig.9). ROR1 transfected cells were manifold in size after five days of differentiation compared to the normal cells not transfected with additional ROR1.

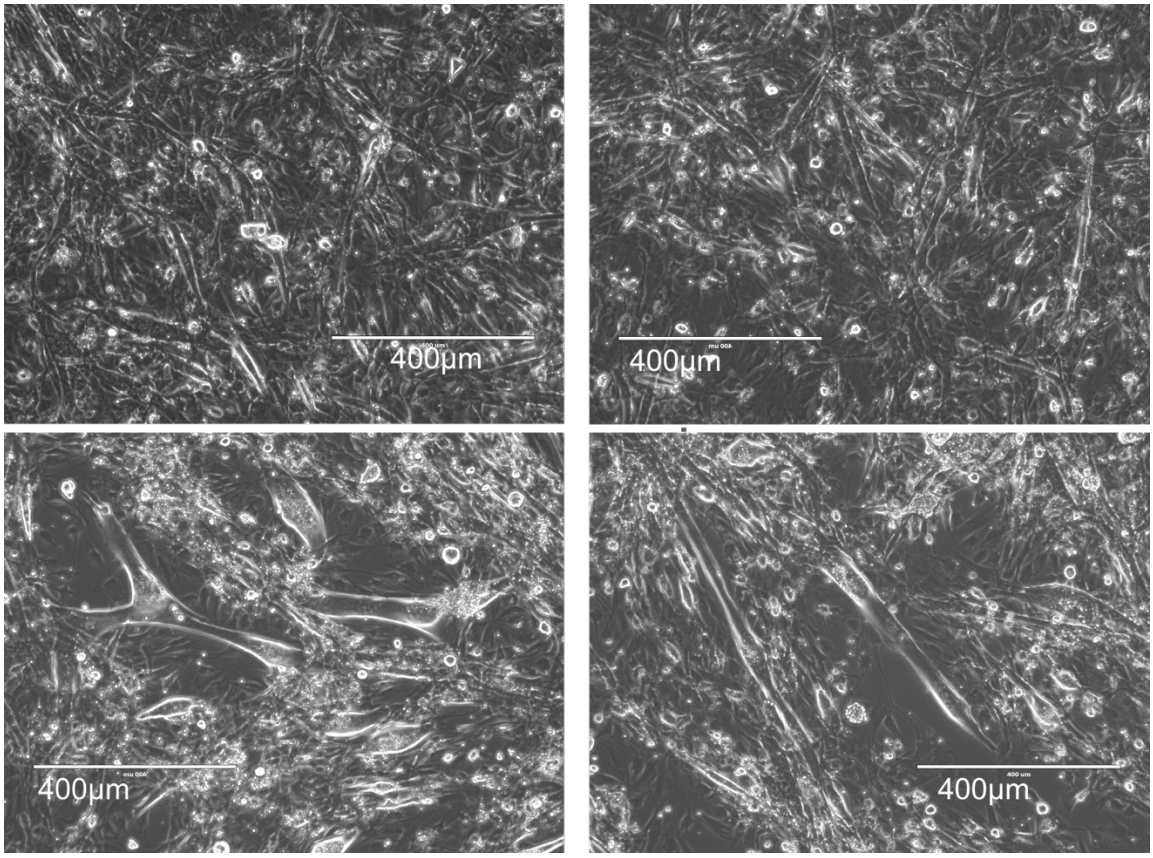

Figure 9. Normal 5 day-differentiated C2C12 myotubes above. ROR1 transfected myotubes after 5 days of differentiation below. The scalebar shown in the pictures for comparison is 400um long.

4 DISCUSSION

ROR1 and MuSK share common structural features being the closest relatives in the receptor tyrosine kinase family. In addition to this, both ROR1 and MuSK have seemed to be robustly abundant during embryonic development, but expressed to lesser extent in adult tissues. Both have also been shown to affect synapse formation in the brain (Garcia-Osta et al., 2006, Paganoni et al., 2010), although examining their possible interaction in the process is yet to be done. Both ROR1 and MuSK are also known to function as receptors for Wnt molecules in Wnt signaling, ROR1 has been shown to bind Wnt-5a (Paganoni et al., 2010) and MuSK to bind several Wnt ligands (Barik et al., 2014), while a common Wnt ligand for the two has not been identified yet. ROR1 was previously thought to be completely absent in adult tissues other

than tumors. Several recent studies have shown a modest ROR1 expression in different adult tissues, including the pancreas, esophagus, duodenum, stomach and parathyroid (Balakrishnan et al, 2017). Here in this study we showed the expression in neurons and muscle as well. The expression pattern of MuSK is similar. MuSK expression levels during embryonic development is high in both brain and muscle compared to the expression levels in adult brain and muscle. Despite the significantly lower levels in adult tissues, MuSK is known to play a vital part in adult muscle tissue and also in memory formation and synaptic plasticity in the brain (Garcia-Osta et al., 2006). This is turn suggests, that although the ROR1 expression levels are low in adult tissues, it is still present and its importance should not be underestimated. ROR1 is still found in several different adult tissues and, as MuSK, may also have an important function. The structural similarity of ROR1 and MuSK combined with a common expression pattern suggested ROR1 might interact with MuSK and have a role of its own in the cellular processes where MuSK functions in crucial roles. In this study, this was demonstrated for the first time. The study shows ROR1 interacts and becomes phosphorylated by MuSK, and also interacts with Dok-7, strongly suggesting ROR1 to have an important role in muscle cell signaling in the process of synapse formation and maintenance. Since only ROR1 and not ROR2 interacts with both MuSK and Dok-7 even though ROR1 and ROR2 are structurally similar, ROR1 must have a very specific role and MuSK a preference for it. In the study in a larger scale, excluded from this thesis, ROR1 was also able to bind Lrp4, which is a receptor for neural Agrin and another protein required for MuSK activation (Karvonen et al., 2018). ROR1 being able to bind Dok-7 and Lrp4 in addition to interacting with MuSK suggests ROR1 might be a part of a larger protein complex in muscle cell signaling. The interaction between ROR1 and Dok-7 was weaker when the three proteins were transfected together compared to their binding in the absence of MuSK, which indicated that ROR1 binds primarily to MuSK.

In another set of experiments, we tested whether disabling the kinase activity of MuSK would affect the ROR1-MuSK interaction and ROR1 phosphorylation (Fig. 4A, Karvonen et al., 2018). ROR1 and mutated MuSK K608A still interacted, in fact

MuSK K608A without kinase activity was able to pull down more ROR1 in co-immunoprecipitation experiments than a MuSK wild type. ROR1 did not become phosphorylated when transfected with MuSK K608A, suggesting MuSK kinase activity is a requirement for ROR1 phosphorylation. These data combined implicates that ROR1 may depart from its interaction with MuSK-Dok-7 complex to have its own function shortly after becoming phosphorylated. Also, Dok-7 is required for the phosphorylation of both MuSK and ROR1 leaving it unclear which one of these two proteins binding ROR1 truly causes the phosphorylation. MuSK phosphorylation and kinase activity is however required for the phosphorylation of ROR1, suggesting MuSK might be the protein mainly responsible for ROR1 phosphorylation.

We also show, that transfecting an additional ROR1 into C2C12 myoblasts before inducing differentiation leads to very fast and robust differentiation of the cells. Overexpression of ROR1 drastically increase the differentiation process, indicating a role for ROR1 in muscle cell differentiation. This would fit in well with the previous findings of ROR1 being vital for muscle cell regeneration after injury (Kamizaki et al., 2017). Although this is interesting and novel information, it requires further studying.

Our studies confirm for the first time that ROR1 and MuSK interact in both *in vitro* transfection experiments and also *in vivo*, in differentiated myogenic cells and that ROR1 becomes phosphorylated upon MuSK phosphorylation. We also show ROR1 interacting with MuSK ligand required for MuSK phosphorylation, Dok-7. These results indicate a signaling role for ROR1 in the formation of the neuromuscular synapses. The ability of ROR1 to bind MuSK ligands as well suggests ROR1 could be a part of a larger protein complex functioning at the NMJ formation and maintenance. We also demonstrated ROR1 being endogenously expressed in differentiated muscle cells and in neurons. These findings add up to previous studies pointing out ROR1 is expressed in adult tissues. Although it is expressed in lesser amounts compared to levels during embryonic development, it may still have an essential role, which should be taken account to while designing cancer treatments targeting ROR1.

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