Arctic charr head development: miR-206 and its targets *tnnc* and *tmyo*

Drífa Hrund Guðmundsdóttir

Líf- og umhverfisvísindadeild
Háskóli Íslands
2015

Arctic charr head development: miR-206 and its targets *tnnc* and *tmyo*

Drífa Hrund Guðmundsdóttir

12 eininga ritgerð sem er hluti af
Baccalaureus Scientiarum gráðu í líffræði

Leiðbeinendur
Zophonías Oddur Jónsson
Kalina Hristova Kapralova

Líf- og umhverfisvísindadeild
Verkfræði- og náttúruvísindasvið
Háskóli Íslands
Reykjavík, maí 2015

Arctic charr head development: miR-206 and its targets *tnnc* and *tmyo*
12 eininga ritgerð sem er hluti af *Baccalaureus Scientiarum* gráðu í líffræði

Líf- og umhverfisvísindadeild
Verkfræði- og náttúruvísindasvið
Háskóli Íslands
Askja, Sturlugötu 7
101 Reykjavík
Sími: 525 4000

Skráningarupplýsingar:
Drífa Hrund Guðmundsdóttir, 2015, Arctic charr head development: miR-206 and its targets *tnnc* and *tmyo*, BS ritgerð, Líf- og umhverfisvísindadeild, Háskóli Íslands, 28 bls.

Prentun: Háskólaprent
Reykjavík, maí 2015

Útdráttur

Í Þingvallavatni er að finna fjögur mismunandi afbrigði Bleikju, formgerð þessara afbrigða er afar ólík, sérstaklega hvað varðar lögun munnsins. Sýnt hefur verið fram á að microRNA spili lykilhlutverk í fósturþroskun dýra með því að stýra tjáningu ýmissa gena sem koma að þroskun. MiR-206 er sérhæft fyrir beinagrindarvöðva og vitað er að það tekur þátt í stjórn tímasetningar vöðvamyndunnar í þroskun. Rannsóknir hafa einnig sýnt að tjáning miR-206 sé mismikil á milli bleikjustofna. Í þessari rannsókn var staðbundin tjáning miR-206 skoðuð á þremur fósturstigum bleikjunnar (150 DD, 161 DD og 200 DD) með *in situ* þáttapörun á heilum fóstrum (WISH). Í ljós kom að miR-206 er tjáð í vöðvum sem tengjast beinum og brjóski kjálkans á öllum fósturstigum. Borin voru kennsl á tvö möguleg markgen (*tnnc* og *tmyo*) með tölvugreiningum. *In situ* þáttapörun leiddi í ljós að bæði genin sýndu sömu tjáningarmynstur og miR-206 og qPCR leiddi í ljós að tjáning genanna einkennist af sveiflum sem eru gagnstæðar við það sem einkennir miR-206 í bleikjunni. Þessar niðurstöður gefa til kynna að að bæði *tnnc* og *tmyo* séu undir stjórn miR-206. Marktækur munur reyndist vera á magni tjáningar *tnnc* á milli tveggja bleikjuafbrigða (LB og AC), sem gefur enn fremur til kynna að miR-206 og markgen þess spili lykilhlutverk í mótun ólíkra svipgerða bleikjunnar.

Abstract

In Þingavallavatn there are four morphs of Arctic charr, differing markedly in morphology, specifically in the structure of their feeding apparatus. It has been established that microRNAs play an essential role in animal development by regulating the expression levels of many developmental genes. MiR-206 is a skeletal muscle-specific miRNA known to play a key role in the regulation of developmental timing of myogenesis. This miRNA has been found to be differentially expressed during the development of contrasting Arctic charr morphs. In this study the spatial expression of miR-206 was analyzed in Arctic charr at three developmental stages (150 DD, 161 DD and 187 DD) using whole mount *in situ* hybridization (WISH). miR-206 was expressed in the muscles supporting the bones and cartilage of the feeding apparatus at all stages. Two candidate target genes were identified (*tnnc* and *tmyo*) using computational approaches. Both genes showed the same spatial expression pattern as miR-206 and qPCR analysis showed that their expression fluctuates in a manner opposite to that of miR-206. The results taken together suggest that both *tnnc* and *tmyo* are likely to be under miR-206 regulation. Significant differences were also detected in the expression levels of *tnnc* between two contrasting morphs (LB and AC), further indicating that miR-206 and its target genes may play a role in the shaping of the morphological differences between the Arctic charr morphs.

Table of contents

Figures

Tables

Abbreviations

AC	Aquaculture
actb	actin B
AGO	Argonaute
AM	Anterior mandebularis
Anti-DIG-AP	Anti-Digoxigenin antibody linked to alkaline Phosphatese
BSA	Bovine serum albumin
cDNA	Complementary DNA
CDS	Coding sequence
DD	Developmental Disabilities
DGCR8	DiGeorge syndrome chromosomal region 8
DMSO	Dimethyl sulfoxide
dsRNA	Double stranded RNA
ets2	v-ets avian erythroblastosis virus E26 oncogene homolog 2
GW182	Glycine-tryptophan protein of 182 kDA proteins
HH	Hyohyoideus
IH	Interhyoideus
IMA	Intermandebularis anterioris
IMP	Intermandebularis posterioris
LB	Large benthivorous
Ldbr	Lariat debranching enzyme
Let-7	Leathal-7
Lin-4	Lineage defective-4
lum	lumican
miRISC	miRNA-induced silencing complex
miRNA	microRNA
mmp2	Matrix metalloproteinase-2
mRNA	Messenger RNA
ncRNA	non conding RNA
pax3	paired box 3

PBT	Phosphate buffer saline with tween
PFA	Paraformaldehyde
PFAGA	Paraformaldehyde/glutaraldehyde
PI	Piskivorous
PL	Planktivorous
pre-miRNA	Precursor miRNA
pri-miRNA	Primary miRNA
Ran	RAs-related Nuclear protein
Ran-GTP	Ran-guanosine triphosphate
RNA	Ribonucleic acid
SB	Small benthivorous
sparc	secreted protein, acidic, cysteine-rich
SSC	Saline-sodium citrate
tmyo	tmyogenin
tnnc	troponin C
TRBP	TAR RNA-Binding Protein
ub2l3	ubiquitin-conjugating enzyme E2 L3
UTR	Untranslated region
WISH	whole mount *in situ* hybridization

Acknowledgements

I would like to think my mentor Zophonías O. Jónsson for the opportunity to learn and work in his laboratory. I would also like to thank Kalina H. Kapralova for all her help, her positivity, patience and teacher skills made this research extremely fun and educational. Last I would like to thank Ehsan Pashay Ahi for his assistance and guidance.

1 Introduction

1.1 microRNA

MicroRNAs (miRNAs) are short endogenous non-coding RNA (ncRNA) molecules, known for their role in post transcriptional gene silencing. These ~22 nucleotide single stranded RNAs bind to a target messenger RNA (mRNA) and prevent translation or mediate their degradation. (Bartel, 2004)

Lin-4 was the first miRNA to be discovered, in the nematode *Caenorhabditis elegans*. *Lin-4* has been found to play a role in the temporal switch from the first larval stage (L1) two the second (L2) in the worm's development by regulating the transcription factor *lin-14.* In 1993 two separate research groups came to the same conclusion, that *lin-4* was not a protein, but a short RNA transcript that regulated gene expression through complementary binding to the target gene mRNA (Wightman, 1993; Lee, 1993). This was thought to be a worm specific mechanism, until the discovery of *let-7* in 2000. Like *lin-4*, *let-7* has a role in a temporal switch between stages in *C. elegans* development (late larva to adult) but it is conserved between distantly related species. This eventually led to the discovery of thousands of miRNA in other organisms. Since their discovery, miRNAs have become known to have a role in various cellular processes including cell differentiation and animal development with their spatial and temporal expression patterns (Reinhart, 2000; Almeida, 2011).

1.1.1 miRNA biogenesis and function

Biogenesis of miRNA (Figure 1) starts with the transcription of primary-miRNA (pri-miRNA). The pri-miRNA transcripts arise from RNA polymerase II transcription of specific miRNA genes (canonical miRNAs) or from spliced introns of protein coding genes (mirtrons) (Westholm, 2011; Ruby, 2007). The pri-miRNAs have a mirrored complementary sequence within them, resulting in the formation of a hairpin structure. In the case of canonical miRNA, the pri-miRNA is cleaved by an RNase III enzyme called Drosha and its dsRNA binding partner DGCR8 to form a ~55-70 nucleotide long miRNA precursor hairpin (pre-miRNA) (Figure 1A). In the case of mirtrons pri-miRNA processing is quite different. The splicing of the pri-miRNA intron occurs with a lariat intermediate before the pre-miRNA is formed with the help of the lariat debranching enzyme (Ldbr) (Figure 1B and C). Sometimes the intron consist solely of the pri-miRNA so the splicing results in the pre-miRNA, in other cases the intron splicing leaves a 5'- or 3'-tailed pri-miRNA product, where the 5'-tailes are trimmed by an enzyme with an unknown function while the 3'-tails are trimmed by RNA exosome (Westholm, 2011). For canonical miRNA and mirtrons the resulting pre-miRNA has a ~2 nucleotide 3'-overhang which is important for the recognition of the molecule by the nuclear export factor Exportin-5 that exports pre-miRNA to the cytoplasm in a Ran-GTP dependent pathway (Westholm, 2011; Bohnsack, 2004). In the cytoplasm the RNase III enzyme Dicer and its binding partner TRBP recognize the 3'-overhang, Dicer cuts the pre-miRNA leaving a miRNA/miRNA* duplex with a 3'-overhang at both ends. One strand of this miRNA duplex is called the guide miRNA and represents the mature miRNA molecule, the other strand which is called the

passenger miRNA or star sequence is degraded. The mature miRNA is finally incorporated into a miRNA-induced silencing complex (miRISC) which consists of the Argonaute protein family (AGO) and glycine-tryptophan repeat-containing protein (GW182) (Westholm, 2011; Braun, 2011). The miRISC binds to the 3'UTR of the target mRNA usually with an incomplete base pairing but always with a perfect match with the seed region which is positioned at nucleotides 2-8 of the 5' end of the miRNA, and acts to either repress translation or to mediate mRNA degradation. The incomplete base pairing between a miRNA and a target mRNA, gives each miRNA molecule the ability to regulate many genes. The decision between degradation and translational repression is thought to lie in the degree of complementarity, where the most complete matches are considered to result in degradation (Westholm, 2011; Hussain, 2012).

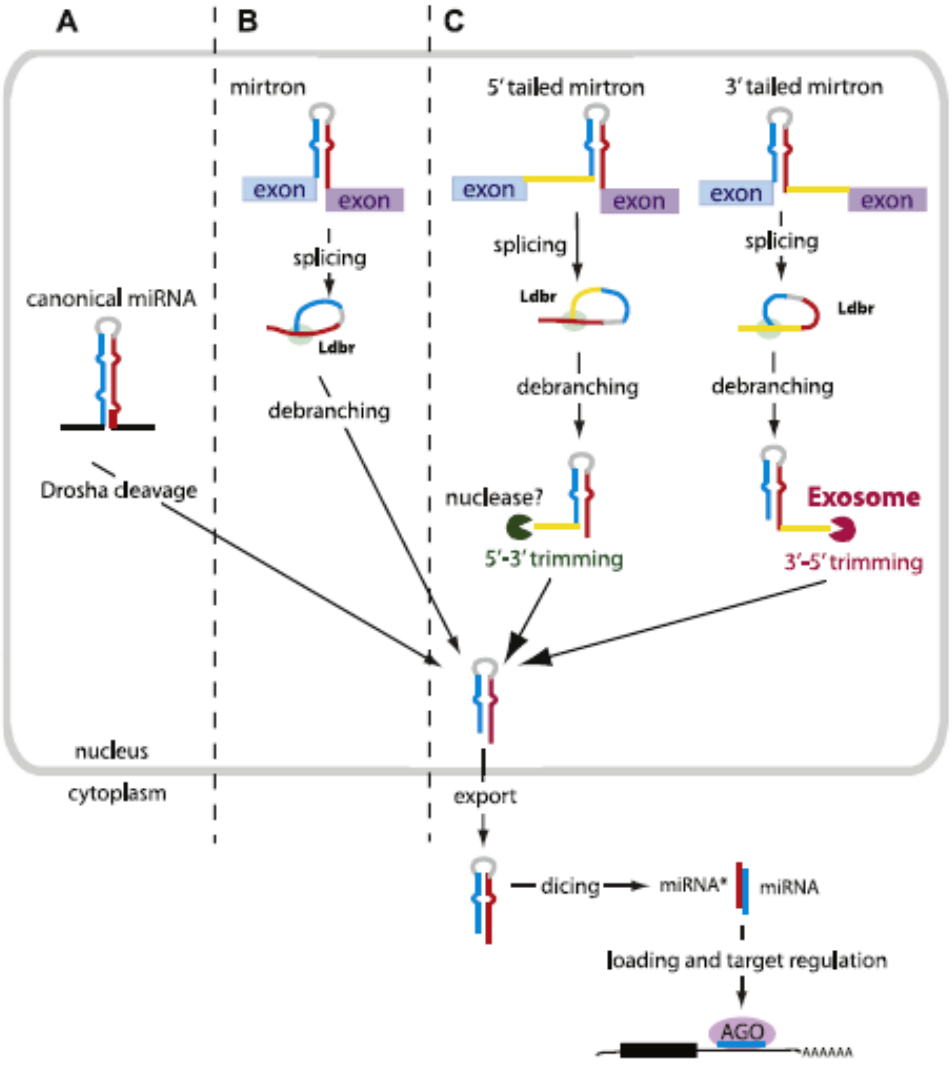

Figure 1 – miRNA biogenesis. *Canonical miRNAs are transcriped as miRNA genes and cleaved by Drosha (A) before they are exported out of the nucleus.Mirtrons are spliced introns of proteincoding genes (B,C), their processing occurs with a lariat intermediate before export (from Westholm, 2011).*

1.1.2 The myomiR; miR-206

Since miRNA discovery in 1993, numerous studies have provided evidence that they are expressed in a tissue specific manner. Seven miRNAs have been identified as striated muscle specific, or so called myomiRs (see details Table 1).

Table 1 – Striated muscle specific myomiRs. *Muscle specificity and origin*.

myomiR	Muscle specificity	Origin
miR-1	Heart and skeletal	miRNA gene
miR-133a	Heart and skeletal	miRNA gene
miR-206	Skeletal	miRNA gene
miR-208a	Heart	intronic
miR-208b	Heart and skeletal	intronic
miR-486	Heart and skeletal	intronic
miR-499	Heart and skeletal	intronic

Since the myomiRs discovery, much interest has been in finding their function in striated muscle. The first myomiR to be discovered was miR-1. The overexpression of miR-1 in HeLa cells and knockout in flies suggested that it had a role in promoting muscle cell identity. It has also been suggested that myomiRs have a function in the stress response following an intense muscle demanding workout and muscle cell proliferation. (McCarthy, 2008; Kirby, 2013).

miR-206 is the only myomiR that is not expressed in cardiac muscle (or at very low levels), and thus the only known skeletal muscle-specific myomiR. It is a member of the miR-1 family along with miR-133 and miR-1, where miR-1 is the closest relative with only a few nucleotide differences. Unlike miR-1, miR-206 has not been found to be expressed in invertebrates and it is enriched in slow-twitch muscle fibers whereas miR-1 does not show fiber-type specificity (McCarthy, 2008; Sweetman, 2008).

In zebrafish several genes that play an important role in embryonic muscle development, are predicted to be miR-206 targets. One of these genes is *pax3*. Research has provided evidence that miR-206 regulation of *pax3* is important in developmental timing of myogenesis (Goljanek-Whysall, 2011).

The important role miR-206 plays in myogenesis and animal development has raised questions about possible roles in adult muscle plasticity and increasing muscle complexity of higher species, which however remains still unexplored for the most part (Kirby, 2013).

1.2 *Salvelinus alpinus* (Arctic charr)

The aim of this project was to examine expression of miR-206 and some of its putative targets in different Arctic charr morphs from Þingvallavatn. There are four Arctic charr morphs present in the lake, grouped into two morphotypes; a limnetic morphotype which include the planktivorous (PL) and the piscivorous (PI) charr and a benthic morphotype

which includes the small benthivorous (SB) and the large benthivorous (LB) charr (Snorrason, 1994). These four morphs show extreme morphological differences in size and shape, especially in the craniofacial structure and the feeding apparatus (Ahi, 2014). The differences between the morphotypes can be seen in Figure 2; fish belonging to the limnetic morphotype are characterized by pointy snouts and evenly protruding upper and lower jaws, while fish belonging to the benthic morphotype have blunt snouts and more protruding upper jaw (Snorrason, 1994). These four sympatric morphs live in different parts of the lake and exhibit different feeding behavior; PL manly feed on zooplankton, PI on threespine stickleback and the benthic morphs mainly feeds on benthos and snails. Such an extreme naturally occurring morphological differences make the Arctic charr a good candidate for studying adaptive divergence and the process of speciation (Kapralova, 2014). These adaptive differences in the morphotypes are thought to arise during development, at least partly through differences in mRNA and miRNA expression. For example the genes *mmp2* and *Sparc* (known for their role in craniofacial development) exhibit differences in the level and timing (heterochrony) of expression during Arctic charr development, which is an indication of their role in the craniofacial morphological differences between Arctic charr morphotypes (Ahi, 2014).

Kapralova *et al*. 2014 found 57 different miRNAs to be differentially expressed between Arctic charr developmental stages and that 72 miRNAs showed differences in expression between two contrasting morphologies (SB and an aquaculture stock from Hólar with a limnetic like morphology (AC)). MiR-206 was one of these miRNAs showing difference in expression between morphs, with higher expression in SB embryos.

Ástrós Skúladóttir 2014 and Kate Ligthart 2014 BSc research projects (Skemman, http://hdl.handle.net/1946/18075 and http://hdl.handle.net/1946/20254) were also a follow up studies of Kapralova et al. 2014, where miR-199a and its targets *lum* and *ets2* were examined in association with head bone and cartilage development. Two putative targets (*lum* and *ets2*) of miR-199a were discovered using computational approaches. These findings were further confirmed using Whole Mount *In situ* Hybridization (WISH): miR-199a, *lum* and *ets2* were found to have the same spatial expression patterns during Arctic charr head development. Further analysis of the tissue specific expression of miR-199a and its targets using cryosectioning suggested that miR-199a and ets2 were both expressed in the perichondrium while *lum* was expressed in both the perichondrium and the cartilage tissues. Heterochronical differences were also detected between Arctic charr morphs for both miR-199a (Kapralova, 2014) and ets2 (Ahi, 2014). These findings strongly suggest that miR-199a and *ets2* as its target may play a role in the Arctic charr morphological differences.

The aim of the project presented here was to examine the spatial expression of miR-206 and its potential targets in the development of the muscles of the feeding apparatus in two contrasting Arctic charr morphs. To this end putative targets of miR-206 were identified using computational approaches. Next the expression of miR-206 and its putative targets was examined using WISH in two different morphs (SB and AC), and by qPCR quantification in four different morphs (SB, LB, PL and AC) at different developmental stages (150 DD, 161 DD and 200DD).

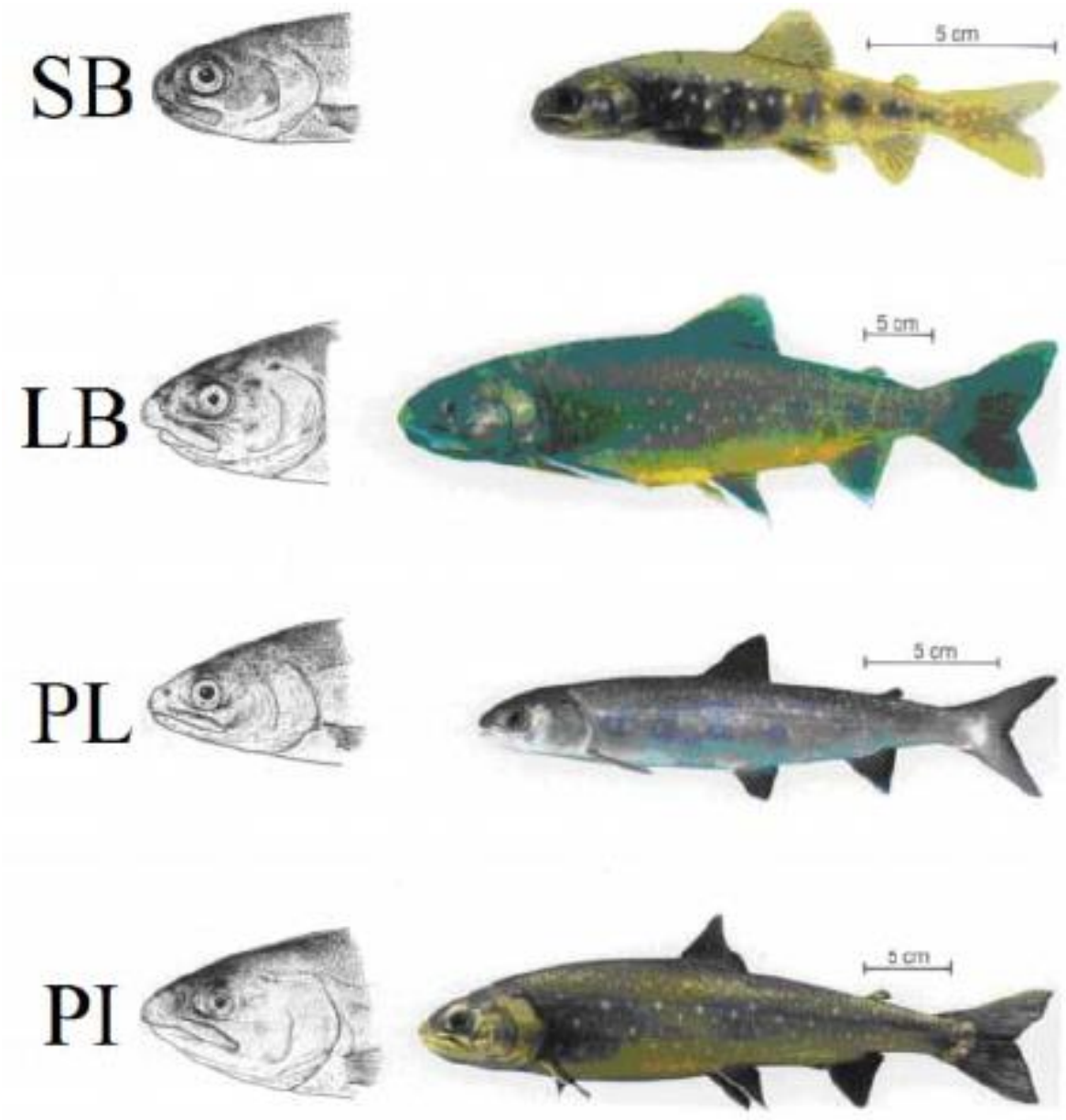

Figure 2 – Arctic charr morphological differences*. The figure shows the size differences, and morphological differences of the head of the four Arctic charr morphs from Þingvallavatn. SB=small benthivorous, LB= large benthivorous, P L=planktivorous and PI=piscivorous (from Sandlund et al. 1992).*

2 Materials and methods

The methods used in this study are summarized in the following flowchart seen in figure 3.

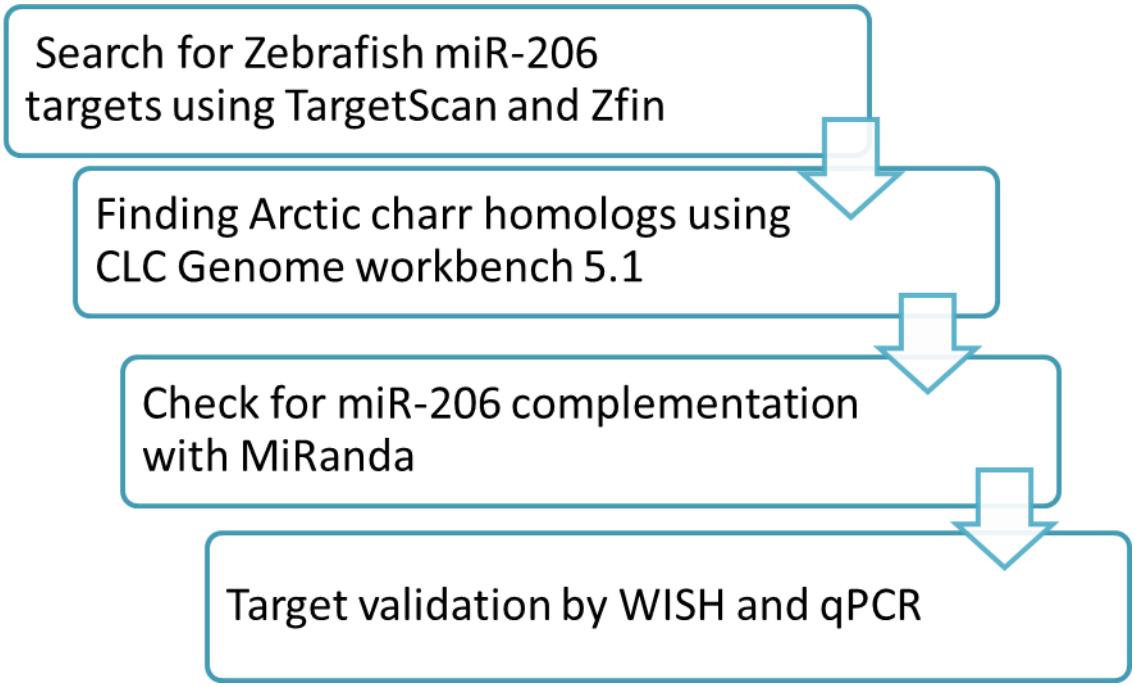

Figure 3 – Method flowchart*. Flowchart of methods used in the search for miR-206 target gene and in target gene validation.*

2.1 Bioinformatics: search for miR-206 target genes and primer design

2.1.1 Search for miR-206 target genes

To find miR-206 targets we used a conservation based approach. As miRNA targets are often conserved between species (Farh, 2005), we searched for known miR-206 targets in zebrafish and targets known to be conserved between fish, mouse and humans. Putative zebrafish miR-206 targets were also taken into consideration. The selected targets were then tested in Arctic charr for mRNA-miR-206 complementarity using miRanda.

A step-by-step description of the approach and databases used is shown below:

1. TargetScan.org was used to find known miR-206 targets in zebrafish since information about Arctic charr is unavailable (http://www.targetscan.org/cgi-bin/targetscan/fish_62). TargetScan identifies possible miRNA targets by finding complementation to the miRNA seed region. To narrow down the search the following criteria were used: genes with known expression in development were selected, while undescribed genes were discarded.

2. miRTarBase (http://mirtarbase.mbc.nctu.edu.tw/) was used to find target genes that are conserved between human, mouse and zebrafish. miRTarBase is a database with a collection of known miRNA targets and a record of target gene literature and experimental target validation. Candidate target genes that had a record of experimental validation were chosen for further analyses.

3. Exiqon.com was used to find out what the miR-206 spatial expression is during fish development (http://www.exiqon.com/gallery-of-in-situ-hybridization-images).

4. Zfin.org was used to examine spatial expression of candidate target genes during zebrafish development (http://zfin.org/search?q=&fq=category%3A%22Expression%22&category=Expression). Zfin.org is a model organism database for zebrafish.

5. 3'-UTR sequences were extracted from the available zebrafish genome, using the following steps:
    - Go to http://www.ensembl.org/index.html, to obtain the genes 3'UTR and coding sequence (CDS).
    - Use the CDS to find the 3'UTR of the candidate targets in Salmonids, using NCBI. Search for somewhat similar sequences (blastn) in Salmonidae. From all available sequences, the ones with complete CDS and a query cover >80% were chosen and their 3'UTR located (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome).

6. CLC genome workbench 5.1 was used to do an alignment of the candidate genes, using Arctic charr transcriptome available in the lab to find Arctic charr homologs.

7. 3'UTR sequences of possible Arctic charr target genes found with the methods listed above were run against miR-206 using MiRanda. MiRanda is an algorithm that gives an alignment score with focus on the complementation between the miRNA seed region and a potential target. The program also gives an estimate of the thermodynamic stability of the alignment.

2.1.2 qPCR and *in situ* hybridization primer design

The following tools and databases were used for primer design: CLC genome workbench 5.1, Salmon DB, reverse complement and OligoAnalyzer 3.1. The goal was to design qPCR primers overlapping exon-exon boundaries. First Salmon DB (http://salmondb.cmm.uchile.cl/) was used to locate exons and CLC genome workbench 5.1 then used to design the primers. Second, 20-25 bp primers were designed and analyzed with respect to melting temperature (T_m) and GC%, using Oligoanalyzer 3.1 (https://eu.idtdna.com/calc/analyzer). Self-primer analysis was done for all primers and hetero-dimer analysis for both reverse and forward primers together. Only primers with a GC% over 50%, a T_m between 57-63°C and with a low ability to form heterodimers (< 7kcal/mol) were considered. After designing forward and reverse primers, the reverse primers were reverse complemented (http://www.bioinformatics.org/sms/rev_comp.html).

The same reverse primers were used for both qPCR and whole mount *in situ* hybridization (WISH), but new forward primers were made for WISH to reach a sufficient product

length to exhibit an optimum sensitivity and specificity of the WISH probes (for primer details see Table 2).

The primers were designed to have the probes overlapping with the miR-206 target site in the 3'UTR. Having the probes include the actual miR-206 target site of the gene eliminates background staining of possible paralogues. Also, it provides a stronger argument for co-localization of the miRNA and the putative target gene with the correct 3'UTR.

Table 2 – qPCR and WISH primers. *Primer length, melting temperature (T_m), GC content (GC %) and product length.*

gene	***qPCR primers***	***length***	***T_m(°C)***	***GC%***	***product length***
tnnc	F_CTCTTCGCCCCTTACTGGTCCATT	24	64.26	54.17	85
	R_GTAACCTCCACCAGGTGCCT	20	61.49	60.00	
tmyo	F_GCAGGGAAACCTACTGTACATTCCA	25	62.55	48.00	132
	R_GCTGTGTGGTGTCCAAGTGCTA	22	63.01	54.55	
	WISH primers				
tnnc	f_CGGAACCATCGACTTCGAGGAGTTC	25	60.80	56.00	442
	R_GTAACCTCCACCAGGTGCCT	20	61.49	60.00	
tmyo	f_TGTCGTCGTCGAGTGAGCAGG	21	61.02	61.90	481
	R_GCTGTGTGGTGTCCAAGTGCTA	22	63.01	54.55	

2.2 Probe synthesis

cDNA synthesis was done with a reverse transcription kit (Applied Biosystems) and the resulting cDNA was purified from gel (Macherey-Nagel, NucleoSpin®). This was followed by *in vivo* cloning (Life technologies, TOPO®cloning) and colony PCR with T3/T7 primers. *In vivo* cloning products were then retrieved from colonies (Life technologies, PureLink® MiniPrep). This was followed by product linearization and gel purification. Then in vitro transcription was performed (Roche, DIG RNA Labelling Kit (SP6/T7)) and the product size and quantity were verified on agarose gel and by nanodrop (Thermo scientific, NanoDrop 1000), respectively.

Probe synthesis was partly done by Ehsan Pashay Ahi.

2.3 Whole mount *in situ* hybridization (WISH)

2.3.1 Buffers and solutions

Phosphate buffer saline/0.1% tween (PBT)

4% paraformaldehyde (PFA) in PBT

25%/50%/75% methanol (MeOH) in PBT

Paraformaldehyde/glutaraldehyde (PFAGA)

Hybridization buffer (2 g blocking reagent (Roche), 1 mL 10% triton X, 1 mL 10% chaps, 25 mL SSC, 50 mL formamide, 500 µL tRNA(10 mg/mL), 100 µL heparin (50 mg/mL), 2.5 mL EDTA(0.2 M), up to 100 mL with MilliQ water)

2x saline-sodium citrate (SSC), 2xSSC/0.1% Tween, 0.2xSSC/0.1% Tween

Blocking solution (4 mL BSA(50 mg/mL), 5 mL sheep serum, 1 mL DMSO(dimethyl sulfoxide), PBT up to 100 mL)

Coloration buffer (10 mL $MgCl_2$(1M), 20 mL NaCl(1M), 20 mL Tris pH 9.5, 200 µL Levamisole(1M), 20 mL 10% Tween 20, up to 200 mL MilliQ water)

2.3.2 Embryo pre-fixation

Embryos were pre-fixed for 30 minutes in 4% PFA in PBT. The chorion was punctured with care not to damage the embryos. Punctured embryos were then incubated in 4% PFA overnight at 4°C. After incubation and two short PBT washes, embryos were dehydrated with increasing concentration of MeOH in PBT, with two final washes in 100% MeOH. Embryos were stored at -20°C in 100% MeOH. This step was done by Kalina H. Kapralova at Hólar.

2.3.3 Probe hybridization

Embryos were rehydrated with short washes with decreasing concentration of MeOH in PBT, and two final PBT washes. Each embryo was dissected, by carefully removing the chorion and the yolk sac. The dissection took place under a stereoscope (Leica S61) in ice cold PBT. Embryos were permeabilized using proteinase K diluted 2:1000 in PBT, the digestion took place at 37°C for 15-30 minutes depending on developmental stage (for details see table 3). After two short PBT washes, embryos were fixed in PFAGA for 20 minutes. Fixation was followed by three extra PBT washes and embryos were then placed in hybridization buffer, after sinking to the bottom they were moved to pre-heated hybridization buffer and incubated for 2 hours. A temperature of 70°C was used for the target genes and 64°C for miR-206. After the pre-hybridization step, embryos were moved to a pre-heated hybridization buffer containing DIG-labeled probes for the candidate target genes and incubated overnight.

2.3.4 Washing, blocking and antibody reaction

Salt washes with increasing stringency were done after overnight hybridization: first two washes with 2xSSC , followed by three washes with 2xSSC/0.1% Tween and three washes with 0,2xSSC/0.1% Tween. Salt washes are done at 64/70°C with pre-heated SSC buffers. This was followed by two PBT washes at room temperature and a 2 hour incubation in blocking solution. Embryos were then incubated overnight in 1:2000 anti-DIG-AP in blocking solution, at 4°C on a rocking platform.

2.3.5 Color reaction

The antibody was removed with eight PBT washes (20 minutes each) followed by 4 short washes (5 minutes each) in the coloration buffer. The color reaction took place in 1.5 mL

of coloration buffer with 30 µL of NBT-BCIP. After this step, embryos were kept in the dark at all times. The coloration reaction took 1-2.5 hours depending on the probes and developmental stage. After staining could be seen, embryos are washed with PBT and then fixed for an hour in 4% PFA. Embryos were stored in PBT at 4°C in the dark.

Table 3 – Proteinase K digestion. *Digestion time for different developmental stages.*

Developm. Stage	Digestion time
150dd	15 min
161dd	15 min
177dd	22 min
187dd	23 min
200dd	30 min

2.4 qPCR

2.4.1 cDNA synthesis

cDNA was made from previously extracted and DNase digested RNA from three embryonic stages (150dd, 161dd and 200dd) of whole Arctic charr embryos.

First the RNA products were diluted to 200ng/µL, and then the RNA was reverse transcribed to cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) as recommended by the manufacturer.

After cDNA synthesis, products were stored at -21°C until qPCR was performed.

2.4.2 qPCR

For the qPCR we made use of the SYBR green amplification detection method. SYBR green is a fluorescent dye that emits a signal as it binds to dsDNA. A Master Mix was prepared with the superSYBRgreen® reagent and the previously designed primers as recommended by the manufacturer (Applied Biosystems), with a 5µM final concentration of each primer and 20-40 ng of cDNA template, and a final reaction volume of 10 µL. The qPCR system that was used for the experiment was ABI 7500 real time PCR system (Applied Byosystems). Each reaction was run with two biological replicates and two technical replicates. The qPCR run started with a 2 minute holding stage at 50°C and hot start for 10 minutes at 95°C, this was followed by 40 cycles of 15 second denaturing at 95°C and 1 minute extension at 60°C. A melting curve was obtained at the end of each qPCR run to verify the product specificity.

2.4.3 Reference genes and data normalization

The housekeeping genes *actb* and *ub2l3* were chosen as reference genes, according to findings in Ahi et al. 2013. The *ub2l3* primers did not work in our hands, they were suspected to be degraded do to contamination, so the normalization was calculated only with the *actb* results (instead of using the geometric mean of both reference genes).

Expression levels and normalization for both genes were calculated as follows:

$\Delta C_{T\ target} = C_{T\ target} - C_{T\ reference}$

$\Delta\Delta C_{T\ target} = \Delta C_{T\ target} - \Delta C_{T\ calibrator}$ (the highest $\Delta C_{T\ target}$ was used as a calibrator)

Relative quantity (RQ)= $2^{-(\Delta\Delta CT\ target)}$

Relative quantity was used to present the data and for statistical analysis.

3 Results

3.1 miR-206 is expressed in muscles of the feeding apparatus throughout Arctic charr development

As previously mentioned miR-206 is involved in craniofacial development. miR-206 is a relatively well-studied miRNA and its expression pattern has been described in zebrafish. Figure 4 shows the expression pattern of mir-206 in zebrafish larvae (left panel), compared to a fluorescent antibody staining of zebrafish larvae jaw muscles (right panel). It can be seen from this figure that miR-206 is expressed in different craniofacial muscles such as the anterior mandebularis (AM), the interhyoideus (IH), the hyohyoideus (HH), the intermandebularis anterioris (IMA) and the intermandebularis posterioris (IMP).

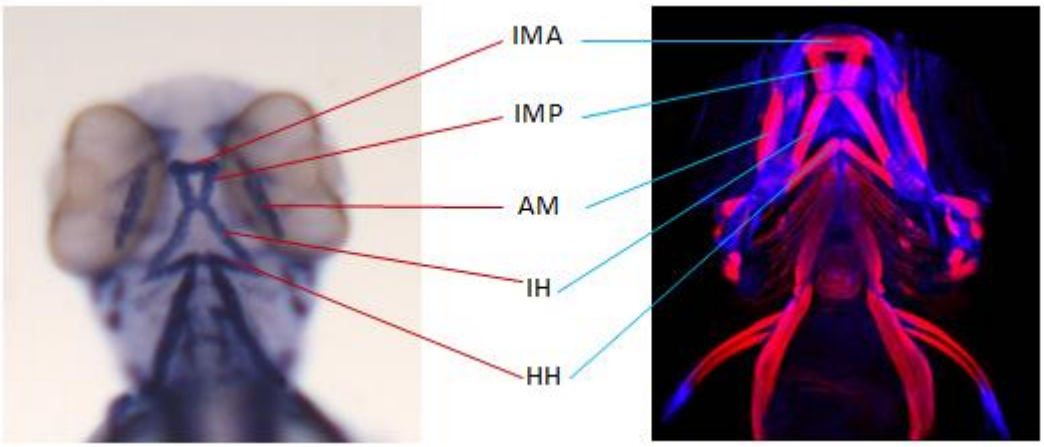

Figure 4 – Muscles of the zebrafish feeding apparatus. *Ventral view of a zebrafish larvae head: miR-206 in situ hybridization in Zebrafish (left) and a fluorescent antibody staining of Zebrafish jaw muscles in red (right). AM: anterior mandebularis; HH: hyohyoideus; IH: interhyoideus; IMA: intermandebularis anterioris; IMP: intermandebularis posterioris. From: http://www.exiqon.com/gallery-of-in-situ-hybridization-images and http://www.sheffield.ac.uk/bms/research/roehl/research.*

The expression profiles of miR-206 throughout development were examined using WISH of three developmental stages (150, 161 and 187 DD) (Figure 5). Because of sample availability, only the AC morph was used for the developmental profiles. For all the stages, staining was detected at the anterior mandebularis (AM), interhyoideus (IH) and intermandebularis posterioris (IMP), although the staining of IMP was vague at 155 DD. Staining was also visible at the intermandebularis anterioris (IMA) for the two later stages (161 DD and 187 DD). The staining pattern of the muscles appears to become clearer as development progresses. For the WISH of miR-206 no negative control was used, since the probe that was used is a commercial LNA probe and miR-206 WISH had already been ran

with scramble RNA as negative control (Kapralova, 2014 unpublished data) on identical samples. Moreover WISH staining patterns of miR-206 were consistent between individuals of all stages and morphs.

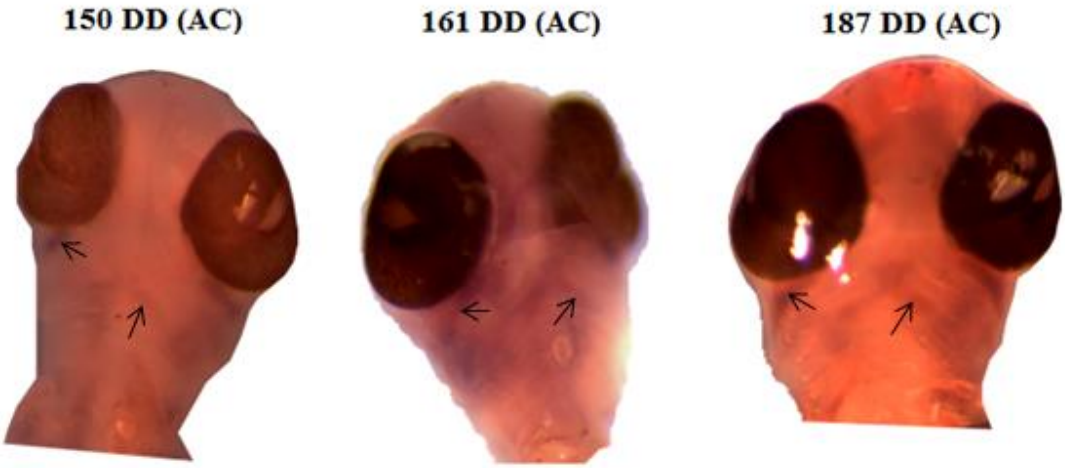

Figure 5 – miR-206 expression throughout Arctic charr development. *miR-206 WISH staining in the Arctic charr morph AC, developmental stages 150 DD, 161 DD and 187 DD.*

3.2 *tmyo* and *tnnc* are a likely miR-206 targets in Arctic charr

3.2.1 miR-206 seed region is complementary to the 3`UTR of *tmyo* and *tnnc*

When searching for miR-206 target genes in Arctic charr (see protocol in chapter 2, materials and methods), 30 candidates were found to be putative targets showing a similar *in situ* hybridization pattern according to the zebrafish database (zfin.org). Of those 30 genes, 12 had sequenced Arctic charr homologs and two of them (*tmyo* and *tnnc*) were found to be putative targets of miR-206 in Arctic charr. (Table A1).

Furthermore miRanda showed a complete seed region complementation between miR-206 and bases belonging to the 3'UTR of the Arctic charr genes *tmyo* and *tnnc* (Figure 6).

Figure 6 – Complementary sequences in seed region and bases in 3'UTR. *miRanda output shows complete complementation between miR-206 seed region and bases belonging to tnnc and tmyo 3'UTR.*

3.2.2 Overlapping expression patterns of miR-206 and the target genes

WISH for both *tmyo* and *tnnc* was performed with two developmental stages (150 DD and 187 DD) and two morphs (AC and SB). Both genes showed very similar expression patterns as miR-206. For *tmyo* both stages showed staining at the AM, IH, IPM and IMA and the pattern was clearer for the earlier developmental stage in both morphs (Figure 7). *Tnnc* showed a similar expression pattern as the one described for *tmyo*, except the IMA was not stained at the earlier developmental stages in either morph (Figure 8). WISH staining patterns of *tmyo* and *tnnc* were consistent between individuals of all stages and morphs.

For the *tmyo* and *tnnc* WISH, sense probes were used as negative control since the expression pattern was not previously known. Sense probe embryos showed no staining (data not shown).

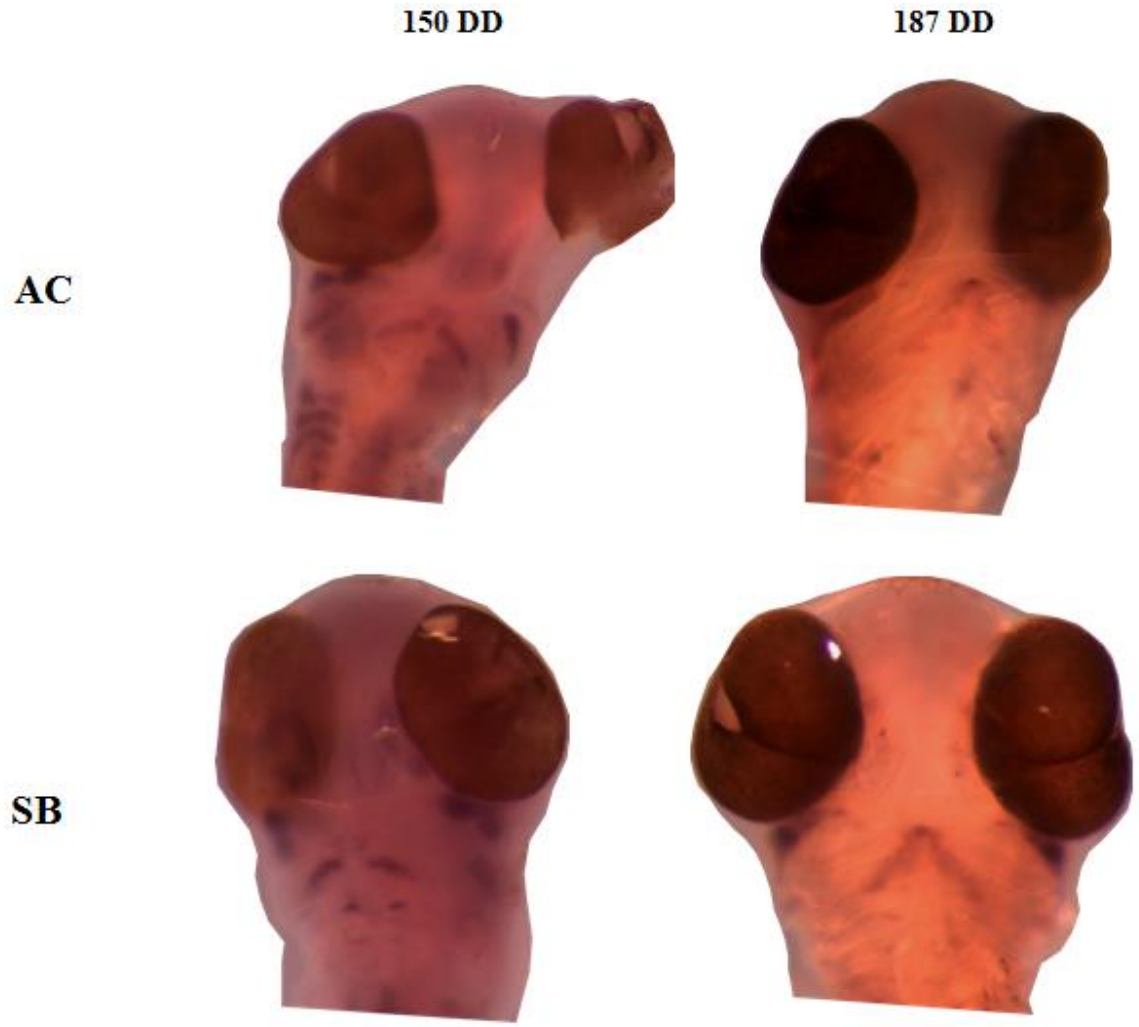

Figure 7 – tmyo expression in two contrasting Arctic charr morphs. *tmyo WISH staining in two Arctic charr morphs (AC and SB) at two developmental stages (150 DD and 187 DD).*

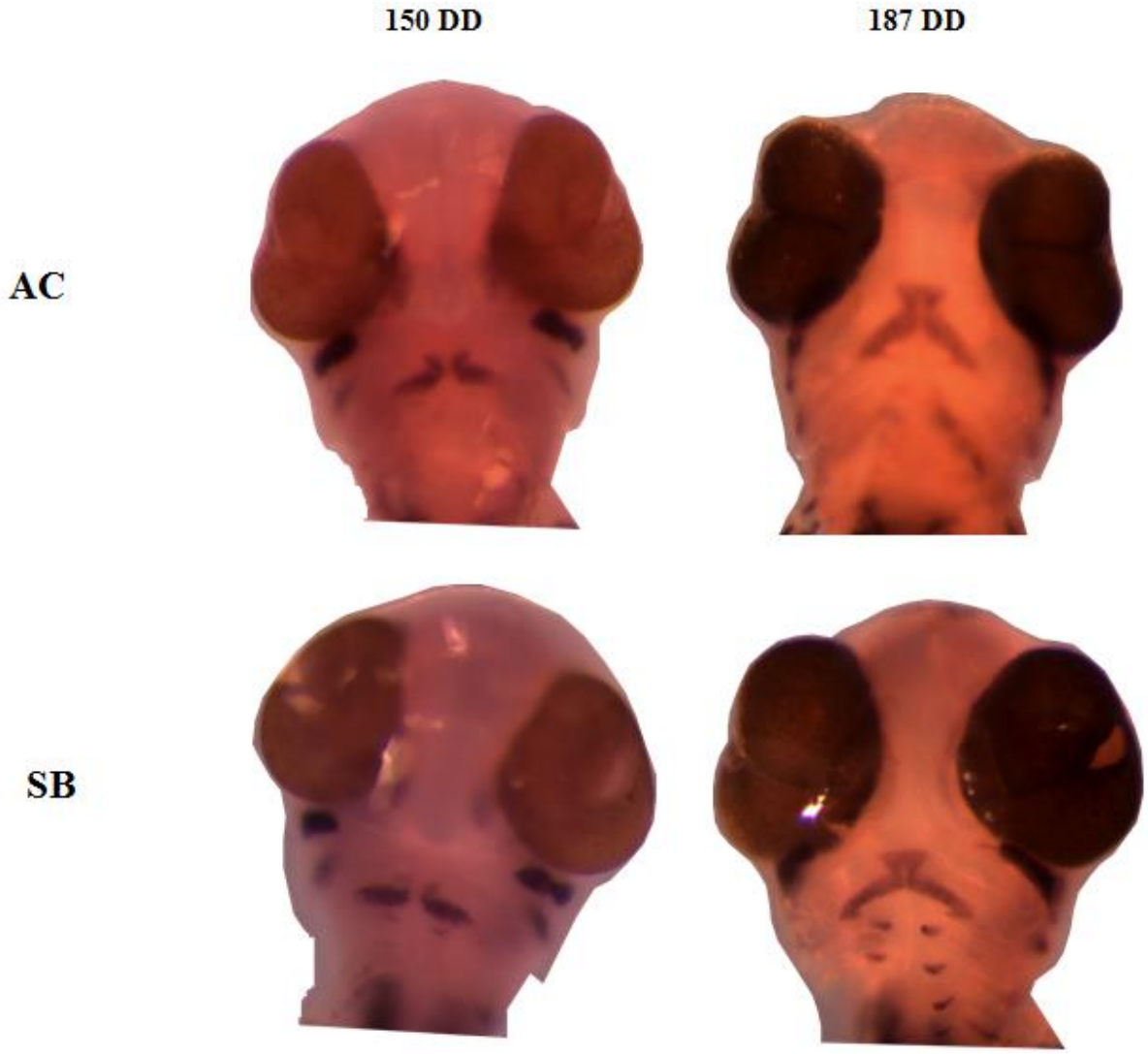

Figure 8 – tnnc expression in two contrasting Arctic charr morphs. *tnnc WISH staining in two Arctic charr morphs (AC and SB) at two developmental stages (150 DD and 187 DD).*

3.2.3 qPCR of miR-206 and its putative targets *tmyo* and *tnnc*

qPCR using LNA primers for miR-206 was done by Kalina H. Kapralova, it was performed with three developmental stages (150 DD, 161 DD and 200 DD) and two morphs (AC and SB). The results for the miR-206 analysis can be seen in Figure 9, where the upper graphs show results for RNA-seq and the lower graphs show the qPCR results. There is a good consistency between the RNA-seq and the qPCR results, except the AC expressional peak is at a later stage in the RNA-seq results. Furthermore, the data showed a significant difference in expression levels between the two morphs. Instead of using a reference gene for this analysis, the geometric mean of all the miRNAs studied by qPCR (miR-196a, miR-199a, miR-181a, miR-140, miR-30b, miR-26a, miR-206 and miR-17) was used as a reference (Kapralova, 2014).

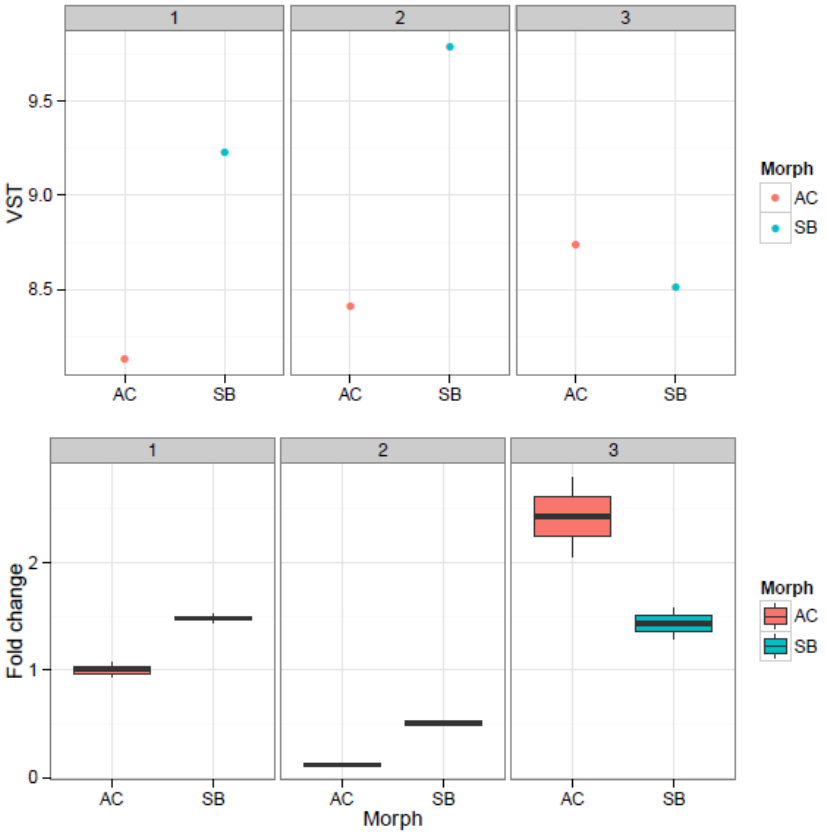

Figure 9 – miR-206 expression in two contrasting Arctic charr morphs. *RNAseq (upper panel) and qPCR (lower panel) results for two contrastin Arctic charr morphs (AC and SB) and three developmental stages (150 DD, 161 DD and 200 DD). Developmental stage 150 DD is market as 1, 161 DD as 2 and 200 DD as 3. AC is in red color and SB in blue.*

qPCR was also performed for the putative targets of miR-206 *tmyo* and *tnnc* for all the morphs and the same three developmental stages (150 DD, 161 DD and 200 DD). The results show that both genes follow the same temporal pattern with expressional peak at 161 DD (Figures 10 and 11). When this is compared to the data from the miR-206 qPCR in Figure 8 it can be seen that miR-206 expression is at a low point at the 161 DD developmental stage, the same stage where *tmyo* and *tnnc* expression peaked. These results, taken together with the WISH results, that show overlapping expressional patterns of miR-206 and the target genes, and the fact that there is a complete complementation between the miR-206 seed region and the putative miR-206 binding sites in the 3'UTRs of the target genes, indicate that both *tmyo* and *tnnc* are miR-206 targets in Arctic charr.

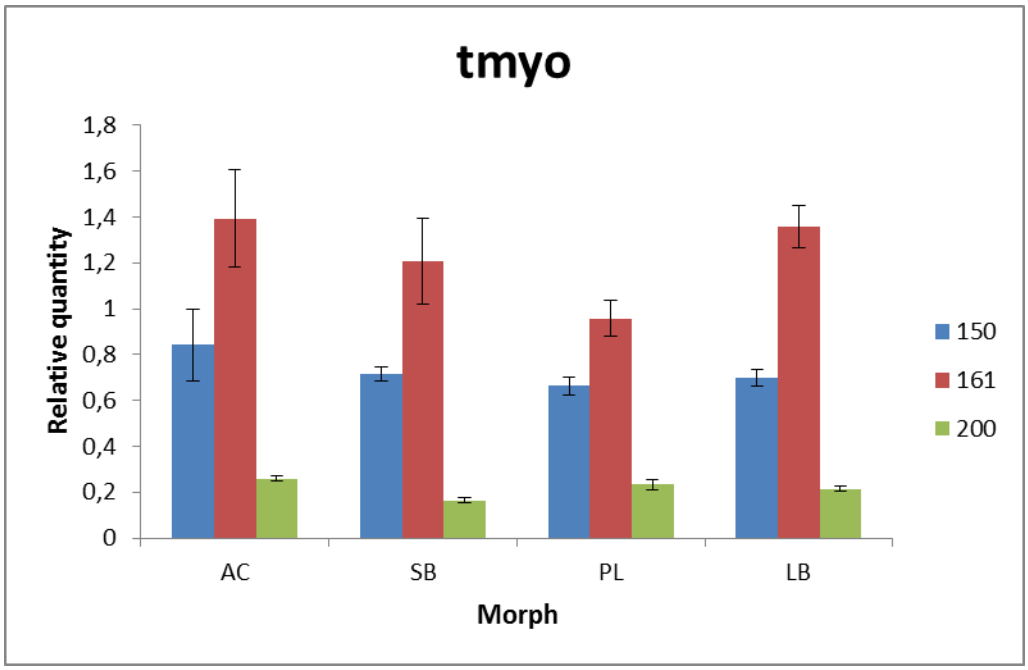

Figure 10 – The relative quantity of tmyo expression. *The relative quantity of tmyo expression for four morphs (AC, SB, PL and LB), at three developmental stages (150 DD, 161 DD and 200 DD). For all morphs expression rises between developmental time points 150 DD and 161 DD, and declines again between 161 DD and 200 DD.*

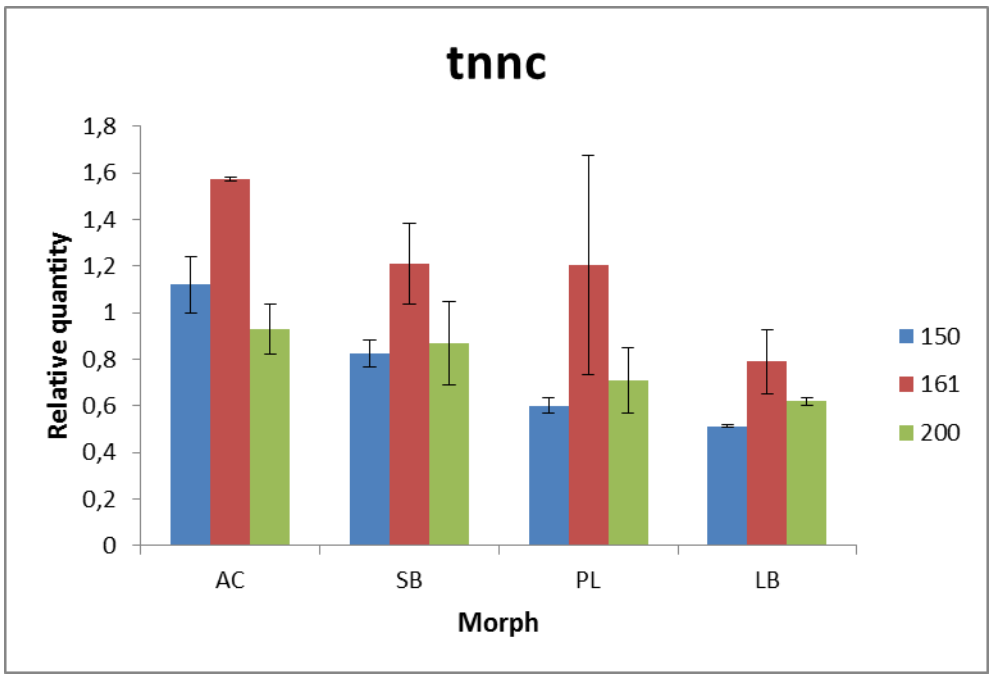

Figure 11 – The relative quantity of tnnc expression. *The relative quantity of tnnc expression for four morphs (AC, SB, PL and LB), at three developmental stages (150 DD, 161 DD and 200 DD). For all morphs expression rises between developmental time points 150 DD and 161 DD, and declines again between 161 DD and 200 DD.*

The difference in expression between morphs and stages for both *tmyo* and *tnnc* was analyzed using two-way ANOVAs, where the three following null hypothesizes were tested for each gene:

H_{0A}: "there is no difference in expression of the gene between morphs"

H_{0B}: "there is no difference in expression of the gene between stages"

H_{0C}: "there is no interaction between morphs and stages that effects expression of the genes"

Null hypothesis A showed significance for *tnnc* (table 3.1), indicating a difference of *tnnc* expression between the four morphs. This was analyzed further with Tukey's post hoc test. The results showed significant difference in expression between AC and LB (Table 3.2) but not between any other two morphs. Null hypothesis A showed no significance for *tmyo* (data not shown).

Table 4 – ANOVA tnnc~morph*stage. **significance, **strong significance*

	df	sum of sq.	mean sq.	F-value	P-value
Morph	3	1.0153	0.3384	5.724	0.01142*
Stage	2	0.9463	0.4732	8.004	0.00619**
Morph:Stage	6	0.1613	0.0269	0.455	0.82851
Residuals	12	0.7094	0.0591		

Table 5 – Tukey's test tnnc~morph*stage. ***strong significance*

Morphs	diff	lwr	upr	p-value (adj)
LB-AC	-0,56641	-0.98319	-0.14964	0.00778**
PL-AC	-0.36906	-0.78583	0.04772	0.08918
SB-AC	-0.23982	-0.65660	0.17695	0.36130
PL-LB	0.19736	-0.21942	0.61413	0.51931
SB-LB	0.32659	-0.09018	0.74337	0.14626
SB-PL	0.12923	-0.28754	0.54601	0.79456

Table 6 – Tukey's test tnnc~morph*stage. **significance*

Stages	diff	lwr	upr	p-value (adj)
161-150	0.42947	0.10513	0.75381	0.01066*
200-150	0.01698	- 0.30736	0.34132	0.98931
200-161	-0.41249	-0.73683	-0.08814	0.01336*

Null hypothesis B showed significance for *tnnc* and *tmyo* (Table 3.1 and Table 3.4 respectively). Tukey's test showed significance between all the stages in both genes except between developmental stages 150 DD and 200 DD for *tnnc* (Tables 3.3 and 3.5).

No significance was detected for null hypothesis C, for either gene.

Table 7 – ANOVA tmyo~morph*stage. ****very strong significance*

	df	sum of sq.	mean sq.	F-value	P-value
Morph	3	0.148	0.0494	2.389	0.120
Stage	2	4.082	20.409	98.726	3.5*10-8***
Morph:Stage	6	0.133	0.0221	1.071	0.431
Residuals	12	0.248	0.0207		

Table 8 – Tukey's test tmyo~morph*stage. ***strong significance, ***very strong significance*

Stages	diff	lwr	upr	p-value (adj)
161-150	0.49813	0.30634	0.68993	4.37*10-5***
200-150	-0.51201	-0.70380	-0.32021	3.34*10-5***
200-161	-0.01014	-0.20193	-0.81835	0.00000**

4 Discussion

It has been suggested that the jaw muscles, play a role in determining the structure of the feeding apparatus by supporting the bones and cartilage of the lower jaw (Schilling, 1997). miR-206 has been found to be expressed in craniofacial muscles during development, indicating that this miRNA may be involved in muscle development. Furthermore miR-206 has been found to regulate mRNAs of genes that are essential in the developmental timing of myogenesis (Goljanek-Whysall, 2011). With that in mind the aims of this project were to examine the spatial expression of miR-206, find potential target genes for this miRNA and study their spatial and temporal expression in Arctic charr. This study yielded three interesting result points, which will be discussed in separate sub-chapters bellow.

4.1 miR-206 is expressed in the muscles of the feeding apparatus in Arctic charr development

In zebrafish miR-206 is expressed in the anterior mandebularis (AM), the interhyoideus (IH), the hyohyoideus (HH), the intermandebularis anterioris (IMA) and the intermandebularis posterioris (IMP). In this study we found that miR-206 is expressed in the same tissue specific manner in charr as in zebrafish. The extreme similarity of the expression pattern between the two species indicates that the function of miR-206 is also conserved.

miR-206 expression was present at all developmental stages examined (150 DD, 161 DD and 200 DD). As development of the jaw muscles progresses and they grow, so does the expression of miR-206 indicating that this miRNA may play a role in the development of the facial muscles. It is also likely that miR-206 plays a role in the maintenance of facial muscles. Functional analysis using knockouts or overexpressing miR-206 in model organisms such as zebrafish will help pinpoint its function.

4.2 *tnnc* and *tmyo* are a likely miR-206 targets in Arctic charr

Two putative miR-206 targets were found using computational approaches: *tnnc* and *tmyo*. miRanda showed a complete seed region complementation between miR-206 and bases in the 3'UTRs of the two genes and both are conserved between zebrafish and Arctic charr.

In the next step the spatial expression of the candidate mRNA targets *tnnc* and *tmyo* was studied using WISH. The probes were designed to include the sequence of the 3'UTR that is complementary to the seed region. This was done to insure that the probes were targeting the miR-206 targets and not possible splice variants of the gene that are not under miR-206 control. The WISH showed that both *tnnc* and *tmyo* are expressed in the same tissue specific manner as miR-206, and they are expressed at the same developmental time points (150 DD and 187 DD).

The *tnnc* and *tmyo* qPCR results showed that the expression of both genes fluctuates in a manner opposite to that of miR-206. Taken together the computational and expression results indicate an interaction between miR-206 and the two genes.

To conform that *tnnc* and *tmyo* are being targeted by miR-206, further studies need to be performed. One possibility is to do an *in situ* hybridization after sectioning of the embryos or a WISH followed by sectioning (Skemman, http://hdl.handle.net/1946/20254). This would allow for more precise localization of expression and would determine whether *tnnc* and *tmyo* are expressed in the same cell type as miR-206. Another possibility would be to verify whether miR-206 and the two genes interact. This is usually done with a luciferase reporter gene assay that uses engineered luciferase genes that contain the predicted miRNA targeting sequence (Jin, 2013).

4.3 Significant differences in miR-206 and *tnnc* expression between morphs

Statistical analysis of the qPCR data showed a significant difference in *tnnc* expression between LB and AC with lower expression in LB. These two morphs belong to two different morphotypes (LB of the benthic morphotype and AC of the limnetic morphotype). However the fact that expressional differences were only detected between LB and AC, indicates that the observed pattern may be morph specific rather than morphotype specific.

miR-206 has been found to be highly expressed in SB compared to AC (Kapralova, 2014). We have no data available for LB and PL and it will be interesting to study the expression of miR-206 in these morphs and test whether the observed patterns of expression patterns holds, i.e. higher expression of miR-206 expression is coupled with lower expression of *tnnc* and vice versa.

These finding are indeed very interesting and show the importance of miR-206 in fish development. However further analyses are needed to study the roles that this miRNA and its expression network play in the evolution of the extreme morphological differences between Arctic charr morphs.

References

Ahi, E. P. et al., 2014. Transcriptional dynamics of a conserved gene expression network associated with craniofacial divergence in Arctic charr. *EvoDevo,* 5(40), pp. 1-19.

Almeida, M. I., 2011. MicroRNA history: Discovery, recent applications, and next frontiers. *Mutation resaerch,* 717(1-2), pp. 1-8.

Bartel, D. P., 2004. MicroRNAs: Genomics, Biogenesis, Mechanism, and Function. *Cell,* 116(2), pp. 281-297.

Bohnsack, M. T., 2004. Exportin 5 is a RanGTP-dependent dsRNA-binding protein that mediates nuclear export of pre-miRNAs. *RNA society,* 10(2), pp. 185-191.

Braun, J. E., 2011. GW182 Proteins Directly Recruit Cytoplasmic Deadenylase Complexes to miRNA Targets. *Molecular Cell,* 44(1), pp. 120-133.

Farh, K. K.-H. et al., 2005. The Widespread Impact of Mammalian MicroRNAs on mRNA Repression and Evolution. *Science,* 310(5755), pp. 1817-1821.

Goljanek-Whysall, K. et al., 2011. MicroRNA regulation of the paired-box transcription factor Pax3 confers robustness to developmental timing of myogenesis. *PNAS,* 108(29), pp. 11936-11941.

Hussain, M. U., 2012. Micro-RNAs (miRNAs): genomic organisation, biogenesis and mode of function. *Cell Tissue,* 349(2), pp. 405-413.

Jin, Y., 2013. Evaluating the MicroRNA Targeting Sites by Luciferase Reporter Gene Assay. *Methods Mol Biol,* Bindi 936, pp. 117-127.

Jin, Y., 2013. Evaluating the MicroRNA Targetingsites by Luciferase Reporter Gene Assay. *Methods Mol Biol,* Bindi 936, pp. 117-127.

Kapralova, K. H. Et al., 2014. Patterns of MiRNA Expression in Arctic Charr Development. *Plos One,* 9(8), pp. 1-13.

Kirby, T. J., 2013. MicroRNAs in skeletal muscle biology and exercise adaptation. *Free Radical Biology and Medicine,* Bindi 64, pp. 95-105.

Lee, R. C., 1993. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell,* 75(5), pp. 843-854.

McCarthy, J. J., 2008. MicroRNA-206: The skeletal muscle-specific myomiR. *Biochimica et Biophysica Acta,* 1779(11), pp. 682-691.

Reinhart, B. J. et al., 2000. The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. *Nature,* 403(6772), pp. 901-906.

Ruby, J. G., 2007. Intronic microRNA precursors that bypass Drosha processing. *Nature,* 448(7149), pp. 83-86.

Schilling, T. F., 1997. Musculoskeletal patterning in the pharyngeal segments of the zebrafish embryo. *Development.*

Schilling, T. F., 1997. Musculoskeletal patterning in the pharyngeal segments of the zebrafish embryo. *Development,* 124(15), pp. 2945-2960.

Snorrason, S. S. e. a., 1994. Trophic specialization in Arctic charr Salvelinus alpinus (Pisces; Salmonidae): morphological divergence and ontogenetic niche shifts. *Biological journal of the Linnean society,* 52(1), pp. 1-18.

Sweetman, D. Et al., 2008. Specific requirements of MRFs for the expression of muscle specific microRNAs, miR-1, miR-206 and miR-133. *Developmental Biology,* 321(2), pp. 491-499.

Westholm, J. O., 2011. Mirtrons: microRNA biogenesis via splicing. *Biochimie,* 93(11), pp. 1897-1904.

Wienholds, E. et al., 2005. MicroRNA expression in zebrafish embryonic development. *Science,* 309(5732), pp. 310-311.

Wightman, B., 1993. Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. *Cell,* 75(5), pp. 855-862.

Appendix A

Table A1 – miR-206 target candidates in Arctic charr. **RNF111/20/145/38/11/5/215 and rnf26/34a/213a/144aa/165b/146/24. **FAM217B/211B and fam21c*

Gene zfin	*Genes from other species*	*Charr homologues*	*miR-206 target in Charr*
Rnf2		*	No
Myf5			No
Ptp4a3		Ptp4A2	No
Six1a			No
Six1b		Six4.3	No
Popdc2			No
Fam20b		**	No
Vgll2a		Vgll4	No
Actn2			No
Actn3		ACTN4	No
Myhz1			No
Myhz2			No
Myog		**tmyogenin**	**Yes**
Tnni1b			No
Rbfox1l			No
Vmhc			No
Smyhc1			No
Smyhc2			No
Smyhc3			No
Stnnc			No

(tnnc)			
Unc45b		Unc45a	No
Tnnt3a			No
Tnna3b			No
ttna			No
myoD1			No
Pax7b			No
	Ccnd2[1]	Ccnd2	No
	Mmd[2]	paqrb	No
	Ptplad1[2]	Ptad1	No
Tnnc2		**tnnc**	**Yes**

[1] Homo sapiens
[2] Mus musculus