Regulatory factors controlling muscle mass: competition between innate immune function and anabolic signals in regulation of atrogin-1 in Atlantic salmon.

Zeynab Heidari[1], Ralph Bickerdike[2], John Tinsley[2], Jun Zou[1], Ting-Yu Wang[3], Tzong-Yueh Chen[3] and Samuel A. M. Martin[1]§

[1]Institute of Biological and Environmental Sciences, University of Aberdeen, Tillydrone Avenue, Aberdeen, AB24 2TZ, UK.
[2]BioMar Ltd, Grangemouth Docks, Grangemouth FK3 8UL, UK.
[3]Institute of Biotechnology, National Cheng Kung University, Tainan 70101, Taiwan, Republic of China

§Corresponding author
Professor Samuel A.M. Martin
Institute of Biological and Environmental Sciences,
University of Aberdeen,
Tillydrone Avenue,
Aberdeen,
AB24 2TZ,
UK.

Sam.martin@abdn.ac.uk

Abstract

Atrogin-1 is a conserved ubiquitin E3 ligase that is central to the early stages of skeletal and cardiac muscle wasting and degradation following starvation and inflammatory diseases. The control of protein turnover is different between endothermic and ectothermic animals reflecting the body energy requirements. Here we have characterised the promoter of the atrogin-1 gene in a phylogenetically diverse group of vertebrates and show conserved FOXO elements are present in all species examined. We have examined the gene expression responses in primary muscle cell culture to key immune modulators (IL-1β, interferon type 1 and interferon γ) and to the anabolic hormone insulin like growth factor (IGF-1). We show that the IL-1β and interferon type 1 increased atrogin-1 mRNA expression whereas IGF-1 suppressed atrogin-1 expression. The proximal promoter of salmon atrogin-1 was used to transfect primary muscle cell cultures and we found all three cytokines increased promoter activity whereas there was a decrease caused by IGF-1 exposure. We hypothesise that the main drivers for atrogin-1 expression are via the conserved FOXO site, but other transcription binding sites such as NFκB, STAT and IRFs may also be involved in a synergistic manner following immune stimulation when free amino acids need to be released for muscle protein reserves.

Introduction

Skeletal muscle is the largest body protein reserve and is under dynamic regulation to control the rate protein of deposition and degradation. Muscle atrophy occurs during fasting and in a variety of diseases such as sepsis, cancer and chronic viral infection (Hasselgren et al., 2005; Lecker et al., 2006; Gonnella et al., 2011). Loss of muscle mass occurs by both increased protein breakdown and decrease in protein synthesis. There are three key pathways of protein degradation, these include lysosomal proteases, calpains and the ubiquitin proteasome route of protein degradation (Ubp). In muscle tissue the Ubp is responsible for the vast majority of protein turnover (Mitch & Goldberg, 1996; Gomes et al., 2001). The Ubp has also been shown to be involved in increased protein degradation during muscle atrophy in fish as in mammals (Seiliez et al., 2008; Tacchi et al., 2011; Fuentes et al., 2012). During the Ubp pathway proteins are targeted for destruction by the proteasome, three enzymatic components are required to link chains of ubiquitin monomers onto proteins which targets them for degradation in the proteasome (Glickman & Ciechanover, 2002) and subsequent release of peptides and free amino acids that can either be used for recycling or further oxidation and gluconeogenesis (Fuentes et al., 2012). E1 (Ub-activating enzyme) and E2s (Ub-carrier proteins) prepare ubiquitin for conjugation but the key enzyme in the process is the E3 (Ub–protein ligase) (Bonaldo and Sandri, 2013; Lecker et al., 2006) which confers specificity to the system. E3 ubiquitin ligases are now recognised as an extended family of proteins that regulate many different cellular processes (Berndsen and Wolberger 2014).

Atrogin-1 has been identified as a key ubiquitin E3 ligase, a protein central in regulation of skeletal muscle mass in both mammals (Gomes et al., 2001) and fish (Tacchi et al., 2010, Bower et al., 2010, Cleveland et al 2010). In mammals the expression of atrogin-1 is suppressed by growth factors such as IGF-1 and insulin via a mechanism involving the AKT/FOXO transcription factor and AKT/mTOR pathways (Tesseraud et al., 2007). When transcriptional suppression is released atrogin-1 expression is increased and results in atrophy in the muscle tissue. During viral and bacterial infection the control of protein deposition is altered, most likely to release amino acids for synthesis of immune related genes or for gluconeogenesis. Viral infection triggers interferon (IFN) production, which activates targeted genes enabling the host to prevent further viral replication and induce an antiviral state (Robertsen, 2006; Berg et al., 2009; Zou & Secombes, 2011). IFN type-1 is one of the main innate antiviral cytokines and is essential for eliciting an effective immune response to viral infection whereas IFN-γ is involved with the development of the acquired immune response. During both bacterial and viral infections IL-1β is released as a major proinflammatory cytokine and induces a large number of responsive genes via conserved signalling pathways often via adaptor molecules such as MyD88 and the transcription factor NFκB. In fish atrogin-1 responds to both starvation (Bower et al., 2010; Cleveland and Evenhuis 2010) and the immune response (Tacchi et al., 2010) by increasing in expression. However, the regulation of this response has not been examined in lower vertebrates, as these animals will have different energy requirements than endothermic mammals.

In this paper we have characterised the proximal promoter of the Atlantic salmon (*Salmo salar*) atrogin-1 gene and identified evolutionary conserved transcription factor binding sites, we have also examined the promoter activity in primary muscle cell culture. Transfected primary myocytes with an atrogin-1 luciferase reporter construct were stimulated with salmonid recombinant cytokines IFN type 1, IFN-γ and IL-1β, in parallel we also used recombinant salmon IGF-1 as an anabolic hormone. We show that the immune regulating cytokines increased atrogin-1 activity whereas IGF-1 has a suppressive effect on the atrogin-1 gene.

Materials and methods

Sequence identification, analysis and generation of reporter plasmid

The proximal promoter of the Atlantic salmon atrogin-1 gene was identified following a BlastN search of the Atlantic salmon genome using the salmon atrogin-1 cDNA sequence (accession number, NM_001185027.1) as query. A contig sequence (accession number, AGKD00000000.3, contig AGKD03111157.1) was identified that had 100% identity of the query sequence. This sequence was analysed for the putative promoter and the transcription start sites. DNA repeats present in the sequence were analysed by DNA Repeats Finder (Benson, 1999) (http://tandem.bu.edu/trf/trf.html). The DNA regulatory sequence motifs were predicted by the Genomatix Software tools "common TFs" and "Matbase TFs" (Cartharius et al., 2005 PMID: 15860560). For comparison to other species the flanking regions were obtained from species with published genomes from Ensemble.

Salmon genomic DNA was extracted from muscle tissue using a genomic extraction kit (Promega). To clone the proximal promoter, primers were designed that included restriction enzyme sites to allow for directional cloning (Table 1). PCR was performed using salmon genomic DNA (20 ng) as template with 2500 U/µl of Taq DNA polymerase (BioTaq, Bioline), 50 µM of each dNTP and 200 nM of each primer in a final volume of 50 µl. The cycling protocol was: initial denaturation of 94 ºC for 5 min; 35 cycles of 94 ºC for 30 s, 55 ºC for 30 s and 72 ºC for 30 s; with a final extension of 72 ºC for 5 min. Ten µl of the PCR product was separated on a 2% agarose gel, stained with web green (Genetics) and analysed in a UV image analysis system. PCR products were digested with the appropriate restriction enzymes, *Kpn* I and *Xho* I (Promega), for 1 h at 37 ºC, following the manufacturer's instructions, and purified using a PCR Purification Kit (Invitrogen). The purified PCR products were ligated into pre-digested pGL4.10 luciferase reporter vector (Promega), using T4 DNA ligase (Promega) at 4 ºC overnight, the ligation reaction was used to transformation of *E. coli* TAM1 cells (Active Motif, Belgium) following the manufacturer's instructions. The plasmid insert sequence was confirmed by PCR using pGL4 RV primer 3 (Promega). Plasmids were purified from overnight bacterial cultures using an Endotoxin Free Plasmid Maxi Kit (Qiagen). Plasmid concentrations were determined by spectrophotometry using a nanodrop machine (Labtech International) and stored at -20 ºC.

Expression and purification of recombinant salmon IGF-1 (saIGF1)

The putative mature peptide of the Atlantic salmon (saIGF-1) (GenBank accession number: NP_001117095.1) was predicted by the SignalP:4.0 (Petersen et al., 2011). The cDNA fragment encoding the mature peptide saIGF-1 was amplified with primers F1 and R1 (Table 1) by PCR as described above and subcloned into the *Bam* HI and *Hind* III sites of pQE30 expression vector (Qiagen). The resultant plasmid was sequenced to confirm the open reading frame and transformed into the *E. coli.* JM109 cells (Promega). Induction and purification of the recombinant proteins under native conditions were performed as described previously (Hong et al., 2001). To eliminate the potential contamination of bacterial endotoxins such as LPS, the purified recombinant protein was loaded onto a polymyxin B column (Sigma-Aldrich) and the collected samples were stored at -80°C before use. The purified recombinant saIGF-1 was analysed by a 4 –12% precast SDS-PAGE gel (Invitrogen Life Technologies) stained with Brilliant Blue G (Sigma-Aldrich) (Fig. S1), and the concentration measured by comparison of the protein band density with a standard protein (trypsin inhibitor; Sigma-Aldrich) in the same SDS-PAGE gel using an Ultra Violet Products gel imaging system and Image Quant TL ver. 3.0 software. To confirm the activity of the saIGF-1 a dose response of the recombinant protein was used to examine the expression of atrogin-1 mRNA which is known to be negatively regulated by IGF-1. A dose response is shown in Fig. S2 which shows a significant response at IGF-1 concentrations >40 ng/ml.

RNA extraction and real time PCR

Total RNA was extracted from primary muscle cell culture by lysis in Tri Reagent (1 ml) (Invitrogen), followed by addition of 200 µl of chloroform and vortexing. The aqueous phase (RNA) separated by centrifugation (15 min, 13,000 g at 4 ^{o}C) was precipitated with equal volume of isopropanol. The RNA pellet was washed twice with 500 µl 80% ethanol, dried and resuspended in RNase / DNase free H_2O (Sigma). Total RNA concentration was determined by spectrophotometry (Nano drop) and RNA integrity determined by Agilent bioanalyser 2100. The RNA was kept frozen at -80 ^{o}C until use. For gene expression studies complementary DNA (cDNA) was synthesised from 1 µg total RNA. RNA was denatured at 70 ^{o}C for 5 min in the presence of 1 µl of Oligo dT_{17} (500 ng/µl) and RNase free water in a total volume of 11 µl, and cooled at room temperature for 5 min. The first strand cDNA was synthesized by adding 1 µl of Bioscript reverse transcriptase (200 U/µl, Bioline), 5 µl of 5x Bioscript reaction buffer, 1 µl of dNTP (10 mM each) and 7 µl RNase free H_2O and incubated at 42 ^{o}C for 1.5 h. The cDNA was diluted to a final volume of 50 µl of RNase/DNase free H_2O (Sigma) and stored at -20 ^{o}C. Real time PCR was performed using 3 µl of cDNA template, 2 µl of primers (each 10 µM) (Table1), 10 µl of 2x iQ SYBR Green supermix (Bio-Rad) and 5 μl RNase/DNase free water. Real time PCR amplifications were performed in white 96 well sealed plates with the following program: initial denaturation at 95 °C for 5 min, then 40 cycles of 95 °C for 5 sec, 55 °C for 30 sec, 72 °C for 30 sec, 78 °C for 5 sec. The melting curve was checked by fluorescence reading from 79 °C to 94 °C, to confirm that a single product was amplified. Three housekeeping genes were used for normalization, including elongation factor 1-α (EF1-α), hypoxanthine phosphoribosyl transferase 1 (HPRT1) and β-actin. The real time PCR was carried out with 3 biological replicates. A dilution series of cDNA (1x, 10 x, 100 x and 1000 x) was made to determine the efficiency of primers. The expression of genes was normalized to the mean arbitrary units of the three housekeeping genes for statistical analysis.

Primary muscle cell culture and stimulation by cytokines and IGF-1

Atlantic salmon (approximately 25 g) were maintained in the aquarium facility at University of Aberdeen, UK, under the national ethical guidelines. Fish were kept in fresh water and fed a commercial diet at 1.5% body weight per day and fish were killed using the schedule one method. Muscle tissue (above the mid line of the fish) was removed sterilely with a scalpel and forceps. Primary muscle cell cultures were performed as previously described (Pooley et al., 2013). The isolated primary muscle cells were cultured in L15 medium supplemented with 10% (v/v) foetal calf serum (FCS, Labtech International), and P/S. Cells were maintained for 7 days in a 12 well plates (NuncTM) at 22 °C until 80% confluence. Cells were either used for direct stimulation or were transfected. Cells were stimulated with recombinant salmonid cytokines IFN-γ (Zou et al, 2005), IFN-1 (Zou et al, 2007), IL-1β (Hong et al, 2001), and recombinant Atlantic salmon IGF-1 and maintained for either 6 h (IGF-1) or 24 h (cytokines) before RNA extraction. The times for stimulations were based on previous findings for cytokines (Martin et al. 2007a, 2007b) and for IGF-1 (Cleveland and Weber 2010).

Transfection of primary muscle cell cultures

To confirm the transfection efficiency prior to performing promoter analysis, cells ($2x10^6$) were transfected with 3 μg pTurbo-GFP (Evrogen) using lipofectamine (Life technologies). FACS analysis was performed to determine the efficiency of transfection of the primary muscle cells. For this, following transfection, cells were detached with trypsin and counted with Trypan blue and 10^6 cells were pelleted by centrifugation for 5 min at 250 g. Cells were resuspended in 250 μl of immunofluorescence medium (0.05% sodium azide + 2% FCS) before incubation for 30 min at 4 ^{o}C. Finally cells were centrifuged for 5 min at 250 g and supernatant was removed. One hundred µl of immunofluorescence medium was added to the cell pellet before reading. A FACSCanto II flow cytometer (Becton Dickinson, San Jose, CA, USA) was used to measure the proportion of cells expressing GFP protein. The data were analysed using FACSDiva software (Becton Dickinson). For the atrogin-1 promoter assay, a similar transfection assay was performed with 2.5 μg of the reporter plasmid and 0.5 μg of the internal control reporter vector pRL-TK by using lipofectamine for each well. Cells were incubated overnight at 22 °C. At 24 h post-transfection, cells were stimulated with IL-1β, IFN-1, IFN-γ or IGF-1. . Following 6 or 24 h the cells were harvested for luciferase reporter activity (Castro et al., 2010). Three replicate wells were used for each experiment and each well measured three times. For transfection analysis both firefly and Renilla luciferase activities were measured using the Dual Luciferase Reporter assay system (Promega). Atrogin-1 firefly luciferase activity was normalized to Renilla luciferase activity and expressed as fold change (mean ± se) by comparison between the promoter plasmid transfected cells with control cells transfected with the Renilla plasmid. Each set of experiments was repeated three times using the same protocol.

Statistics

Data were analysed by t-test and all analysis was performed using IBM SPSS statistic 21software package. Differences between means were considered significant if the P value was <0.05.

Results

Atrogin-1 gene promoter analysis cloning and sequence analysis

The blastN search of the Atlantic salmon genome (NCBI version releaseAGKD00000000.3) identified a contig (accession number, AGKD03111157.1) 8185bp in length which contained the first 4 exons of the atrogin-1 gene and the putative promoter sequence. Sequence alignment of the contig sequence (reverse complement sequence) with the reported mRNA sequence (accession number, GU456729.1) revealed an upstream region of beyond the transcription start site of 3153 bp, this region was composed of 5' flanking DNA and also a number of repeat elements until the end of the contig. The promoter region contained a predicted TATA box located 57 bp upstream of the start of mRNA transcript and two DNA repeat sequences, one with 25 copies of CATAACACATCACATCATAACACAT followed by 103 copies of GA and then repeating GAAGTGTACATTTGACTGG to the end of the sequence. Therefore, in this study we used non repeating 590 bp region of the 5’ flanking DNA as proximal promoter region for further analysis. The atrogin-1 gene proximal promoter region was analysed for transcription factor binding sites using Genomatix Software. This revealed a number of highly conserved sites that may be involved in regulation of gene transcription (Fig.1). The promoter region contained several well conserved transcription factor binding sites, these included a highly conserved FOXO site that was located 51 bp upstream of the predicted transcription start site, other conserved sites include a TATA box at -23 bp and several forkhead binding sites as represented by FOXP1 (3 elements), FOXJ and FHXB. We also found several TF binding sites associated with inflammatory and interferon responses including several STAT binding sites which can be defined as gamma interferon activated sites (GAS) and interferon regulatory factor binding elements (IRF). Also present was a conserved NFκB site. When the promoter sequence and predicted transcription factor binding sites were compared across other vertebrate atrogin-1 proximal promoters we find a highly conserved location of the FOXO site in all species examined (Fig 2a.). In all the fish and the amphibian the conserved sequence is GATAACA, with the mammals having a single bases change at the 5’ base, which is recognised as being a FOXO element. In all the species there is also a CEBP site located at -122 bp from the transcription start site (TSS) in salmon, in all species these two sites are 10-20bp apart and in the same orientation. All the species examined have varying numbers of forkhead binding sites, STAT elements and most have at least one IRF site, the position of these is conserved between Atlantic salmon and rainbow trout, but there is no detectable conservation of location between the more distantly related species.

Atrogin-1 mRNA responses to cytokine and IGF-1 stimulation

Our next step was to examine the gene expression response of the salmon primary muscle cells to the recombinant proteins. The initial part of this was to validate the response of genes known to respond to the cytokines or IGF-1 in other cell types as confirmation of responsiveness in our primary muscle cell system. For saIGF-1 activity, expression of IGF-1 mRNA was examined as a negative feedback, we found that concentrations of 50 and 200 ng of the recombinant protein significantly reduced IGF-1 mRNA expression. For activity of IL-1β we chose to use IGF binding protein 6 (IGFBP6) previously shown to be highly responsive to IL-1β in salmon muscle cells (Pooley et al 2013), this gene was highly increased in expression in response to the IL-1β. For the interferons, the classically responsive genes Mx and chemokine CXCL11_L1 were used to assess response to IFN-1 and IFN-γ respectively (Fig. 3) showing large increases in expression 24 h following stimulation.

Following confirmation of the response in the muscle cells to our recombinant proteins we examined the expression of the atrogin-1 mRNA following the same stimulation. Atrogin-1 mRNA expression was significantly decreased following 6 h saIGF-1 exposure but not 24 h (data not shown), whereas both IL-1β and IFN-1 resulted in an increase in atrogin-1 gene expression following 24 h, however we did not detect a significant change in expression in response to IFN-γ (Fig. 4).

Analysis of atrogin-1 gene promoter in primary muscle cell culture

To initiate the transfection studies, preliminary experiments were performed with a control plasmid to determine the transfection efficiency as there are few protocols for transfection into primary myocytes. Our protocol showed we obtained approximately 20% transfection efficiency when the pturboGFP plasmid was used (Fig. S3).

To examine the luciferase activity of the atrogin-1 promoter construct transfected cells were cultured for under similar regime as for gene expression studies. All three cytokines induce a significant increase in luciferease activity driven by the atrogin-1 promoter, however the response is only significant at the higher recombinant protein concentrations (200 ng/ml for IL-1β and 80 ng/ml for both interferons). The saIGF-1 resulted in a small decrease in activity compared to the unstimulated control (Fig. 5).

Discussion

The Atlantic salmon atrogin-1 promoter was used to examine the regulation of this key gene that can be regarded as a marker for catabolic activity in muscle tissue. The proximal promoter was identified from the Atlantic salmon genome and used as a starting point to generate a luciferase reporter plasmid. The plasmid was used for transfections using primary muscle cell cultures, following this the cells were exposed to three key cytokines, IL-1β, IFN-1, and IFN-γ and, in addition the cells were stimulated with IGF-1 which is a well characterised anabolic hormone. We showed that the three cytokines increased luciferase activity following 24 h exposure whereas the IGF-1 caused a marginal decrease in reporter assay compared to control. Gene expression of atrogin-1 was increased by IL-1β and IFN-1, but was unaffected by IFN-γ, whilst IGF-1 resulted in a large decrease in atrogin–1 mRNA expression. Although the mRNA levels were not significantly increased by the IFN-γ stimulation we did find an increase in the reporter assay. This potentially could be a reflection of the dynamics of the IFN-γ response and could be with either mRNA / protein turnover or due to the presence of negative regulator elements in the upstream region of the atrogin-1 promoter analysed in the present study. The control of atrogin-1 expression will help explain the interactions between immune stimulation and anabolic signals. The cytokines used in the experiments are all salmonid recombinant proteins and have been previously characterised (Zou et al., 2005, 2007 and Hogen et al., 2001) whereas we produced the salmon IGF-1 as a recombinant protein during this work and confirm its activity by the effect on atrogin-1 gene expression as has previously been shown in mammals (Sandri et al., 2004).

Rapid muscle protein loss occurs in mammals under a variety of physiological circumstances including sepsis (Lecker et al., 1999), cancer cachexia (Evans et al., 2008; Tisdale, 2009), and starvation (Lecker et al., 1999; Jagoe and Goldberg, 2001), with the animal releasing amino acids from the muscle protein stores. The regulation of these biological processes is becoming more understood and a number of key factors regulate the reallocation of energy stores during transition from normal to immune response (Lochmiller, 2000). During infection especially an inflammatory response, an acute phase response occurs, during this time cytokines are responsible for reprogramming the transcriptional activity of the liver to produce high levels of acute phase reactants such as complement and serum amyloid proteins. In parallel, muscle tissue proteins are broken down by controlled proteolysis to provide amino acid substrates for protein synthesis in the liver. In mammals this occurs rapidly within 24 h (Bonaldo and Sandri, 2013), and believed to be driven by IL-

1β and TNFα, however the action within the muscle tissue is not fully elucidated as yet. It is hypothesised that signalling targeting genes such as atrogin-1 which identifies specific proteins to be ubiquitinated and destroyed by the proteasome. During cancer cachexia, where there is sustained muscle mass loss TNFα is believed to be a principal driver of proteolysis (Donohoe et al., 2011). On the converse, during periods of anabolic growth and rapid deposition of muscle mass atrogin -1 expression is very low, indicating little protein degradation / turnover (Nakashima et al., 2006 and 2013).

During anabolic growth there is a decrease in Ubp pathways and is regulated by the growth hormone/ IGF system. In fish the dynamics of the response is slower than in mammals probably as a result of the energy requirements of ectotherms compared to warm blooded mammals. A number of recent reports have described how atrogin-1 is altered during starvation (Tacchi et al., 2012), hormonal (Cleveland and Weber., 2010) and viral infection (Heidari et al 2015) in salmonids, however these papers have not examined the promoter region of the gene and speculated on the regulatory control.

The promoter used in this paper was identified from the recently released salmon genome, a single contig (8185bp) contained the 5' end of the gene including 590 bp upstream flanking DNA from the transcription start site. Upstream of this 590 bp was a series of repeat sequences (>1800bp) that continued to the very end of the assembled contig, we made several unsuccessful attempts to walk along the flanking DNA by cloning approaches (Castro et al., 2008), in case the repeat was a sequencing artefact. We also observed in the closely related rainbow trout genome that the atrogin-1 promoter was at the end of a contig, most potentially because it too had a repeating element. From this we concluded the proximal promoter may have most of the key transcription binding factors for gene regulation. One of the key transcription factors controlling protein synthesis and degradation are members of the forkhead transcription factors, especially the FOXO family and are regarded as central to the regulation of muscle atrophy. In mammals the FOXO transcription factors are increased in parallel to catabolic stimuli (Sandri et al., 2004) and transfection of FOXO transcription factors results in increased expression of atrogin-1 mRNA (Mcloughlin et al., 2009). Akt signals negatively regulate FOXO by phosphorylating them and promoting export to the cytoplasm and thus preventing binding to gene promoters. The IGF/Akt/mTOR pathway enhances anabolic muscle phenotype and decreases protein degradation, this pathway also phosphorylates the FOXO protein. Catabolic signals result in dephosphorylation of FOXO and translocation to the nucleus and activation of target genes. We carried out *in silico* analysis of common TF binding sites for several teleosts, amphibian and mammals and show that a conserved FOXO site is located within 400 bp of the translation start site. The consensus sequence for FOXO is $D_{(T/A/G)}R_{(G/A)}W_{(T/A)}M_{(A/C)}AACA$ we find this TF binding site in all the promoters we examined with the most common sequence of GATAAACA which is completely conserved for all teleosts and the amphibian Xenopus. In Atlantic salmon and rainbow trout there are three other closely conserved sites but do not fully adhere to the consensus sequence. It is of interest that this conserved FOXO site is consistently 3' (within 50 bp) to a conserved CEBP transcription factor binding site, hence it may be that there is selection to maintain these sites in close proximity. The conservation of the FOXO site strongly suggests evolutionary selection for its maintenance. This can be further seen as in mammals there is evidence that both FOXO1 and CEBP are required for expression of genes involved in gluconeogenesis such as G6Pase and PEPCK, with promoter elements for both FOXO and CEBP being required for promoter activity of these genes (reviewed by Gross et al., 2008), suggesting these promoter element positions may be conserved across other genes.

In addition to the forkhead binding sites we also found other TF binding sites that are often targeted following immune activation following viral and bacterial infection. Specifically we find NFκB binding sites in most of the species, these are the final effectors following cytokine and toll like receptor activation that occurs via intermediates such as MyD88 and TRAF 6 (Bonaldo and Sandri, 2013) which could be an additional mechanism by which the proinflammatory cytokine IL-1β regulates atrogin-1. Interferon regulatory factor binding sites are found in the teleost and Xenopus flanking regions and could contribute to regulation by the interferon molecules. Finally we found STAT binding sites, these often represent binding elements for IFN-γ and for interferon gamma activated sequences (GAS) that are highly conserved and key regulatory sites for responses in salmonid fish (Castro et al., 2008 and 2010). The positioning of these TFs is not as conserved as the FOXO and the closely linked CEPB site. So we would hypothesise that the key drivers for expression are via the FOXO site with additional levels of regulation by other factors directly downstream of the classic cytokine signalling.

The control of anabolic signalling occurs at different levels. Recent work shows that IGF binding protein 6 (IGFBP-6) is strongly up regulated in primary muscle cell cultures of salmon in response to IL-1β (Pooley et al., 2013), so an additional level of control could be suppression of IGF activity by up regulation of the IGFBP6. Here we show that IGFBP6 expression is also increased following IL-1β stimulation, but decreased following IGF-1 stimulation at 50 ng/ml^{-1}. With this in mind additional control could be that the IL-1β interferes with IGF signalling (i.e. prevents IGF-1 / receptor binding) and hence the PI3K, AKT/ FOXO pathway resulting in FOXO being de phosphorylated and binding to the atrogin-1 promoter. Further work using these models would be required to determine how the IL-1β affects the atrogin-1 gene.

In conclusion we show that in vertebrates from teleosts to mammals there are conserved FOXO transcription factor binding sites of the proximal atrogin-1 promoter, in all cases this site is closely linked to a CEBP binding site. We show that the cytokines IL-1β and interferons type I and γ all increase atrogin-1 promoter activity whereas IGF-1 stimulation decreases both gene expression of atrogin-1 and reduces the luciferase reporter activity. These results add to the growing evidence linking the immune response to the control of anabolic and catabolic signalling in muscle cells. Further experiments will target specific sites within the promoter to clarify the regulatory pathways.

Acknowledgements

The research was supported by an industrial PhD studentship between University of Aberdeen and by BioMar Ltd, for Z. Heidari.

References

Benson,G.,1999. Tandem repeats finder: a program to analyze DNA sequence. Nucl. Acids Res. 27 (2), 573-580.

Berg, K., Svingerud, T., Sun, B., Robertsen, B., 2009. An antiserum against Atlantic salmon IFNα1 detects IFN and neutralizes antiviral activity produced by poly I:C stimulated cells. Dev. Comp. Immunol., 33, 638-645.

Berndsen,C.E ., Wolberger, C., 2014. New insights into ubiquitin E3 ligase mechanism. Nat. Struct. .Mol. Biol., 21, 301–307.

Bonaldo, P., Sandri, M., 2013. Cellular and molecular mechanisms of muscle atrophy. Disease models and Mechanisms. 6, 25-39.

Bower, N.I., de la Serrana, D.G., Johnston, I.A., 2010. Characterisation and differential regulation of MAFbx/Atrogin-1 alpha and beta transcripts in skeletal muscle of Atlantic salmon (*Salmo salar*). Biochem. Biophys. Res. Comm. 396, 265–271.

Cartharius, K., Frech, K., Grote, K., Klocke, B., Haltmeier, M., Klingenhoff, M.,.Frisch, M., Bayerlein, M., Werner, T., 2005. MatInspector and beyond: promoter analysis based on transcription factor binding sites. Bioinformatics. 21, 2933–2942.

Castro, R., Martin, S.A.M., Zou, J., Secombes, C.J., 2010. Establishment of an IFN-γ specific reporter cell line in fish. Fish & Shellfish Immunol. 28, 312-319.

Castro, R., Martin, S.A.M., Bird, S., Lamas,N J.S., Secombes, C.J., 2008. Characterisation of interferon responsive promoters in fish. Mol. Immunol. 45, 3454–3462.

Cleveland, B.M., Evenhuis J.P., 2010. Molecular characterization of atrogin-1/Fbox protein-32 (FBXO32) and F-box protein-25 (FBXO25) in rainbow trout (*Oncorhynchus mykiss*): Expression across tissues in response to feed deprivation. Comp Biochem Physiol B Biochem Mol Biol. 157, 248–257.

Cleveland, B.M., Weber, G.M., 2010. Effects of insulin-like growth factor-I, insulin, and leucine on protein turnover and ubiquitin ligase expression in rainbow trout primary myocytes. Am. J. Physiol. Regul. Integr. Comp. Physiol. 298: R341-50.

Donohoe, C.L., Ryan, A.M., Reynolds, J.V., 2011. Cancer Cachexia: Mechanisms and Clinical Implications. Gastroenterology Research and Practice. 2011: 601434.

Evans, W.J., Morley, J.E., Argiles, J., Bales, C., Baracos, V., Guttridge, D., Jatoi, A., Kalantar-Zadeh, K., Lochs, H., Mantovani, G., Marks, D., Mitch, W.E., Muscaritoli, M., Najand, A., Ponikowski, P., Rossi, F.F., Schambelan, M., Schols, A., Schuster, M., Thomas, D., Wolfe, R., Anker, S.D., 2008. Cachexia: a new definition. Clin Nutr. 27,793-799.

Fuentes, F.N., Ruiz, P., Valdes, J.N., Molin, A., 2012. Catabolic Signaling Pathways, Atrogenes, and Ubiquitinated Proteins Are Regulated by the Nutritional Status in the Muscle of the Fine Flounder. PLoS ONE. 7, 44256.

Glickman, M.H., Ciechanover, A., 2002. The ubiquitin-proteasome proteolytic pathway: Destruction for the sake of construction. Physiol Rev. 82, 373-428.

Gomes, M.D., Lecker, S.H., Jagoe, R.T., Navon,A., Goldberg, A.L., 2001. Atrogin-1, a muscle-specific F-box protein highly expressed during muscle atrophy. Proc.Nat. Acad. Sci. U.S. A. 98, 14440-14445.

Gonnella, P., Alamdari, N., Tizio, S., Aversa, Z., Petkova, V., Hasselgren, P.O., 2011. C/EBPβ regulates dexamethasone –induced muscle cell atrophy and expression of atrogin-1 and MURF-1. J. Cell Biochem. 112, 1737-1748.

Gross, D.N., van den Heuvel, A.P. and Birnbaum, M.J., 2008. The role of FoxO in the regulation of metabolism. Oncogene. 27: 2320-2336

Hasselgren, P.O., Menconi, M.J., Fareed, M.U., Yang, H., Wei, W., Evenson, A., 2005. Novel aspects on the regulation of muscle wasting in sepsis. Int. J. Biochem. Cell Biol. 37, 2156–2168.

Heidari, Z., Tinsley, J., Bickerdike, R., McLoughlin, M.F., Zou, J., Martin, S.A.M., 2015. Antiviral and metabolic gene expression responses to viral infection in Atlantic salmon (*Salmo salar*). Fish and Shellfish Immunol. 42, 297-305.

Hong, S., Zou, J., Crampe, M., Peddie, S., Scapigliati, G., Bols, N., Cunningham, C. Secombes, C.J., 2001. The production and bioactivity of rainbow trout (*Oncorhynchus mykiss*) recombinant IL-1β. Vet. Immunol. Immunopathol. 81, 1–14.

Jagoe, R.T., Goldberg, A.L., 2001. What do we really know about the ubiquitin-proteasome pathway in muscle atrophy? Curr. Opin Clin. Nutr. Metab. Care. 4, 183–190.

Lecker, S. H., Goldberg, A. L., Mitch, W. E., 2006. Protein degradation by the ubiquitin-proteasome pathway in normal and disease states. J. Am. Soc. Nephrol. 17, 1807–1819

Lecker, S.H., Solomon, V., Mitch, W.E., Goldberg, A.L., 1999. Muscle protein breakdown and the critical role of the ubiquitin-proteasome pathway in normal and disease states. J. Nutr.129, 227S–237S.

Lochmiller, R.L., Deerenberg, C., 2000. Trade-offs in evolutionary immunology: just what is the cost of immunity? OIKOS 88, 87–98.

Martin, S.A.M., Taggart, J.B., Seear, P., Bron, J.E., Talbot, R., Teale, A.J., Sweeney, G., Hoyheim, B., Houlihan, D.F. and Secombes C.J., 2007. Interferon type 1 and type II responses in an Atlantic salmon (*Salmo salar*) cell line SHK-1 using the salmon TRAITS/SGP microarray. Physiological Genomics. 32, 33-44.

Martin, S.A.M., Zou, J., Houlihan, D.F., and Secombes, C.J., 2007. Directional responses following recombinant cytokine stimulation of rainbow trout (*Oncorhynchus mykiss*) RTS 11 macrophage cells as revealed by transcriptome profiling. BMC Genomics 8: 150.

McLoughlin, T.J., Smith, S.M., Delong, A.D., Wang, H., Unterman, T.G. and Esser, K.A., 2009. FoxO1 induces apoptosis in skeletal myotubes in a DNA-binding-dependent manner. Am. J. Physiol. Cell Physiol. 297, 548-555.

Mitch, W.E., Goldberg, A.L., 1996. Mechanisms of muscle wasting. The role of ubiquitin-proteasome pathway. N. Engl. J. Med. 335, 1897-1905.

Nakashima, K., Ishida, A., Katsumata, M., 2013. Atrogin-1/MAFbx, a muscle- specific ubiqiuitin ligase, is highly expressed in the smooth muscle of chicken gizzard. Biosci. Biotechnol. Biochem.77, 1092-1095.

Nakashima, K., Yarkabe, Y., Yamazaki, M., Abe, H., 2006. Effects of fasting and refeeding on expression of atrogin-1 and AKT/FOXO signalling pathway in skeletal muscle of chicks. biosci. Biotechnol.Biochem. 70, 2775-2778.

Pooley, N.J., Tacchi, L., Secombes, C.J., Martin, S.A.M., 2013. Inflammatory responses in primary muscle cell cultures in Atlantic salmon (*Salmo salar*). BMC Genomics.14, 747.

Robertsen, B., 2006. The interferon system of teleost fish. Fish & Shellfish Immunol., 20, 172-191.

Sandri, M., Sandri, C., Gilbert, A., Skurk, C., Calabria, E., Picard, A., Walsh, k., Schiaffino, S., Lecker, S.H., Goldberg, A.L., 2004. Foxo Transcription Factors Induce the Atrophy-Related Ubiquitin Ligase Atrogin-1 and Cause Skeletal Muscle Atrophy. Cell. 117, 399–412.

Seiliez, I., Panserat, S., Skiba-Cassy, S., Fricot, A., Vachot, C., Kaushik, S., Tesseraud, S., 2008. Feeding status regulates the polyubiquitination step of the ubiquitin-proteasome-dependent proteolysis in rainbow trout (*Oncorhynchus mykiss*) muscle. The Journal of Nutrition. 138, 487-491.

Tacchi, L., Bickerdike, R., Secombes, C.J., Martin, S.A.M., 2011. Muscle-specific RING finger (MuRF) cDNAs in Atlantic salmon (*Salmo salar*) and their role as regulators of muscle protein degradation. Mar. Biotechnol. 14, 35-45.

Tacchi, L., Bickerdike, R., Secombes, C.J., Pooley, N.J., Urquhart, K.L., Collet, B., Martin, S.A.M., 2010. Ubiquitin E3 ligase atrogin-1 (Fbox-32) in Atlantic salmon (*Salmo salar*): sequence analysis, genomic structure and modulation of expression. Comp. Biochem. Physiol. 157, 364-373.

Tesseraud, S., Metayer-Coustard, S., Boussaid, S., Crochet, S., Audouin, E., Derouet, M., Seiliez, I., 2007. Insulin and amino acid availability regulate atrogin-1 in avian QT6 cells. Biochemical and Biophysical Research Communications. 357,181–6.

Tisdale, M.J., 2009. Mechanisms of cancer cachexia. Physiol. Rev. 89, 381-410.

Zou, J., Secombes, C.J., 2011. Teleost fish interferons and their role in immunity. Dev. Comp. Immunol. 35, 1376-1387.

Zou, J., Carrington, A., Collet, B., Dijkstra, J.M., Yoshiura, Y., Bols, N., Secombes, C.J., 2005. Identification and bioactivities of IFN-gamma in rainbow trout *Oncorhynchus mykiss*: the first Th1-type cytokine characterized functionally in fish. J. Immunol.175, 2484-94.

Zou, J., Tafalla, C., Truckle, J., Secombes, C.J., 2007. Identification of a second group of type I IFNs in fish sheds light on IFN evolution in vertebrates. J. Immunol. 179, 3859-71.

Figure legends

Figure 1

Nucleotide sequence of Atlantic salmon atrogin-1 determined from genomic contig AGKD03111157.1. The key transcription factor core binding sites as determined by Genomatrix TF search (Cartharius et al., 2005) are in bold and underlined. The name of the TF binding site is above the sequence. The sequence in grey indicates the beginning of the repeat units that continue to the end of the sequence contig. The sequence highlighted in yellow represents the 5' untranslated region of the mRNA. TSS indicates the transcription start site and the ATG for translation are both shown by arrows.

Figure 2

Transcription factor binding sites in the 5' flanking proximal promoter of atrogin-1 genes from different vertebrate groups. All number are given from 1 which represent the ATG translation start site, this was done as the precise transcription start site is not known for all the genes. The sequences were obtained from ensemble for Stickleback (ENSGACT00000008171), Fugu (ENSTRUG00000005123), Xenpous (ENSXETG00000023228), Mouse (ENSMUSG00000022358) and Human (ENSG00000156804). Rainbow trout promoter was obtained from the rainbow trout genome and Atlantic salmon from NCBI as described above.

Figure 3

mRNA expression in primary muscle cell culture of IGFBP-6, MX and CXCL11 in response to 24h stimulation with (a) IL-1β, (b) IFN-1 (c), IFN-γ, and IGF-1 following 6 h stimulation with saIGF-1 (d). All genes were normalized to 3 housekeeping genes: ELF-1α, β actin and HPRT. The expression is presented as arbitrary units with unstimulated control given a value of 1.00. The bars represent mean ± se (n=3), asterisks indicate a significant response compared to non-stimulated cells ($^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$).

Figure 4:

Gene expression response of atrogin-1 to (a) IL-1β, (b) IFN-1, (c) IFN-γ after 24 h and to IGF-1 (d) following 6h stimulation in primary muscle cell culture. All genes were normalized to 3 housekeeping genes: ELF1, β actin and HPRT. The expression is presented as arbitrary units with unstimulated control given a value of 1.00. The bars represent mean ± se (n=3), asterisks indicate a significant response compared to non-stimulated cells ($^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$).

Figure 5:

Luciferase production of primary muscle cells transfected with the atrogin-1 reporter construct and vector containing the Renilla luciferase gene in response to (a) IL-1β, (b) IFN-1, (c) IFN-γ for 24h and (d) saIGF-1 for 6h. Data are representative of three independent experiments and show the mean RLU±se. (n= 3). Asterisks indicate a significant response compared to non-stimulated cells ($^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$).

Supplementary Figures

Figure S1:

Production and purification of recombinant saIGF-1 from the *E. coli*. JM109 cells. Purified SaIGF-1 protein (300 ng) was separated on a SDS-PAGE gel under denaturing conditions.

Figure S2:

Does response effect of recombinant saIGF-I on the expression of atrogin-1 mRNA in primary muscle cells. The cells were incubated with increasing doses of recombinant saIGF-I (0-200 ng/ml) for 6 h. The expression is presented as arbitrary units and the bars indicated mean ± se (n=3). Atrogin-1 expression was normalized to 3 housekeeping genes: ELF-1α, β actin and HPRT. Asterisks indicate a significant response compared to non-stimulated cells ($^*p<0.05$).

Figure S3:
A. Efficiency of primary muscle cells transfection as determined by FACS. The images are representative of two independent experiments. B. Detection of GFP expression was observed under fluorescence microscopy 24 h after transfected primary muscle cell culture.

Table 1: Primers used for Atlantic salmon atrogin-1 expression and promoter construction.

Primer name	**Sequence 5'-3'[a]**	**Accession No[b]**	**size (bp) [c]**
	Cloning into pGL4		
Atrogin-1_PF	*GGTACCA*TTATACCTGGGAAGAAAATACTT	AGKD03111157.1	590
Atrogin-1_PR	*CTCGAG*TGATGTGTTGTCTGGTATTGTGAC		
	IGF-1 recombinant protein		
sa IGF-1_RF	*CGCGGATCC*GGGCCCGAGACCCTGTGTGG	NM_001123623.1	213
sa IGF-1_RR	C*CCAA*GCTTTCAAGCTGCCTTGCCAGAC		
	Real time PCR		
EF1-α_QF	CAAGGATATCCGTCGTGGCA	AF321836	327
EF1-α_QR	ACAGCGAAACGACCAAGAGG		
β-actin_QF	TGACCCAGATCATGTTTGAGACC	AF012125	146
β-actin_QR	CTCGTAGATGGGTACTGTGTGGG		
HPRT1_QF	CCGCCTCAAGAGCTACTGTAAT	EG866745	255
HPRT1_QR	GTCTGGAACCTCAAACCCTATG		
CXCL11_QF	AAGGCCAAGTGGGGTCATTCTAA	DR696064	320
CXCL11_QR	AACGTATTCAGGCAGTCTTCAGG		
MX_QF	TGAGGACTCGGCAGAAAGGATGTA	U66475.1	415
MX_QR	CTTCGCGGATTTCAGGAGGAGGTTAGG		
IFN-γ_QF	AGGACACGTTTGAGGACAGTGA	AJ841811.1	198
IFN-γ_QR	CTCAGGTATCCTCTTCAGGA		
IL-1β_QF	GCTGGAGAGTGCTGTGGAAGAACATATAG	AJ223954.1	179
IL-1β_QR	CCTGGAGCATCATGGCGTG		
IGF-1_QF	CCTGTTCGCTAAATCTCACTT	EF432852.2	185
IL-1β_QR	TACAGCACATCGCACTCTTGA		
IGF-BP6_QF	GCTCAATAGTGTTCTGCGTGG	DQ190459.2	118
IGF-BP6_QR	CTTGGAGGAACGACACTGCTT		
Atrogin-1_QF	CGAGTGCTTCCAGGAGAATCTG	GU456729.1	384
Atrogin-1_QR	CCATCAAGGAGCTCCTTCAGAC		

The restriction sites added to sequence are in italics: [a]Atrogin-1-KpnI (GGTACC), atrogin-1-XhoI (CTCGAG), saIGF- BamHI (GGATCC) and saIGF1-HindIII (CCCAA). [b]Accession number are from NCBI. [c]Product size (bp).