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### **► To cite this version:**

Iban Seiliez, Jean-Charles Gabillard, Marine Riflade, Bastien Sadoul, Karine Dias, et al.. Amino acids downregulate the expression of several autophagy-related genes in rainbow trout myoblasts. *Autophagy*, 2012, 8 (3), pp.1-12. <10.4161/auto.18863>. <hal-01205048>

**HAL Id: hal-01205048**

**<https://hal.science/hal-01205048v1>**

Submitted on 29 May 2020

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1 **Amino acids down-regulate the expression of several autophagy related genes in**  
2 **rainbow trout myoblasts**

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18 **Running title: Amino acids control the expression of autophagy genes**

19

20 **Abbreviations used:** AA, Amino acids; AAR, Serum deprived medium containing amino  
21 acids plus Rapamycin; AMPK, AMP-activated protein kinase; Atg4b autophagy-related 4b;  
22 Atg12l, autophagy-related 12-like; Baf A<sub>1</sub>, bafilomycin A<sub>1</sub>; CM, Complete Medium; EF1α,  
23 Elongation Factor-1α; eIF2α, eukaryotic initiation factor-2α; 4E-BP1, eukaryotic  
24 initiation factor 4E binding protein; FoxO, forkhead box-O transcription factor; Gabarap11,  
25 gamma-aminobutyric acid type A (GABA(A)) receptor-associated protein-like 1; GCN2,

26 **general control nonderepressible 2**; IGF1, insulin-like growth factor-1; LC3B, microtubule-  
27 associated light chain 3B; MM, Minimal Medium; PI3K, phosphatidylinositol 3-kinase; **R**,  
28 **Serum and amino acids deprived medium containing Rapamycin**; S6, ribosomal protein S6;  
29 TOR, target of rapamycin; **TORC1, TOR complex 1**.  
30

## 31 ABSTRACT

32

33 Many fish species experience long periods of fasting often associated with seasonal  
34 reductions in water temperature and prey availability or spawning migrations. During periods  
35 of nutrient restriction, changes in metabolism occur to provide cellular energy via catabolic  
36 processes. Muscle is particularly affected by prolonged fasting as proteins of this tissue act as  
37 a major energy source. However, the molecular components involved in muscle protein  
38 degradation as well as the regulatory networks that control their function are still incompletely  
39 defined in fish. The present work aimed to characterize the response of the autophagy-  
40 lysosomal degradative pathway to nutrient and serum availability in primary culture of  
41 rainbow trout myoblasts. In this aim, 4-day old cells were incubated in a serum and amino  
42 acid-rich medium (complete medium), a serum and amino acid-deprived medium (minimal  
43 medium) or a minimal medium plus amino acids, and both the transcription-independent  
44 short-term response and the transcription-dependent long-term response of the autophagy-  
45 lysosomal degradative pathway were analyzed. We report that serum and amino acids  
46 withdrawal is accompanied by a rapid increase of autophagosome formation but also by a  
47 slower induction of the expression of several autophagy-related genes (*LC3B*, *gabarapl1*,  
48 *atg4b*). We also show that this later response is controlled by amino acid (AA) availability  
49 and that both TOR-dependent and TOR-independent pathways are involved in this effect.  
50 Together these results suggest an important role for AA released by muscle proteolysis during  
51 the fasting period in regulating the subtle balance between energetic furniture and muscle  
52 protein sparing.

53

54 **Key words:** Fish, myoblast, Amino acids, autophagosome, gene expression, signaling

55

## 56 INTRODUCTION

57

58 Many fish species migrate long distances to the spawning grounds. Extreme examples  
59 are the temperate eel species (2000 – 6000 km)<sup>1, 2</sup> and salmonid species (up to 3000 km)<sup>3, 4</sup> In  
60 the course of the migration, fish enter into a long term starving phase and degrade almost all  
61 their lipid and about half their white muscle mass, thereby reducing their ability to burst  
62 swim.<sup>5</sup> Not all degradation products are funneled into oxidative pathways, but substantial  
63 amounts provide the building blocks for the developing oocytes, some of them mediated  
64 through hepatic vitellogenin biosynthesis. Fuel usage changes drastically, with protein  
65 increasingly important as migration progresses, and likely the sole energy source for the last  
66 half of the migration and the energetically costly spawning process itself. Carbohydrates are  
67 used intermittently, and rebuilt from amino acids for spawning.<sup>5, 6</sup> Overall, these data  
68 highlight the importance of a tight control of muscle protein degradation in these species not  
69 only to fuel migration but also to fuel gonadal maturation and spawning. Individual variations  
70 in this control could therefore lead to differences in reproductive success or life span.  
71 However, the molecular components involved in muscle protein degradation as well as the  
72 regulatory networks that control their function are still incompletely defined.

73

74 Protein degradation in the skeletal muscle of vertebrates is thought to be essentially  
75 mediated by the activity of two highly conserved pathways, the ubiquitin-proteasomal  
76 pathway and the autophagic/lysosomal pathway. While ubiquitin-proteasome-dependent  
77 degradation has been investigated in depth and its contribution to muscle loss has been  
78 already well documented in mammals,<sup>7-11</sup> the role of autophagy has only recently begun to be  
79 investigated and new evidence has demonstrated that this pathway plays a critical role in  
80 controlling muscle mass.<sup>12, 13</sup> In fish, the ubiquitin-proteasome system has been intensively

investigated in recent years and significant progresses have been made in understanding the mechanisms that control this system.<sup>14-20</sup> In contrast, limited studies have focused on the autophagic/lysosomal pathway which is recognized to be up-regulated during muscle wasting,<sup>19, 21, 22</sup> and then the regulatory networks that control this pathway remains little understood in fish.

The autophagic/lysosomal pathway is an evolutionary conserved process that is responsible for the degradation of long-lived proteins and for the elimination of redundant or damaged cellular structures, e.g., mitochondria. During autophagy, portions of cytoplasm and cell organelles are sequestered into vacuoles, called autophagosomes, with subsequent fusion of autophagosomes with lysosomes and digestion of the content of the vacuoles by lysosomal hydrolases.<sup>23</sup> In mammalian models, two main mechanisms have been identified for the induction of the autophagosome formation in response to nutrient and growth factor starvation. The first is a rapid and transient transcription-independent induction mediated by mammalian target of rapamycin (mTOR) and AMP-activated protein kinase (AMPK) complexes, the major nutrient and energy sensors in eukaryotic cells, respectively.<sup>24</sup> The second regulatory pathway is a slower mechanism requiring gene expression and thought to allow the persistent generation of autophagosomes during prolonged fasting.<sup>13, 25</sup> Recent studies in mammals have shown that the transcription factors Forkhead box-O (FoxO3a and FoxO1) are necessary and sufficient to activate autophagy by controlling the expression of some critical autophagy-related genes (*LC3b* and *Gabarapl1* (the mammalian Atg8-related proteins), *Vps34*, *Ulk2*, *atg12*).<sup>26, 27</sup> FoxO proteins are also known as downstream targets of the insulin-like growth factor-1 (IGF1) / insulin – phosphatidylinositol 3-kinase (PI3K) – protein kinase Akt signalling and are thus considered to be major mediators of growth factor effect on autophagy-related gene expression.<sup>26, 27</sup>

106

107       Recently, we have shown that fasting rainbow trout (*Oncorhynchus mykiss*) for 14  
108 days strongly induces the expression of several major genes involved in autophagy  
109 (*gabara11*, *LC3b*, *atg4b*, *atg12l*) in white muscle.<sup>22</sup> These results are consistent with the  
110 strong activation of the lysosomal degradative system under catabolic situations, as observed  
111 by the induction of the expression and activity of cathepsins **L, B, D and S** during salmonid  
112 migration, maturation and starvation,<sup>19, 21</sup> and provide new evidence for the importance of the  
113 autophagic/lysosomal pathway for muscle atrophy in rainbow trout. However, **although recent**  
114 **studies have shown the well conservation of the above mentioned signalling proteins (Akt,**  
115 **TOR, AMPK, FoxO) in rainbow trout,**<sup>15, 22, 28-32</sup> little is known about the mechanisms  
116 involved in the induction of the autophagosome formation in response to nutrient and growth  
117 factor starvation in fish. To our knowledge, the only available data suggest that the regulatory  
118 networks that control the expression of autophagy-related genes have probably diverged  
119 throughout evolution.<sup>22, 33</sup> Indeed, in contrast to what is described in mammalian models, our  
120 recent results failed to demonstrate any insulin or IGF1 effect on the expression of autophagy-  
121 related genes.<sup>22, 27</sup> Therefore, the aim of the present study was to characterize both the  
122 transcription-independent short-term response and the transcription-dependent long-term  
123 response of the autophagy-lysosomal degradative pathway to nutrient and serum withdrawal  
124 in primary culture of rainbow trout muscle cells. A particular attention was paid to the nature  
125 of the stimuli that control the latter response, which would be of particular importance in  
126 salmonid species that, in the course of their spawning migration, enter into a long term  
127 starving phase.

128

## RESULTS

### **Serum and amino acid withdrawal induces autophagosome formation in trout myoblasts.**

Rapid changes in macroautophagy have long been known to occur in response to changes in nutrient and/or hormonal (Insulin/IGF1) supply.<sup>34</sup> To test whether serum and amino acid withdrawal was also accompanied by an increase of autophagosome formation in our cell culture model, we monitored by immunofluorescence the subcellular localisation of the LC3 protein in cells incubated in a serum and amino acid-rich (Complete Medium, CM) or -deprived medium (Minimal Medium, MM) for 4 h. As shown in Figure 1A, immunofluorescence of CM incubated cells showed LC3 staining as a weak diffuse cytoplasmic pool. In contrast, MM incubated cells revealed the appearance of punctate LC3 staining that represent autophagosomes,<sup>35</sup> indicating that serum and amino acid withdrawal induced autophagosome formation in our cell culture model. Furthermore, the addition of bafilomycin A<sub>1</sub>, a vacuolar ATPase inhibitor that inhibits autophagosome-lysosome fusion and prevents the degradation of LC3,<sup>36</sup> had a weak effect in CM incubated cells but strongly induced the number of punctate LC3 structures in MM incubated cells, further supporting the evidence of a short term induction of autophagosome formation by serum and amino acid withdrawal in our cell culture model.

Given that autophagy is a highly dynamic and complex process that is tightly regulated at multiple steps, and in order to strengthen our conclusion, we performed in lysates from CM and MM incubated cells western blots for LC3B, which is cleaved and conjugated to phosphatidylethanolamine during autophagosome formation to generate a faster-migrating form, LC3-II.<sup>37</sup> As shown in Figure 1B, the serum and AA withdrawal was associated with an increase of the ratio LC3-II/LC3-I (the non-cleaved form of LC3B) (compare lanes CM and



MM) which is a hallmark of autophagy induction. Furthermore, the LC3-II/LC3-I ratio in the presence of bafilomycin A<sub>1</sub> is higher in MM incubated cells (compare lanes CM+Baf A<sub>1</sub> and MM+Baf1A<sub>1</sub>), indicating that autophagic flux is increased during serum and AA withdrawal.

Finally, in order to better characterize the autophagy flux in our cell culture model, we monitored by western blotting the LC3 lipidation in 4-day old cells cultivated in CM and MM for 1, 2.5, 4, 6, 12 and 24 h. As shown in Figure 1C, the ratio of LC3-II to LC3-I was significantly increased after 2 h in MM ( $P<0.05$ ) and remained significantly elevated until 6 h before declining to the level of the CM incubated cells.

Overall, these results demonstrated that serum and amino acid withdrawal was accompanied by a rapid and transient increase of autophagy in our cell culture model, indicating that these serum and AA-deprived cells can serve as a relevant model to characterise the factors involved in the induction of this proteolytic system.

## **Serum and amino acid withdrawal induces the expression of several autophagy related genes in trout myoblasts.**

To test if a serum and AA withdrawal might also induce the expression of autophagy related genes in our cell culture model, we analysed by qRT-PCR the mRNA levels of several autophagy related genes in CM and MM incubated cells. As shown in Figure 2, among the four genes studied, *atg12l* was the only gene whose expression was not significantly affected by serum and amino acid withdrawal. The mRNA levels of *gabap1l* and *LC3B* significantly increased after 24 h of serum and AA removal. Those for *atg4b* reached significantly higher levels than those of CM incubated cells as early as 2.5 h after serum and AA removal and remained higher until 24 h. Altogether, these results demonstrate that in addition to the short-term (4 h) effect on the autophagosomal formation demonstrated above, the serum and AA

withdrawal allows also a long-term (24 h) induction of the expression of several autophagy related genes in our cell culture model.

## **AA availability controls the expression of several autophagy related genes and the activity of several AA and growth factors sensing signalling pathways in trout myoblasts.**

There is growing evidence that AA play an important role in controlling gene expression<sup>28, 29, 38-40</sup> In order to test if AA play a role in the above demonstrated induction of autophagy related gene expression in MM incubated cells, we analysed by qRT-PCR the mRNA levels of the autophagy related genes in CM, MM or MM plus AA (AA) incubated cells for 24h (Fig 3). As presented above, serum and AA removal induced the expression of *gabap11*, *LC3B* and *atg4b* and had no effect on *atg12l*. The addition of AA pool into the MM medium reduced the levels of transcripts of the first three genes to that observed in CM cells, but had no effect on that of *atg12l*. These results demonstrated for the first time that the expression of autophagy related genes are regulated by the availability of AA in the cells.

In order to clarify the mechanisms involved in the observed effect of AA on the autophagy-related gene expression we then monitored the activation of several AA and growth factors sensing signalling pathways in CM, MM and MM plus AA incubated cells for 30 min (Fig 4) and 4 h (data not shown). The phosphorylation of the two TOR effectors S6 and the Eukaryotic Initiation Factor 4E Binding Protein (4E-BP1) was strongly induced after AA addition to reach levels similar (S6) or higher (4E-BP1) that those observed in CM incubated cells. In contrast, the phosphorylation status of eIF2 $\alpha$ , known to play an essential role in the amino acid regulation of a large number of genes, was not affected by the treatments in the times monitored. Finally, we examined the phosphorylation status of FoxO1 and FoxO3a, which are considered to play a key role in the control of autophagy-related gene expression in

mammals. As shown in Figure 4, serum and AA removal decreased their phosphorylation (compare CM and MM) but the addition of AA pool into the MM medium had no effect (compare MM and AA). To further exclude FoxO action on the target genes, we then monitored by immunofluorescence the localization of FoxO1 in CM, MM and MM plus AA incubated cells for 4 h. As shown in Figure 4B, immunofluorescence of MM and MM plus AA incubated cells revealed that FoxO1 co-localizes with Hoescht staining, indicating that AA addition do not affect the nuclear localization of the studied protein. In contrast, CM incubated cells showed the loss of nuclear staining for FoxO1, indicating that this treatment prevents nuclear translocation of FoxO1.

Overall, these results demonstrated that AA availability controls the expression of several autophagy related genes as well as the activation of the protein TOR in our cell culture model. These results ask the question of the involvement of this protein in the above demonstrated effect of AA on the autophagy-related gene expression.

## **AA control the expression of several autophagy related genes via TOR-dependent and -independent mechanisms.**

Recent findings demonstrated the involvement of the complex TORC1 in the regulation of the expression of several metabolic- and growth-related genes.<sup>41</sup> In order to determine whether TORC1 mediates the above presented effect of AA addition on the expression of autophagy related genes, MM incubated cells were stimulated with AA in presence or absence of the TOR inhibitor rapamycin for 30 min, 4 h and 24 h. We first checked the specificity of rapamycin treatment in our cell culture model by monitoring by western blot the activation of several TOR-dependent and -independent signalling pathways in cells stimulated for 30 min (Fig 5) and 4 h (data not shown). Whatever the time monitored, the AA mediated induction of phosphorylation of the two TOR effectors S6 and 4EBP1 was strongly abolished in presence

228 of rapamycin. In contrast, the phosphorylation of eIF2 $\alpha$  and FoxO proteins was not affected  
229 by rapamycin treatment. Overall, these results demonstrated that rapamycin treatment  
230 specifically inhibits the activity of TOR in our cell culture model.

231 We then analysed by qRT-PCR the mRNA levels of autophagy related genes in CM and MM  
232 incubated cells stimulated with or without AA in presence or absence of rapamycin for 24 h  
233 (Fig 6). The expression of *atrogen1* has been previously shown to be downregulated by AA  
234 via a TOR-dependent mechanism<sup>39, 40</sup> and was therefore monitored as control. As expected,  
235 *atrogen1* was strongly down regulated (> 2-fold) after 24 hours of stimulation with AA but  
236 not with AA plus rapamycin. In contrast, the effect of AA on the expression of *gabapll* and  
237 *LC3B* genes was not abolished in presence of rapamycin, suggesting that AA act on these  
238 genes via a TOR-independent way. Finally, the AA mediated down-regulation of the  
239 expression of *atg4b* gene was significantly affected by rapamycin treatment supporting an  
240 involvement of the TORC1 complex in the observed effect of AA on the expression of this  
241 gene. Taken together, these results demonstrate for the first time that AA regulates the  
242 expression of several autophagy-related genes via mechanisms involving both TOR-  
243 dependent and TOR-independent pathways.

244

## DISCUSSION

In mammals, new evidence has demonstrated the important role of the autophagic/lysosomal pathway in regulating muscle mass and identified the transcription factors FoxO as a key factor of the control of this proteolytic system in response to nutrient and growth factor starvation by inducing several autophagy-related genes.<sup>26, 27</sup> In rainbow trout, our previous findings showed that fasting fish for 14 days or serum depletion of trout myoblasts strongly induces the expression of several autophagy-related genes.<sup>22</sup> However, we failed to link the phosphorylation status of FoxOs with the expression of autophagy-related genes, suggesting a moderate role for this transcription factor on the autophagic/lysosomal pathway in this species. The aim of the present study was therefore to clarify the signal transduction pathway involved in the regulation of the expression of autophagy-related genes in rainbow trout and monitor the potential role of amino acids in this control by exploring the regulation of the expression of *gabap11*, *LC3B*, *atg12l* and *atg4b* genes in culture of rainbow trout myoblasts.

***Short- and long-term induction of the autophagy-lysosomal degradative pathway by serum and AA withdrawal in trout myoblasts*** – In mammals, rapid changes in the autophagy-lysosomal degradative pathway have long been known to occur in response to changes in nutrient and/or hormonal (Insulin/IGF1) supply.<sup>34, 42</sup> In the present study we demonstrated that serum and amino acid withdrawal was also accompanied by a short term increase of autophagosome formation in our cell culture model, as evidenced by the appearance of punctate LC3 structures as well as the increase of LC3 processing (a reliable marker of autophagosome formation<sup>43</sup>) in MM incubated cells. This rapid induction of autophagosome formation in starved cells could be explained by its critical role in counteracting nutrient

deprivation by delivering damaged or long-lived proteins, macromolecules and organelles within the lysosomal degradative machinery to generate nutrients and energy levels compatible with cell survival.<sup>44, 45</sup>

In addition to this rapid transcription-independent induction of autophagosome formation in response to nutrient and growth factor starvation, a slower mechanism requiring gene expression has been described.<sup>26, 27</sup> Here, we demonstrated that several autophagy-related genes are induced in MM incubated cells well after (24h) the short term (4h) increase of autophagosome formation. These results are consistent with previous findings in mammals showing that during fasting, skeletal muscle shows a persistent generation of autophagosomes that continues for days.<sup>25</sup> Such prolonged autophagic induction requires transcriptional control in order to replenish LC3 and Gabarap, critical proteins that are destroyed during autophagosome fusion and lysosome. Altogether, these results demonstrated an evolutionary conservation of the mechanisms involved in the induction of the autophagy-lysosomal system between lower and higher vertebrates and indicated that these serum and AA-deprived trout myoblasts may serve as a relevant model to characterise the factors involved in the transcriptional regulation of autophagy genes.

***Role of AA in the control of the expression of autophagy related genes*** – Amino acids are not only substrates for various metabolic pathways, but can also serve as signaling molecules controlling signal transduction pathways.<sup>46, 47</sup> In addition, there is growing evidence indicating that amino acids play an important role in controlling gene expression through different pathways including at least GCN2 and TORC1.<sup>28, 29, 38-40, 48, 49</sup> In a previous study, we reported that a pool of AA was able to regulate the expression of several carbohydrate and lipid metabolism related genes in rainbow trout hepatocytes.<sup>29</sup> Here, we demonstrated that AA availability controls also the mRNA levels of several autophagy-related

genes in trout myocytes. These results indicate that in addition to the short-term inhibitory effect previously described<sup>44, 50</sup> AA exert also a long-term effect on the autophagy proteolytic system. Consequently, they suggest that the amount of factors controlling the formation of the autophagosome is tightly tuned to and by environmental conditions. Whether this long-term effect of AA on autophagy-related genes is specific to the studied species, which displays very unusual features of its protein metabolism compared to mammals (i.e. a high dietary amino acid requirement and a continuous growth of the skeletal muscle throughout their life<sup>51, 52</sup>) is worth investigating.

***Mechanisms underlying the action of amino acids on the expression of autophagy-related genes*** – To our knowledge, no findings have been published to date on the effects of AA on the regulation of the expression of autophagy-related genes and the mechanisms involved remained to be determined. In this aim, we monitored the effect of AA availability on several key proteins of the main AA and growth factors sensing signalling pathways in our cell culture model. Recent studies in mammals have shown that FoxO1 and FoxO3a are necessary and sufficient to activate autophagy by controlling the expression of some critical autophagy-related genes<sup>26, 27</sup> We therefore monitored their phosphorylation status in our cell cultures and did not observe any effect of AA supplementation, excluding a possible involvement of these proteins in the observed inhibition of expression of studied genes by AA. Similarly, our results show no effect of AA availability on the phosphorylation status of eIF2 $\alpha$  at the monitored time, also rejecting a possible contribution of the GCN2 pathway in the AA mediated down regulation of autophagy-related genes. In contrast, we demonstrate here that AA addition leads to induce the phosphorylation of the two TOR effectors S6 and 4E-BP1, suggesting that the TOR protein is activated in these conditions and making possible its involvement in the observed effect of AA on the expression of autophagy-related genes.



Indeed, numerous previous studies revealed the importance of the TOR protein in mediating the effect of AA on the expression of several metabolic and growth-related genes<sup>28, 29, 39, 40, 49</sup>. Here, we demonstrated that the treatment of trout myocytes with the TOR inhibitor rapamycin did not prevent the inhibition of the expression of *gabapll* and *LC3B* genes by AA but significantly affected that of *atg4b*. This indicates that AA act on the expression of autophagy-related genes via both TOR-dependent and –independent ways. In this regard, new findings have provided direct evidence on the role of several other factors in mediating the induction of the expression of autophagy related genes in muscle wasting<sup>53</sup>. Collectively, our results and those previously published highlight the complexity of mechanisms involved in the control of the expression of autophagy-related genes. Further studies are warranted to follow these different mechanisms as affected by AA availability in trout myocytes.

**Conclusions and Physiological significance** – In conclusion, in the present study we demonstrated the existence of both short- and long-term control of the autophagy-lysosomal degradative system in rainbow trout myoblasts. From an evolutionary adaptative point of view, this is of particular importance in salmonids that, in the course of their spawning migration, enter into a long starving phase and degrade about half of their white muscle mass. Such a prolonged degradation of muscle proteins requires transcriptional control in order to replenish critical proteins that are destroyed during autophagosome fusion with lysosome. We also demonstrated that AA availability controls the mRNA levels of several autophagy-related genes in this species. To our knowledge, such effects of AA on the regulation of the expression of autophagy-related genes have not been demonstrated so far and whether this effect is specific to the studied species is worth investigating. However, such a regulation system could originate from an evolutionary adaptation of migrating salmonids that have to deal between energy supply (mainly coming from muscle protein bound AA) and muscle



345 protein sparing to achieve their spawning migration. Amino acids released by muscle  
346 proteolysis would thus regulate themselves this subtle balance that allows salmonids to finally  
347 arrive to spawning grounds. Studies along these lines either with migrating salmonids or those  
348 under experimentally induced starvation are necessary to verify our hypotheses.  
349

## MATERIALS AND METHODS

### *Animals*

Juvenile immature rainbow trout (~ 5 g) were maintained in our own experimental facilities (INRA, Donzacq, France) at 18°C under natural photoperiods (12 h/12 h). All experiments were carried out in accordance with legislation governing the ethical treatment of animals (Decret N° 2001-464, May 29, 2001), and investigators were certified by the French Government to carry out animal experiments (N° agrément 35-47). All animal work was approved by the Ministère de l'Enseignement Supérieur et de la Recherche (Autorisation N°A352386).

### *Myosatellite cell isolation and culture*

Myoblasts were carried out as follows: for each culture, 30 to 60 animals, each weighing approximately 5 g, were killed by a blow to the head and then immersed for 30 s in 70% ethanol to sterilize external surfaces. Cells were isolated, pooled, and cultured following previously described protocols.<sup>22, 54</sup> Briefly, after removal of the skin, dorsal white muscle was isolated under sterile conditions and collected in Dulbecco's modified Eagle's medium (DMEM) containing 9 mM NaHCO<sub>3</sub>, 20 mM HEPES, 15% horse serum, and antibiotic-antimycotic cocktail (100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 g/ml fungizone) at pH 7.4. After mechanical dissociation of the muscle in small pieces, the tissue was enzymatically digested with a 0.2% collagenase solution in DMEM for 1 h at 18°C and gentle shaking. The suspension was centrifuged (300 g for 5 min at 15°C) and the resulting pellet was subjected to two rounds of enzymatic digestion with a 0.1% trypsin solution in DMEM for 20 min at 18°C with gentle agitation. After each round of trypsinization the suspension was centrifuged and the supernatant was diluted in 2 volumes of cold DMEM supplemented

with 15% horse serum and the same antibiotic-antimycotic cocktail mentioned above. After two washes with DMEM, the cellular suspension was filtered through 100- and 40- $\mu$ m nylon filters. All experiments were conducted three times with cells seeded at a density of 1.5 to 2 x 10<sup>6</sup> per well, in six-well plastic plates (Nunc, 140675). Plates and coverslips were previously treated with poly-L-lysine and laminin to facilitate satellite cell adhesion. Cells were incubated at 18°C, the optimal temperature for culture of trout origin, with a complete medium (CM) containing Earle's Balanced Salt (EBSS) culture medium (Sigma, E7510) supplemented with 10% fetal bovine serum (Sigma, F7524), MEM vitamins solution (Invitrogen, 11120-037), MEM essential amino acid mixture (Invitrogen, 11130-036) and MEM non-essential amino acid mixture (Invitrogen, 11140-035) and antibiotic-antimycotic cocktail under an air atmosphere. The medium was renewed every 2 days and observations of morphology were regularly made to control the state of the cells.

## *Treatment conditions*

In the first experiment, 4-day old cells (myoblasts, as verified by visual microscopy) were incubated in a serum and amino acid-rich (Complete Medium) or -deprived medium (Minimal Medium) with or without bafilomycin A<sub>1</sub> (a vacuolar ATPase inhibitor that inhibits autophagosome-lysosome fusion and prevents the degradation of LC3), **for different times specified in figure legends**. In the following experiments, after 4 days of culture, the cells were subjected or not to serum and amino acid deprivation in a minimal medium (MM) for 1h. Then, according to each experimental design, cells were incubated in either amino-acids free (MM) or amino acids (AA) containing medium (MEM essential and non-essential AA mixtures) for different times specified in figure legends. In experiments involving rapamycin (specific TOR-inhibitor), the inhibitor was added 30 min prior stimulation with AA. At the end of the stimulation periods, the mediums were removed, the wells were washed with ice-

cold PBS and the cells were used for immunofluorescence, gene expression or western blot analysis. Each experiment was performed three times.

## *Immunofluorescence analysis*

Cells on glass coverslips were briefly washed two times by PBS and fixed 10 min with methanol. For permeabilization cells were incubated 3 min in 0.1% TritonX100/PBS. After three washes, cells were saturated for 1 h with 3% BSA, 0,1% Tween20 in PBS (PBST). Cells were incubated 3 h with the **primary** antibody anti- LC3B (Cell Signaling Technologies, 2775) diluted in blocking buffer. The secondary antibody anti-Rabbit Alexa488 (Invitrogen, A-21441) was diluted in PBST and applied for 1 h. Cells were mounted with Mowiol 4-88 (Calbiochem, 475904) containing Hoescht (0.5 µg/ml). Cells were photographed using a Canon digital camera coupled to a Canon 90i microscope.

## *Gene expression analysis*

Total RNA samples were extracted from muscles using Trizol reagent (Invitrogen, 15596018) according to the manufacturer's recommendations. One microgram of the resulting total RNA was reverse transcribed into cDNA using the SuperScript III RNaseH- Reverse Transcriptase kit (Invitrogen, 18080085) and oligo dT (Promega, C1101) or Random primers (Promega, C1181) according to the manufacturer's instructions.

Target gene expression levels were determined by real-time quantitative RT-PCR (qRT-PCR) with 300 nM of each primer. Primers were designed to overlap an intron if possible (Primer3 software) using known sequences in trout nucleotide databases (<http://www.sigenae.org/>) as previously described.<sup>22</sup> Real-time RT-PCR was carried out on an iCycler iQ TM real time PCR detection system using iQ TM SYBR® Green Supermix (BIO-RAD, 172-5006). Relative quantification of the target gene transcripts with a chosen reference gene transcript

(EF1 $\alpha$  and Rps29) was made following the Pfaffl method with the Relative Expression Software tool (REST $\text{C}$ )<sup>55, 56</sup> In experiments involving rapamycin Rps29 was used as reference transcripts because EF1 $\alpha$  RNA was not stable during the experimental conditions. PCR was performed using 10  $\mu$ l of the diluted cDNA mixed with five pmoles of each primer in a final volume of 25  $\mu$ l. The PCR protocol was initiated at 95°C for 3 min for initial denaturation of the cDNA and hot-start iTaq<sup>TM</sup> DNA polymerase activation and continued with a two-step amplification program (20 s at 95°C followed by 30 s at specific primer hybridization temperature) repeated 40 times. Melting curves were systematically monitored (temperature gradient at 0.5°C/10 s from 55 to 94°C) at the end of the last amplification cycle to confirm the specificity of the amplification reaction. The different PCR products were initially checked by sequencing to confirm the nature of the amplicon. Each PCR run included replicate samples (duplicate of reverse transcription and duplicate of PCR amplification) and negative controls (reverse transcriptase-free samples, RNA-free samples).

## *Protein extraction and Western blotting*

Protein homogenates from cells were prepared as previously described<sup>31</sup> Protein concentrations were determined with Bradford reagent method<sup>57</sup> Cell lysates (10  $\mu$ g of protein) were subjected to SDS-PAGE and Western blotting using the appropriate antibody: Anti-phospho FoxO1 (Thr24)/FoxO3a (Thr32) (Cell Signaling Technologies, 9464), anti-FoxO1 (Epitomics, 1874-1), anti-anti-phospho S6 (Ser235/Ser236) (Cell Signaling Technologies, 4856), anti-carboxyl terminal S6 (Cell Signaling Technologies, 2217), anti-phospho 4E-BP1 (Thr37/Thr46) (Cell Signaling Technologies, 9459), anti-4E-BP1 (Cell Signaling Technologies, 9452), anti-phospho eiF2 $\alpha$  (Ser51) (Cell Signaling Technologies, 3398), anti-carboxyl terminal eiF2 $\alpha$  (Cell Signaling Technologies, 9722), anti-LC3B (Cell Signaling Technologies, 2775), anti- $\beta$ -actin (Santa-Cruz Biotechnology, sc-47778), anti- $\beta$ -

Tubulin (Cell Signaling Technologies, 2146). Anti-phospho FoxO1 (Thr24)/FoxO3a (Thr32), anti-FoxO1, anti-phospho S6 (Ser235/Ser236), anti-carboxyl terminal S6, anti-phospho 4E-BP1 (Thr37/Thr46), anti-4E-BP1, anti- $\beta$ -tubulin and anti- $\beta$ -actin have been previously validated in trout.<sup>15, 20, 22, 28, 29, 31-33</sup> For anti-phospho eIF2 $\alpha$ , anti-carboxyl terminal eIF2 $\alpha$  and anti-LC3B the amino acid sequences of the corresponding proteins were monitored in the SIGENAE database<sup>58</sup> to check for well-conservation of the antigen sequences with the corresponding sequences from mammals, ensuring a good specificity of the used mammalian antibodies in our samples. Preliminary experiments were also performed with murine C2C12 cell line as control. After washing, membranes were incubated with an IRDye infrared secondary antibody (LI-COR Inc, 956-32221). Bands were visualized by Infrared Fluorescence using the Odyssey® Imaging System and quantified by Odyssey infrared imaging system software (Application software, version 1.2).

## *Statistical analysis*

The results of gene expression and protein phosphorylation analyses were expressed as means  $\pm$  SEM ( $n = 3$  independent observations, each the mean of 6 and 3 replications for gene expression and protein phosphorylation, respectively) and analyzed by one-way ANOVA followed by Student-Newman-Keuls test. For all statistical analyses, the level of significance was set at  $P < 0.05$ .

471 **ACKNOWLEDGEMENTS**

472 We thank F. Terrier, Y. Hontang and F. Sandres for fish rearing in the INRA experimental  
473 farm (Donzacq, France).

474

475 **GRANTS**

476 This study was supported by the French National Research Agency (ANR-08-JCJC-0025,  
477 Low utilisation of dietary carbohydrates in carnivorous rainbow trout: role of amino acids,  
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## FIGURE LEGENDS

**Figure 1. Effect of serum and AA withdrawal on autophagosome formation in trout myoblasts.** 4-day old cells were incubated in a serum and amino acid-rich (Complete Medium, CM) or -deprived medium (Minimal Medium, MM) with or without bafilomycin A<sub>1</sub> (Baf A<sub>1</sub>). (A), Immunolocalization of LC3B (green) was performed after 4 h incubation as described in MATERIALS & METHODS. Nuclei were stained with Hoescht (blue). Graph represents the quantification of the number of LC3 puncta per cell. Results are expressed as means  $\pm$  SEM, n=4-5 (mean of 4-5 replications) and were analysed by one-way ANOVA followed by Student Newman Keuls test for multiple comparison ( $P<0.05$ ). Different letters represent significantly different values. (B), After 4 h incubation, cell lysates (10  $\mu$ g) were analysed by Western blot with an anti-LC3B antibody.  $\beta$ -actin was used as loading control. The ratio of LC3-II to LC3-I is shown quantitatively as a graph. (C), Cells were incubated in a serum and amino acid-rich (Complete Medium, CM) or -deprived medium (Minimal Medium, MM) for 0.5, 2, 4, 6, 12, 24 h. Cell lysates (10 $\mu$ g) were analyzed by Western blot with the indicated antibodies. A representative blot is shown. Graphs represent the ratio of LC3-II to LC3-I. Results are means  $\pm$  SEM, n=3 (mean of 3 replications) and were analyzed using one way ANOVA followed by the Student-Newman-Keuls test for multiple comparisons ( $p<0.05$ ). Different letters represent significantly different values.

**Figure 2. Effect of serum and AA withdrawal on autophagy-related gene expression in trout myoblasts.** 4-day old cells were incubated in a serum and amino acid-rich (Complete Medium, CM) or -deprived medium (Minimal Medium, MM) for 1, 2.5, 4, 8 or 24 h. *Gabarapl1*, *LC3B*, *atg4b* and *atg12l* mRNA levels were estimated using real-time RT-PCR. Expression values were normalised with elongation factor 1 $\alpha$  (EF1 $\alpha$ )-expressed transcripts.

Results are expressed as means  $\pm$  SEM, ( $n = 3$  independent experiments, each the mean of 6 replications) and were analysed by one-way ANOVA followed by Student Newman Keuls test for multiple comparison ( $P < 0.05$ ). Different letters represent significantly different values.

**Figure 3. Effect of AA on autophagy-related gene expression in trout myoblasts.** 4-day old cells were incubated in a serum and amino acid-rich (Complete Medium, CM) or -deprived medium (Minimal Medium, MM) for 1 h. The Minimal Medium was then replaced for 24 h with an amino acid-free (MM) or amino acids (AA) containing medium (MEM essential and non-essential AA mixtures). *LC3B*, *gabarapl1*, *atg12l* and *atg4b* mRNA levels were estimated using real-time RT-PCR. Expression values were normalised with elongation factor 1 $\alpha$  (EF1 $\alpha$ )-expressed transcripts. Results are expressed as means  $\pm$  SEM, ( $n = 3$  independent experiments, each the mean of 6 replications) and were analysed by one-way ANOVA followed by Student Newman Keuls test for multiple comparison ( $P < 0.05$ ). Different letters represent significantly different values.

**Figure 4. Effect of AA on the phosphorylation of S6, 4E-BP1, eIF2 $\alpha$ , FoxO1 and FoxO3a proteins in trout myoblasts.** 4 days cultivated cells were incubated in a serum and AA deprived medium for 1 h. Afterwards, the culture medium was replaced with a serum and AA rich medium (Complete Medium group CM), a serum and AA-free medium (Minimal Medium group MM) or a medium containing amino acids (Amino acids group AA) for 30 min before harvest. Cell lysates (10 $\mu$ g) were analyzed by Western blot with the indicated antibodies. A representative blot is shown. Graphs represent the ratio between the phosphorylated protein and the total amount of the targeted protein. For FoxO proteins, filled and dashed columns represent FoxO1 and FoxO3a, respectively. Results are means  $\pm$  SEM,



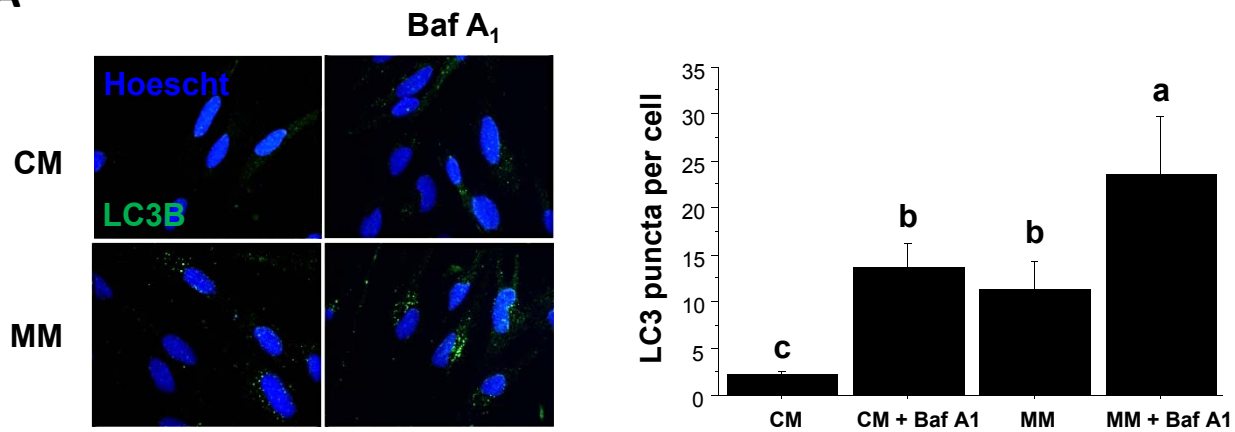
n=3 (mean of 3 replications) and were analyzed using one way ANOVA followed by the Student-Newman-Keuls test for multiple comparisons ( $p<0.05$ ). Different letters represent significantly different values. (B), Immunolocalization of FoxO1 (red) was performed as described in MATERIALS & METHODS. Nuclei were stained with Hoescht (blue).

**Figure 5. Effect of AA with or without rapamycin on the phosphorylation of S6, 4E-BP1, eIF2 $\alpha$ , FoxO1 and FoxO3a proteins in trout myoblasts.** 4 days cultivated cells were incubated in a serum and AA deprived medium for 30 min and then preincubated for 30 min with or without 100 nM rapamycin. Afterwards, the culture mediums were replaced for 30 min with a serum and AA rich medium (Complete Medium group CM), a serum and AA-free medium (Minimal Medium group MM and Rapamycin group R) or a medium containing amino acids (Amino acids group AA and Amino acids plus Rapamycin group AAR). Cell lysates (10 $\mu$ g) were analyzed by Western blot with the indicated antibodies. A representative blot is shown. Graphs represent the ratio between the phosphorylated protein and the total amount of the targeted protein. For FoxO proteins, filled and dashed columns represent FoxO1 and FoxO3a, respectively. Results are means  $\pm$  SEM, n=3 (mean of 3 replications) and were analyzed using one way ANOVA followed by the Student-Newman-Keuls test for multiple comparisons ( $p<0.05$ ). Different letters represent significantly different values.

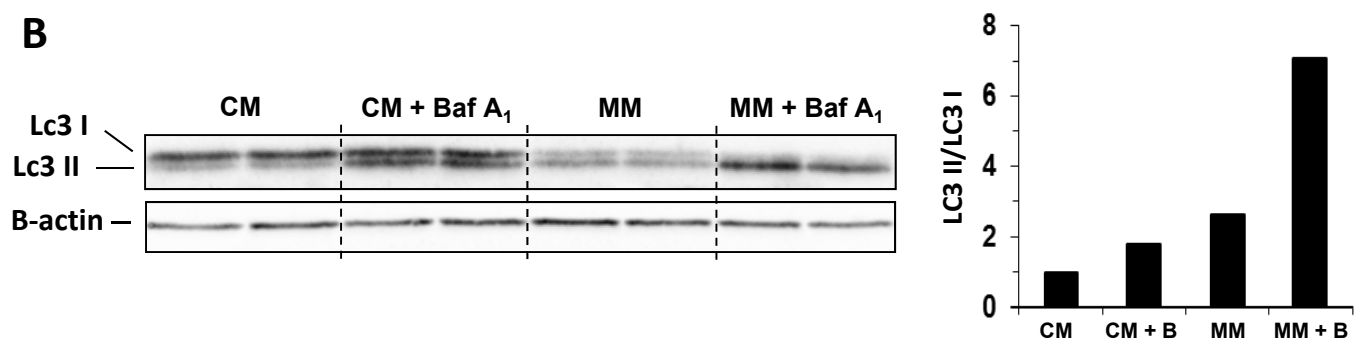
**Figure 6. Effect of AA with or without rapamycin on the expression of several autophagy-related genes in trout myoblasts.** 4 days cultivated cells were incubated in a serum and AA deprived medium for 30 min and then preincubated for 30 min with or without 100 nM rapamycin. Afterwards, the culture mediums were replaced for 24 h with a serum and AA rich medium (Complete Medium group CM), a serum and AA-free medium (Minimal Medium group MM and Rapamycin group R) or a medium containing amino acids (Amino

704 acids group AA and Amino acids plus Rapamycin group AAR). *Atrogin1*, *LC3B*, *gabarapl1*,  
705 *atg12l* and *atg4b* mRNA levels were estimated using real-time RT-PCR. For each treatment,  
706 six replicate were performed. Expression values were normalised with *Rps29* expressed  
707 transcripts. Results are expressed as fold of the CM group and presented as means  $\pm$  SEM,  
708 n=6 (mean of 6 replications). They were analysed by one-way ANOVA followed by Student  
709 Newman Keuls test for multiple comparison ( $P < 0.05$ ). Different letters represent significantly  
710 different values.

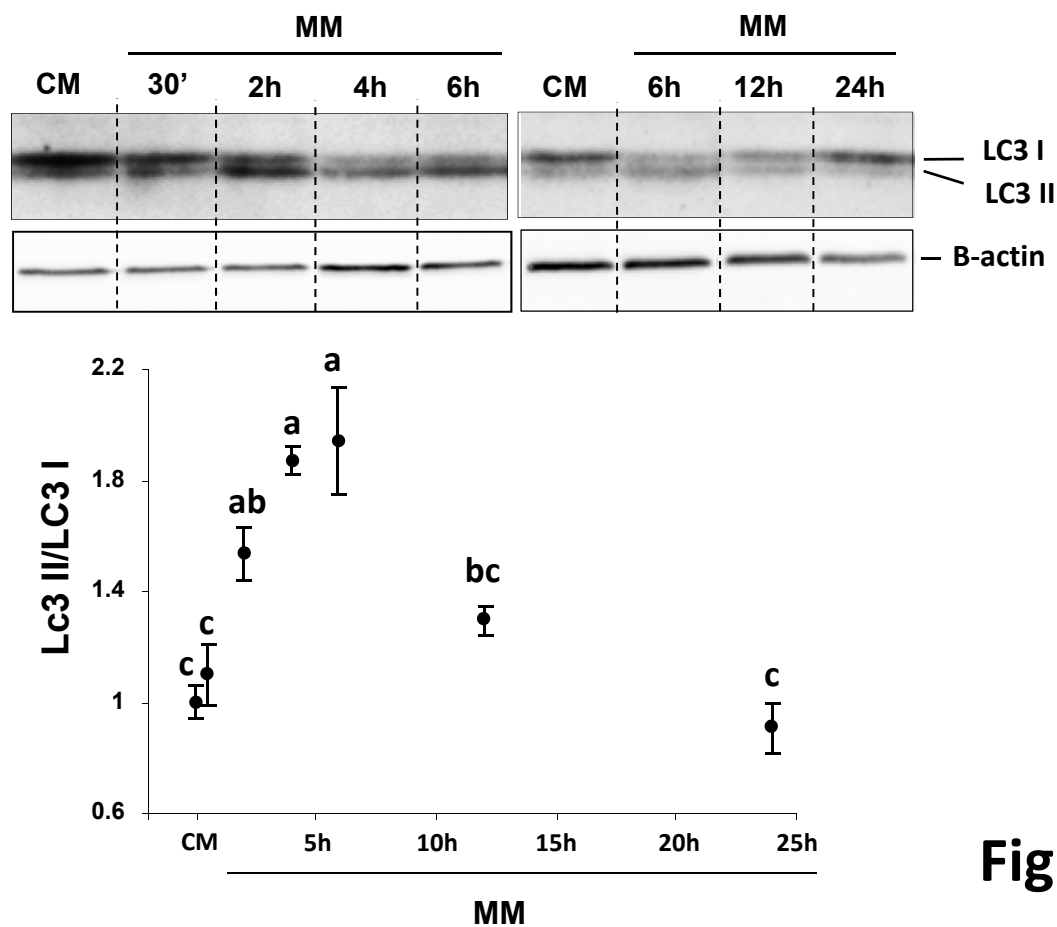
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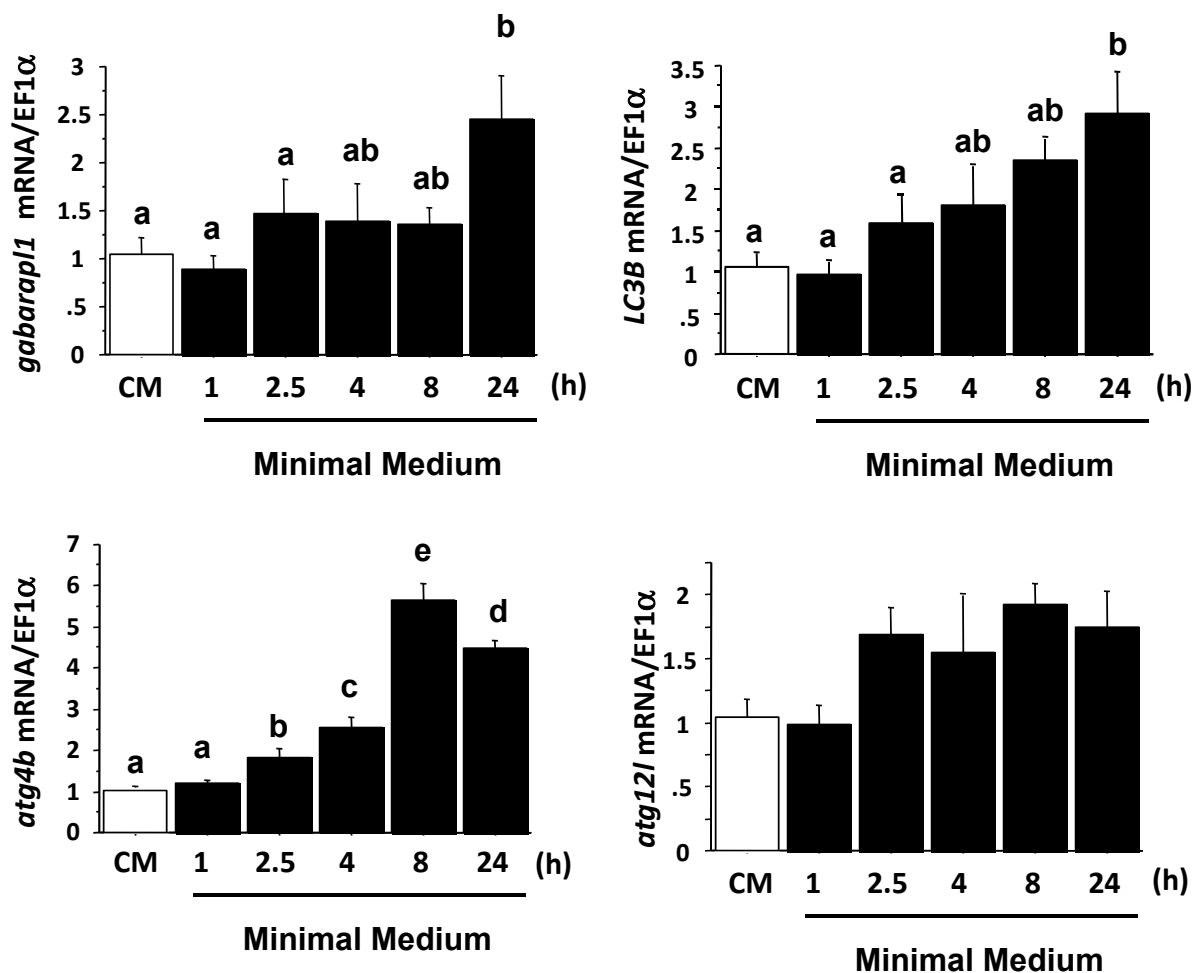
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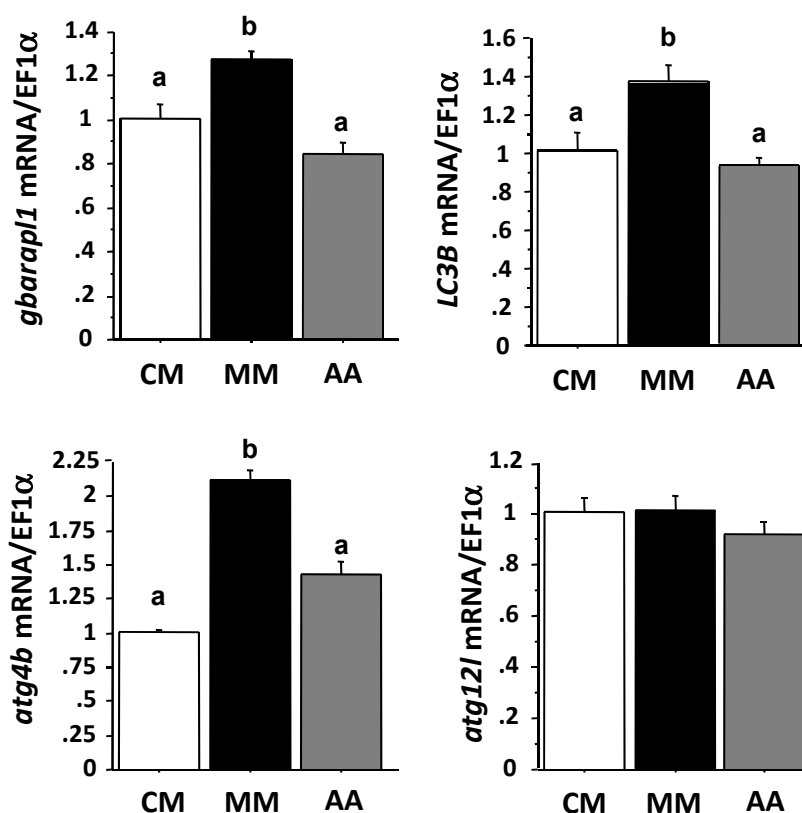
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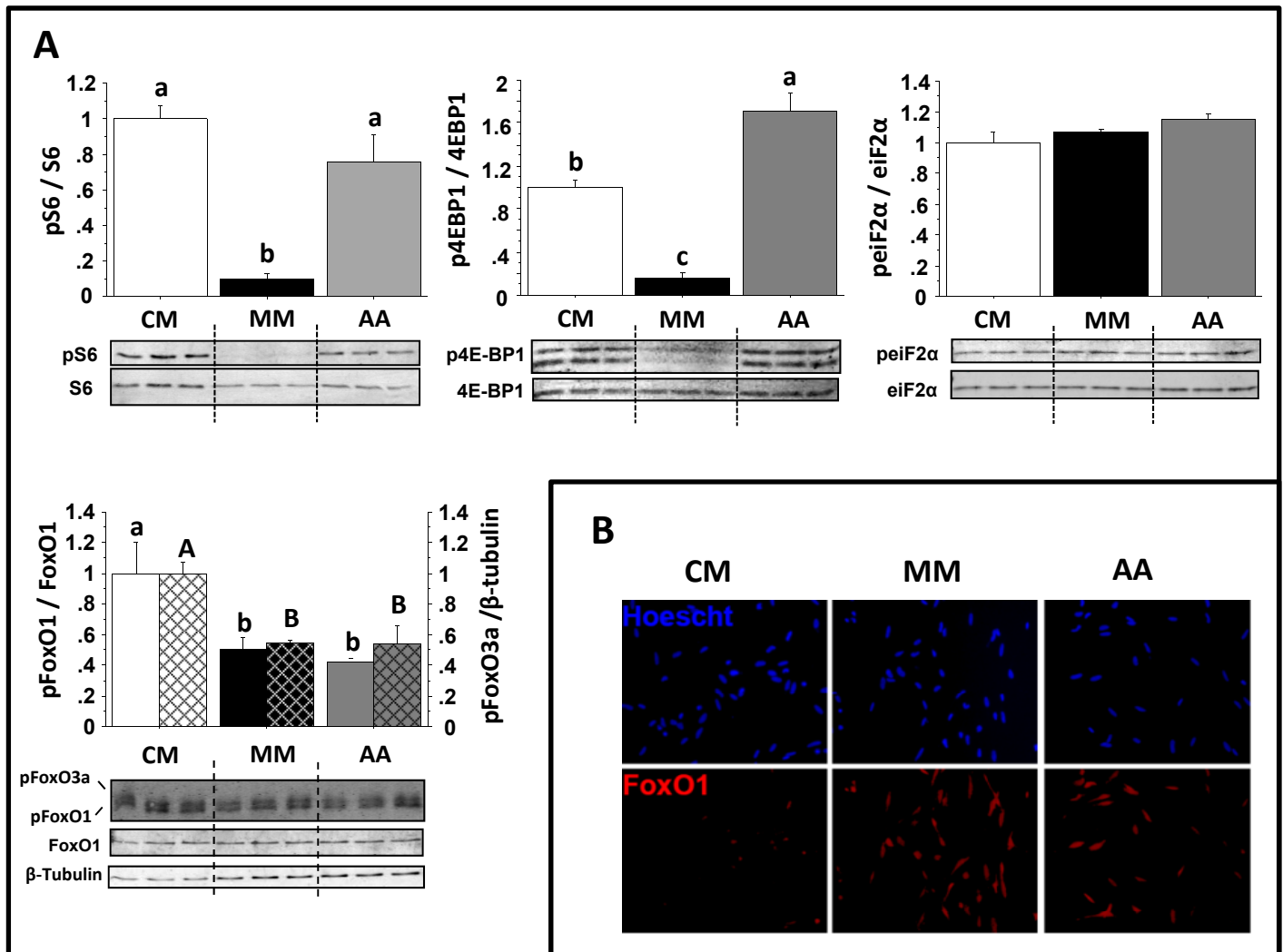
**Fig. 1**



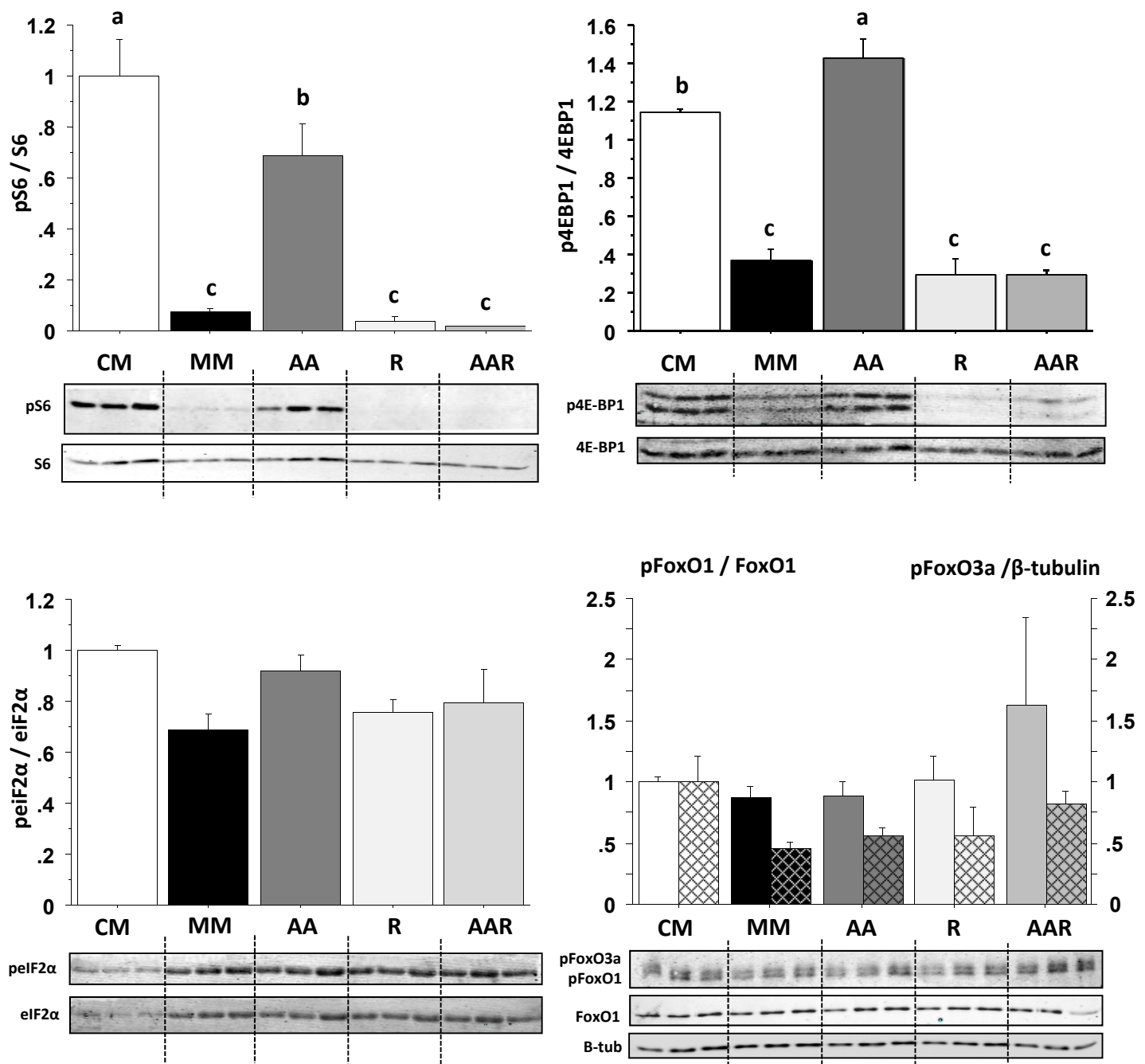
**Fig. 2**



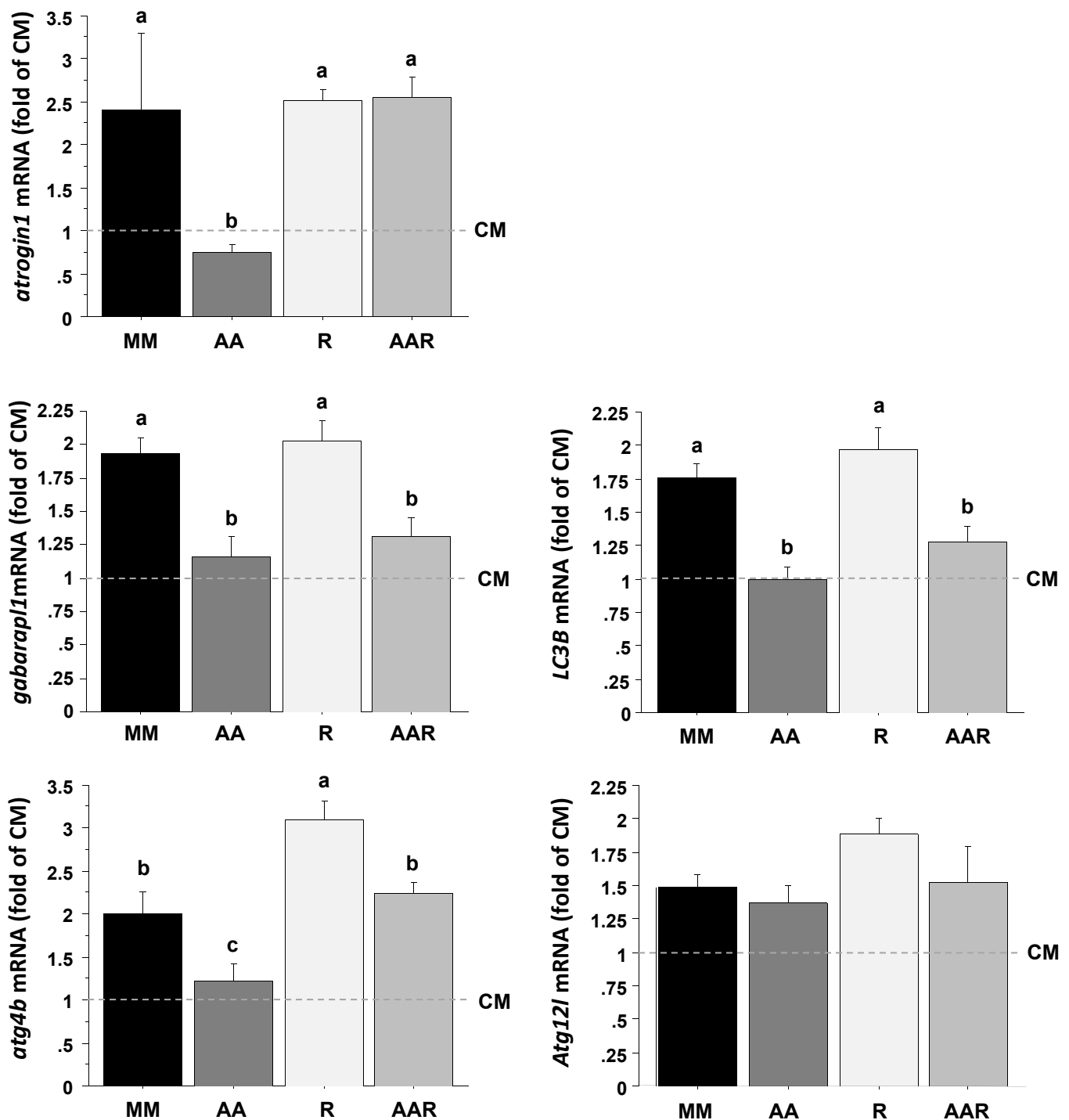
**Fig. 3**



**Fig. 4**



**Fig. 5**



**Fig. 6**