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Unacylated ghrelin activity on satellite cells and myoblasts: molecular and cellular mechanisms.

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INTRODUCTION (part I)

Ghrelin (acylated ghrelin - AG) is an octanoyl-acylated peptide hormone that binds to the growth hormone secretagogue receptor 1a (GHSR1a), promoting food intake, growth hormone release, and adiposity (Andrews *et al.,* 2011). The acylation of ghrelin is necessary for the GHSR1a activation; indeed, the unacylated form of ghrelin (UnAG) lacks GHSR1a-dependent activities such as GH release (Kojima *et al.,* 1999). However, AG and UnAG share several biological activities, for instance on the skeletal muscle where they impair atrophy in mice (Porporato *et al.,* 2013). Moreover, AG and UnAG act on myoblasts promoting differentiation *in vitro* and muscle regeneration *in vivo* (Filigheddu *et al.,* 2007, Togliatto *et al.,* 2013), suggesting the presence of a common not yet identified receptor. The AG-UnAG common receptor is likely a G protein-coupled receptor (GPCR), as the anti-atrophic activity of the two peptides is Gαs-dependent (Porporato *et al.,* 2013) and AG and UnAG treatment of pancreatic cells increase intracellular levels of cyclic AMP (cAMP) (Granata *et al.,* 2007).

GPCRs represent the largest family of transmembrane receptors in human genome (more than 800 identified) and consist in seven transmembrane regions, among which the first one and the last one extend in the extracellular and intracellular compartment, and are involved respectively in the binding to the ligand and in the cellular signaling response (Ritter and Hall, 2009). Three proteins are associated to GPCR and form a hetero-trimer: the α, β, and γ subunits. The binding of the ligand to the receptor triggers the dissociation and subsequent activation of the Gαs subunit and the Gβ-γ heterodimer. While the Gα subunit mainly regulates ACs activity, the Gβ-γ heterodimer induces activation of the PI3Kβ and PI3Kγ enzymes, leading to the production of phosphatidylinositol (3,4,5)-trisphosphate (PIP3), thus activating the Akt/mTOR pathway (Vanhaesebroeck *et al.,* 2010).

Moreover, PI3Kγ is involved in a fine-tuning feedback regulation of the GPCRs signaling, such as in β-adrenergic receptor (β-AR). Indeed, activation of PI3Kγ leads to β-AR kinase 1 (βARK1) recruitment to the plasma membrane and subsequent phosphorylation of the β-AR. Phosphorylated β-AR eventually undergoes endocytosis in a β-arrestin/clathrin-dependent manner, leading to receptor internalization (Naga Prasad *et al.,* 2002).

The PI3Kγ activity is strictly dependent from the balance between the two regulatory subunits of the enzyme: the p84/87 and the p101 (Perino *et al.,* 2011). p84/87 subunit functions as a scaffold protein that links p110γ to the cAMP-dependent protein kinase A (PKA). When is in complex with p110γ, PKA phosphorylates p110γ and inhibits its catalytic activity, thus preventing β-AR internalization (**Figure 1 – Left**).

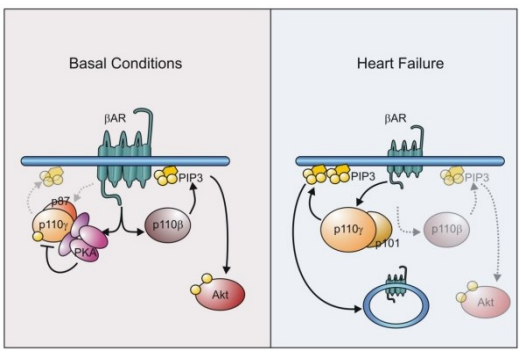

Figure 1. Schematic representation of PI3Kγ-dependent adrenergic receptors internalization.
PI3Kγ-dependent regulation of β-AR density on plasma membrane in normal vs. pathological (heart failure) conditions (from Perino 2012); see text for details.

Otherwise, p101 subunit lacks any PKA-scaffolding property. Indeed, when the p110γ is associated with the p101 subunit, PKA-dependent p110γ inhibition is lacking, and β-AR is internalized (**Figure 1 – Right**).
When the balance between the two regulatory subunits is altered, the feedback regulation of β-AR can assume a pathological relevance. In heart failure, the p101 subunits expression is increased, leading to enhanced activation of p110γ and subsequent receptor internalization. This mechanism links GPCRs-mediated PKA activation to a PI3Kγ-dependent receptor internalization and it is associated with the impairment of the physiological heart contractility (Perino *et al.,* 2011).

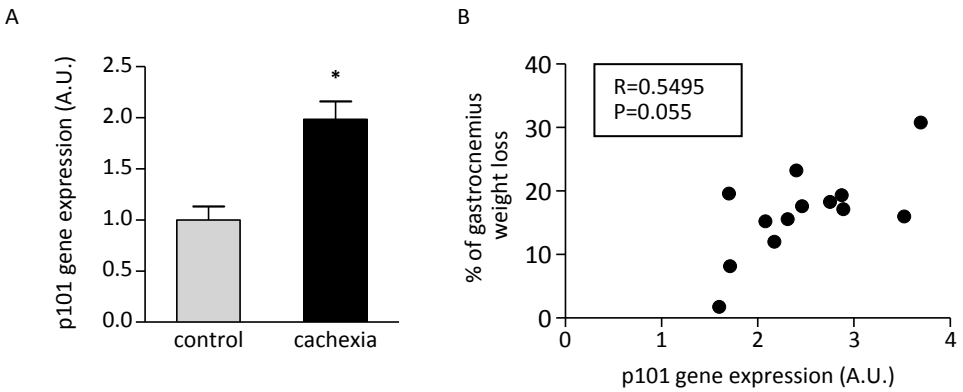

Figure 2. The p101 expression increases in cachectic muscles proportionally to the cachexia grade. ***(A)*** *p101 expression in gastrocnemii from non-tumor-bearing (control) and cachectic mice.* ***(B)*** *Correlation between p101 expression and gastrocnemii weight loss percentage. *P < 0.01. (modif. from Michele Ferrara PhD thesis)*

Our previous data show that p101 subunit expression is increased during cancer cachexia, a multi-organ syndrome mainly characterized by a severe skeletal muscle wasting, and that this expression correlates with the atrophy severity (**Figure 2**). Interestingly, during cachexia, UnAG anti-atrophic activity is weaker compared to the effects on denervation- and starvation-induced atrophy (**Figure 3**).
Consistently, raising levels of plasmatic AG and UnAG are detected in oncological patients during the pathology progression, suggesting a substantial downregulation of AG and UnAG signaling (Argilès and Stemmler, 2013).
As the mechanism of PI3Kγ-induced receptor internalization can be ideally applied to all the GPCRs that associate with a Gαs subunit triggering cAMP response, we raised the hypothesis that the AG/UnAG unknown GPCR is subject to the same desensitization occurring for the β-AR, leading to the establishment of AG/UnAG resistance in muscle during cancer cachexia.

Based on this consideration, the aims of my project (I) are:
- To verify if the overexpression of the p110γ/p101 subunits in muscle cells affects the AG/UnAG activities.
- To elucidate the mechanism through which AG/UnAG exert their biological activities.

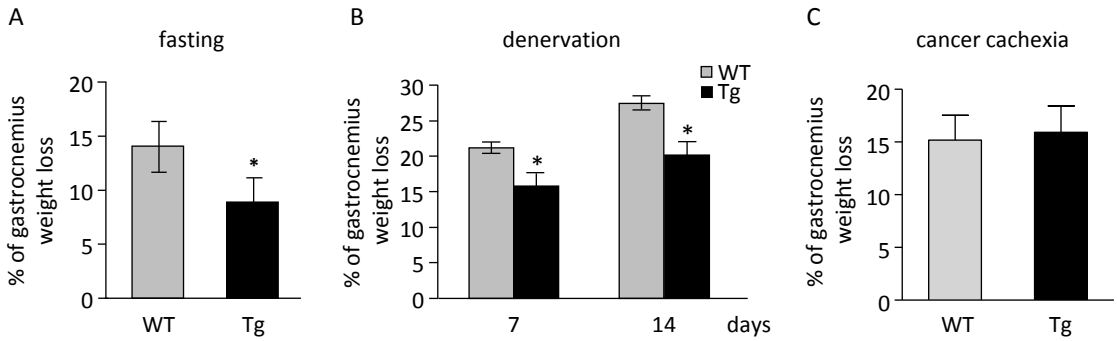

Figure 3. In vivo UnAG anti-atrophic activity in different conditions. *Percentage of gastrocnemius weight loss in WT animals vs. Myh6/Ghrl mice (Tg) characterized by 50-folds increase of plasmatic UnAG in fasting-* ***(A)****, denervation-* ***(B)****, and cancer cachexia-* ***(C)*** *induced atrophy. (Modif. from Porporato et al 2013 (A and B) and from Michele Ferrara PhD thesis (C))*

METHODS (I)

Intracellular cAMP measurement

C2C12 myotubes were serum-deprived for 18h, and then treated for 5 min with 1 µM AG and UnAG in the presence of 100 µM IBMX (3-Isobutyl-1-methylxanthine, phosphodiesterase inhibitor). 1 µM CGS, the agonist of adenosine receptor A2A, was used as positive control. Enzyme immunoassay (cAMP EIA kit - Cayman Chemical) was performed following the producer's protocol.

Myoblasts transfection

Myoblasts at 70% of confluence were transfected with the indicated plasmids in a 100 mm plate using Lipofectamine-3000 (Life technologies) following the manufacturer's indication. The next day, cells were detached and plated in a 6-wells plate in DMEM containing 20% FBS (at a confluence of 450,000 cells per well). After 3 hours, cells were serum-deprived for 18h and then treated at the indicated time with 1 µM AG, UnAG, and CGS, and with 10 ng/ml IGF-1.

Immunoprecipitation and western blot

C2C12 myoblasts were serum-deprived overnight and then treated with 1 µM AG, UnAG, CGS, and with 10 ng/ml IGF-1. Cells were washed with PBS containing 1 mM Na_3VO_4, and then homogenized in RIPA buffer (1% Triton X-100, 1% sodium-deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM EGTA, 59 mM NaF, 160 mM NaCl and 20 mM Tris-HCl pH 7.4) containing 1 mM DTT, *Protease Inhibitors Cocktail*, and 1 mM Na_3VO_4. The homogenates were centrifuged at 14,000 *g* for 15 min to remove debris and nuclei.

For Immunoprecipitation, cell lysates were incubated with anti-p110γ antibody (kind gift of Emilio Hirsch) using Dynabeads-Protein G (ThermoFisher scientific) kit, following the producer's protocol.

For immunoblot, lysates were mixed with SDS loading buffer 3X (187.5 mM Tris-HCl pH 6.8, 6% w/v SDS, 30% glycerol and 0.03% w/v bromophenol blue) and boiled at 95°C for 5 min. The electrophoresis was performed on 8-10% polyacrylamide gel, and then the proteins were transferred on PVDF membranes (Hybond-P, GE Healthcare). After 1-hour of saturation in 3% BSA at room temperature, the membranes were incubated with primary antibody overnight at 4°C, and then with secondary antibody for 1h at room temperature. Finally, the proteins were visualized with Western Lightning Chemiluminescence Reagent Plus (PerkinElmer Life Sciences) and their levels analyzed with Quantity One software (Bio-Rad).

RESULTS (Part I)

AG and UnAG treatment increases cAMP intracellular levels in myoblasts.

As AG and UnAG increase intracellular cAMP levels in pancreatic cells and the anti-atrophic activity of the two peptides is Gαs-dependent, the AG/UnAG common receptor is likely a Gαs-coupled GPCR. To test the hypothesis that these hormones induce cAMP increase in myoblasts, we treated C2C12 with 1 µM AG and UnAG. After 5 minutes of treatment, we measured a twofold increase in the intracellular cAMP levels in AG- and UnAG-treated myoblasts (**Figure 4**).

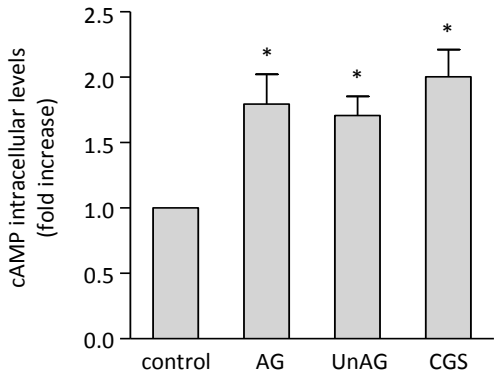

Figure 4. AG and UnAG increase cAMP intracellular levels in C2C12 myoblasts.
*cAMP intracellular levels (fold increase) in myoblasts treated for 5 minutes with 1 µM AG, UnAG, and CGS (A2A receptor agonist, used as positive control). n=3 independent experiments. *P<0.05.*

PI3Kγ overexpression reduces AG- and UnAG- mediated AKT activation.

To test the hypothesis of a PI3Kγ-dependent AG/UnAG resistance, we checked if the overexpression of the p110γ catalytic and the p101 regulatory subunit affected AG/UnAG signaling by co-transfecting p110γ (pcDNA3-myc-p110γ) and p101 (pcDNA3-HA-p101) subunits. After 48h we checked the expression and the association of the two PI3Kγ subunits. Both proteins were expressed and were associated in a complex (**Figure 5A**).

As PI3Ks-produced PIP3 activates AKT/mTOR pathway, we set the conditions (i.e. the p110γ plasmid amount to be transfected) to avoid a basal AKT activation (**Figure 5B**). Co-transfection of 0.125 µg p110γ and 1 µg p101 (1:8 ratio) per 500,000 cells blunted the AG/UnAG-induced phosphorylation of AKT, suggesting that the increased activity of PI3Kγ in myoblasts resulted in an impairment of the AG/UnAG-activated signaling (**Figure 5C**).

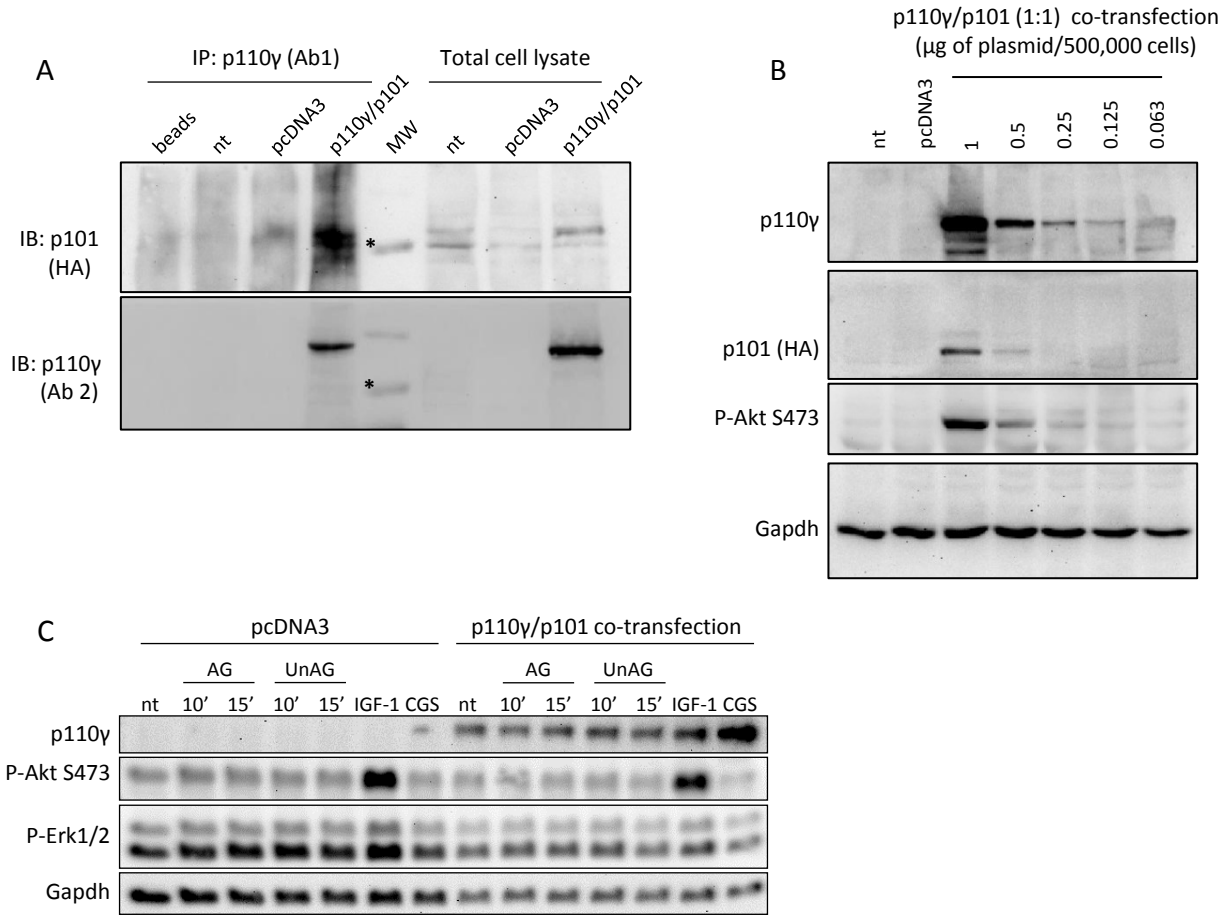

Figure 5. p110 and p101 overexpression blunts AG- and UnAG-induced AKT activation.
(A) *pcDNA3-p110-Myc and pcDNA3-p101-HA were co-transfected (1 µg/500,000 cells each) then lysate were immunoprecipitated with anti-p110γ antibody (clone 1). Immunoblot analysis shows that p101 protein co-precipitate with p110γ.* ***(B)*** *Different amount of p110γ and p101 plasmids induces a dose-dependent AKT phosphorylation on S473.* ***(C)*** *p110γ and p101 co-transfection blunts AG- and UnAG-induced AKT and Erk1/2 activation (IGF-1 and CGS were used as controls, see methods for details).*

CONCLUSION (I)

The purpose of this project is to understand the molecular mechanism underling ghrelin-resistance in skeletal muscle during cancer cachexia. Indeed, while UnAG acts as a strong anti-atrophic factor in starvation- and denervation- induced atrophy (Porporato *et al.*, 2013), the effects of UnAG on muscle wasting during cancer cachexia seems to be impaired. Moreover, the correlation between the plasmatic AG/UnAG levels in cancer patient and the muscle wasting severity suggest that the muscle responsiveness to these hormones is compromised (Argilès and Stemmler, 2013). We raised the hypothesis that ghrelin resistance could depend on a dysregulation of the feedback mechanisms leading to receptor internalization. The PI3Kγ enzyme plays a crucial role in this signaling. In particular, when the balance between the two regulatory subunits of the enzyme (p84/85 and p101) is altered, the β-AR is over-internalized and the adrenergic response is diminished (Perino *et al.,* 2011). Since this mechanism could be ideally applied to all the Gαs-associated GPCRs, AG/UnAG response on skeletal muscle could involve the same mechanism. During cachexia, the p101 subunit muscle expression is increased and its expression levels negatively correlates with the UnAG protection from muscle wasting.

In this annual report, I showed preliminary data indicating that over expression of p110γ and p101 subunits blunts the AG/UnAG-induced activation of AKT. Together with this signaling read-out, we are investigating if the overexpression of p110γ and p101 subunits affects also cAMP response induced by AG/UnAG treatment.

Based on our hypothesis, reduction of the AG/UnAG-induced response depends on abnormal receptor internalization. Thus, the determination of the AG/UnAG receptor identity, will consent a direct investigation of the PI3Kγ-dependent changes in the AG/UnAG receptor density on plasma membrane.

Introduction (part II)

Satellite cells (SCs) are muscle progenitor cells located between the basal lamina and the myofiber that sustain growth and repair of skeletal muscle (Zammit *et al.,* 2004). In the adult skeletal muscle, SCs are in a quiescent state and express the transcription factor paired-box 7 (Pax7) (Seale *et al.,* 2000). After injury or intense exercise, SCs undergo activation, inducing the expression of myogenic genes, such as the myogenic factor 5 (Myf5) and the myoblasts determination factor (MyoD), then they enter the cell cycle (Singh and Dilworth, 2013), and, after several cellular divisions, myoblasts turn off Pax7 expression and start to express other key genes that orchestrate the final stages of differentiation. At this stage, myoblasts exit the cell cycle and fuse into existing myofibers or to each other to form de novo myofibers (Yin *et al.,* 2013). Moreover, SCs are able to maintain the myogenic pool through the mechanism of self-renewal. In particular, a small portion of SCs, during the first division, generates two distinct daughter cells: one committed progenitor, and the other with stem-like features, through asymmetric division (Kuang *et al.,* 2007).

UnAG acts directly on SCs inducing their proliferation, thus promoting skeletal muscle regeneration *in vivo* (Togliatto *et al.,* 2013). Moreover, in the last annual report, I described other direct action of UnAG on SCs. In particular, we isolated single fibers from EDL muscles, thus maintaining SCs in an original niche-like environment (Zammit *et al.,* 2004), and we cultured them in the presence or absence of 100 nM UnAG, then analyzed SCs on the fiber for Pax7 and MyoD expression at different time-points.

SCs undergo activation turning on MyoD expression. Upon 6h incubation, UnAG expanded the portion of activated Pax7+/MyoD+ SCs above the total number of SCs (**Fig. 6A**). After 72 h, several clusters of myoblasts originated from a single SC are visible on myofibers and UnAG increased the number of cells in each cluster (**Fig. 6B**). During this phase, the majority of activated SCs turns off Pax7 and undergoes terminal differentiation, while a small subset undergoes self-renewal retaining Pax7 but not MyoD. After 96h, UnAG significantly raised the portion of quiescent Pax7+/MyoD- SCs (**Fig. 6C**).

To verify that this effect on self-renewal applied also *in vivo*, we administered BrdU to WT and *Myh6/Ghrl* transgenic mice (characterized by high plasmatic levels of UnAG) during the phase of intense myoblasts proliferation post-injury (**Fig. 6D**). Since BrdU is incorporated in every cycling cell, when muscle regeneration is fully achieved (thus all SCs are in a quiescent state), any cell positive for both BrdU and Pax7 (**Fig. 6E**) is a SC that cycled at least once and then underwent self-renewal (Shea *et al.,* 2010). The number of Pax7+/BrdU+ SCs – normalized on SC number in the controlateral, non-injured muscle – was higher in *Myh6/Ghrl* than in WT muscles (**Fig. 2F**), demonstrating that upregulation of UnAG enhanced SC self-renewal also *in vivo*. We decided to investigate in deeper the cellular and molecular mechanism through which UnAG induces self-renewal of SCs.

Based on this consideration, the aims of my project (II) are:

- To understand the cellular mechanism through which UnAG induces self-renewal of SCs
- To elucidate the molecular mechanism involved in UnAG-induced self-renewal of SCs.

METHODS (II)

Myofibers isolation and culture.

EDL muscles from WT FVB mice were digested in 0.2% collagenase type-I (Sigma) in DMEM for 60–70 minutes at 37°C with shaking. Muscles were mechanically dissociated and single fibers liberated. After extensive washing, myofibers were cultured in F12 medium supplemented with 15% HS and 1 nM FGF-2 in the presence or absence of 100 nM UnAG.

For experiments with the chemotherapic drug AraC (Cytosine β-D-arabinofuranoside, Sigma-Aldrich), myofibers were cultured for 72h, incubated with or without 100 µM AraC for further 48 h, and then fixed (day 5). For asymmetric division experiments, myofibers were fixed after 48h of incubation in the presence or absence of 100 nM UnAG.

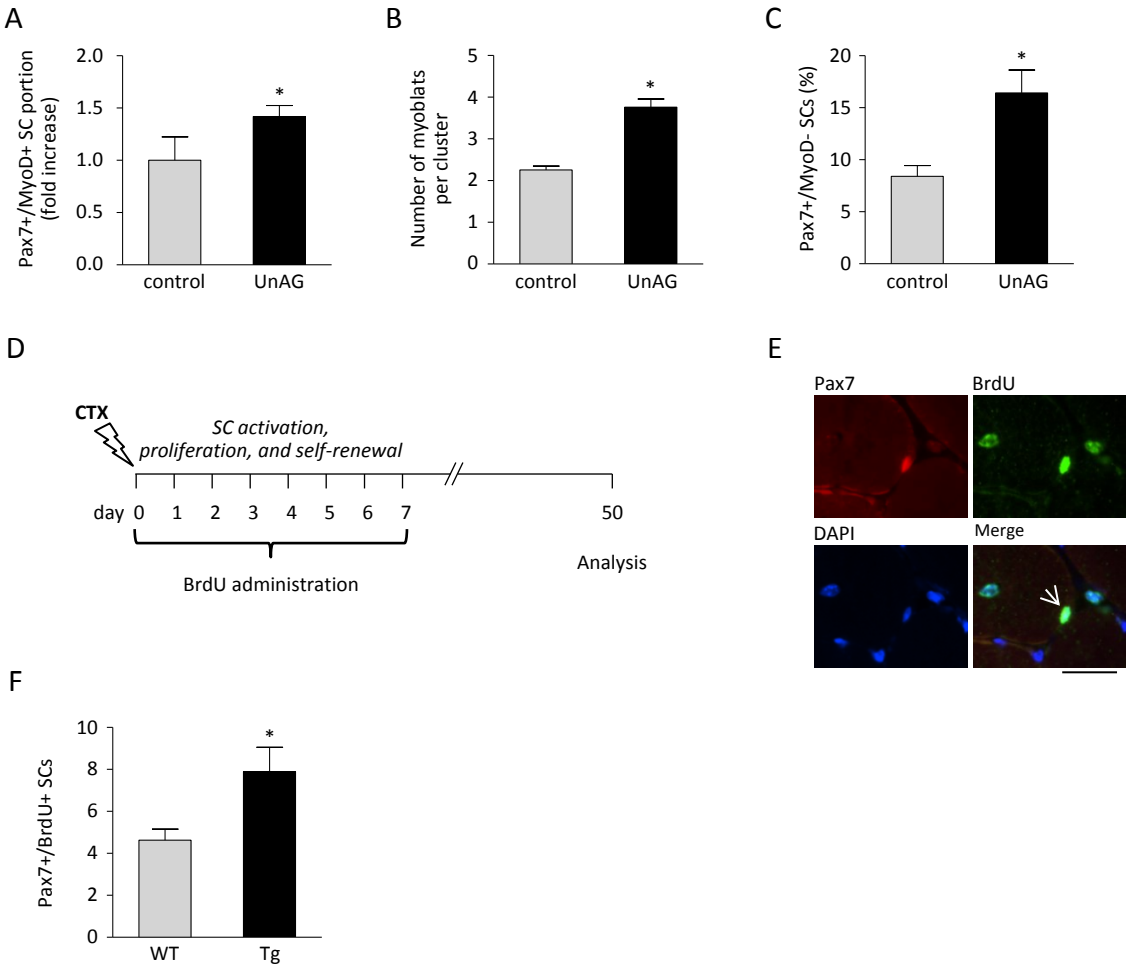

Figure 6. UnAG induces activation, proliferation, and self-renewal of SCs.

*(**A-C**) Percentage of MyoD+ SCs (**A**) after 6h of UnAG treatment. (**B**) Cells per cluster after 72h of treatment. (**C**) Percentage of Pax7+/MyoD- SCs after 96h of treatment. Means±s.e.m. *P<0.05; ≥25 myofibers/treatment; n=3 independent experiments. (**D**) Experimental design schematic for BrdU treatment. (**E**) Representative images of TA transverse sections, arrow indicates a Pax7+/BrdU+ nucleus. Scale bar, 40 µm. (**F**) Pax7+/BrdU+ nuclei, normalized to the controlateral SC number. Means±s.e.m. *P<0.05; n≥8.*

Immunofluorescence and analysis.

For immunofluorescence on isolated fibers and on cultured SCs, samples were fixed in 4% PFA for 10 min, permeabilized with 0.5% triton for 6 min and blocked in 4% BSA for 30 min. Primary antibodies to detect Pax7 (1:100 Developmental Studies Hybridoma Bank) and MyoD (1:500; Santa Cruz) were incubated overnight at 4°C and the secondary antibodies for 45 min at RT, followed by 5 min of DAPI staining. Images were acquired with a Leica CTR5500 B fluorescent microscope with the Leica Application SuiteX 1.5 software and quantification was performed by ImageJ v1.49o software. To evaluate the asymmetric division events in SC pairs, MyoD levels were obtained by subtracting the background from the nuclear fluorescence intensity (determined by overlap with DAPI staining). Asymmetric cell pairs were scored when the nuclear intensity of one daughter cell was ≤ 1 (“MyoD-”) and the other one was >1 (“MyoD+”).

RESULTS (II)

UnAG increases self-renewal acting on the first division of SCs.

Asymmetric division the main mechanisms through which SCs undergo self-renewal and it generally occurs during the first cellular division, when MyoD expression is segregated in only one of two daughter cells (Troy *et al.,* 2012). Thus we investigated if UnAG effect on self-renewal occurred during the first SC division. Treatment with AraC, which selectively kills cycling cells, during the first three days of myofibers culture results in a total SC pool depletion, indicating that all SCs enter the cell cycle during this phase (data not show).

However, when the mitotoxin AraC was added from day 3 to day 5 of myofibers culture few Pax7+/MyoD- SCs were evident. Since at day 3 all SCs enter the cell cycle, these AraC-resistant SCs are cells that underwent quiescence after one cell division (Bernet *et al.,* 2014, **Fig. 7A**). In the presence of 100 nM UnAG in this experimental setting, the number of Pax7+/MyoD- AraC-resistant SCs was increased (**Fig. 7B**), indicating that UnAG acts during the first replication of SCs and suggesting that it might regulate their asymmetric division.

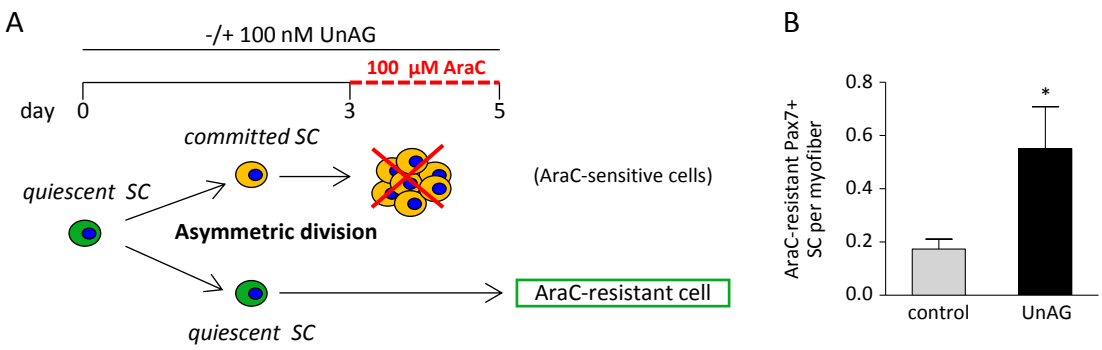

Figure 7. UnAG increases self-renewal acting on the first SCs division.

(A) *Schematic of experiments with AraC.* ***(B)*** *Pax7+/AraC-resistant cells per myofibers, comparing control vs. UnAG treatments. Means±s.e.m. *P<0.05; ≥25 myofibers/treatment; n=3 independent experiments.*

UnAG increases asymmetric division of SCs.

To evaluate the ability of UnAG to induce SCs asymmetric division, we treated myofibers-associated SCs with 100 nM UnAG for 48h and we scored the percentage of the asymmetric division events, seen as daughter cell pairs containing one cell negative and one cell positive for MyoD nuclear staining (Troy *et al.,* 2012, **Fig. 8A**).

Treatment with UnAG quadruplicates the percentage of asymmetric division events, indicating that UnAG promotes asymmetric division of SCs (**Fig. 8B**).

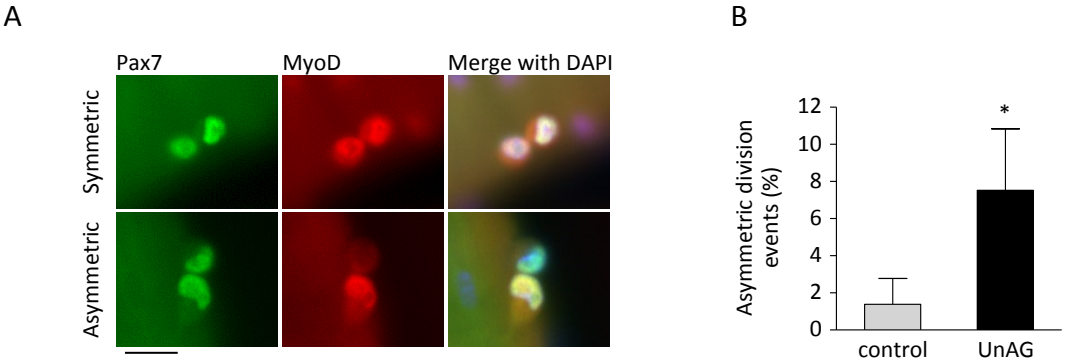

Figure 8. UnAG increases asymmetric division events of SCs.

*(**A**) Representative images of SCs that underwent symmetric (top) or asymmetric (bottom) division fixed after 48h in culture and stained for Pax7 (green), MyoD (red) and DAPI (blue). Scale bar, 20 µm. (**B**) Asymmetric division events in SC doublets. Means±s.e.m. *P<0.05; ≥22 doublets/treatment; n=3 independent experiments.*

UnAG-induced self-renewal is mediated by p38 activity.

Asymmetric division of SCs requires p38. Indeed, the Par-complex-dependent asymmetric activation of p38a/b MAPK controls self-renewal of SCs (Troy *et al.*, 2012). As UnAG activates p38 in muscle cells (Filigheddu *et al., 2007*; Porporato *et al., 2013*), we raised the hypothesis that UnAG induces self-renewal through activation of p38. Treatment of myofibers-associated SCs with UnAG for 96h increased the percentage of quiescent Pax7+ SCs, however this effect was blunted by the p38-specific inhibitor (SB203580) (**Fig.9**). This result indicates that UnAG-induced self-renewal is mediated by p38 activity.

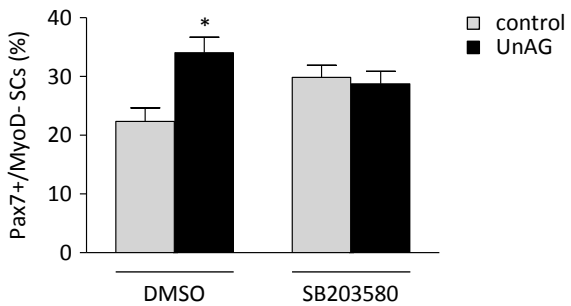

Figure 9. UnAG induces self-renewal through activation of p38.

*Percentage of Pax7+/MyoD- SCs after UnAG treatment in the presence/absence of SB203580 (Sigma-Aldrich), a p38-specific inhibitor. Means±s.e.m. *P<0.01 vs. DMSO-treated control; ≥100 clusters of ≥25 myofibers/treatment; n=3 independent experiments.*

CONCLUSION (II)

Self-renewal of SCs is the cellular mechanism that permits to maintain the original pool of myogenic precursors even after several rounds of regeneration (Collins *et al.,* 2005). In some pathological context, such as in dystrophies, skeletal muscle undergoes continuous rounds of degeneration and regeneration, and this leads to gradual depletion of SCs and to deposition of fat and fibrotic tissue, resulting in loss of structure and function of skeletal muscle (Jiang *et al*., 2014). Understanding the cellular and molecular mechanisms that control self-renewal of SCs is crucial to improve dystrophy treatment. Asymmetric division of SCs appears to be the most relevant mechanism through which SCs exert self-renewal (Kuang *et al*., 2007). This mechanism requires a proper activation of the atypical PKCι-ζ during the SC activation (Troy *et al*., 2012). Activation of atypical PKC leads to asymmetric phosphorylation of p38 and segregation of P-p38 in one daughter cell during cell division (Bernet *et al.,* 2014; Troy *et al*., 2012). The data herein presented demonstrate that UnAG increases self-renewal of SCs through p38 activation, inducing their asymmetric division. It remains still unknown if UnAG induces asymmetric activation of p38 and what is the signaling controlling this. Future experiments will focus on the elucidation of the mechanism through which UnAG acts on asymmetric division of the SC.

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Angelino, E., Reano, S., Ferrara, M., Agosti, E., Graziani, A., Filigheddu, N. (2015). **Antifibrotic activity of acylated and unacylated ghrelin**. Int J Endocrinol. doi: 10.1155/2015/385682.

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POSTERS

Simone Reano, **Elia Angelino,** Michele Ferrara, Hana Sustova, Emanuela Agosti, Sara Clerici, Nicoletta Filigheddu. Unacylated ghrelin stimulates satellite cells self-renewal and skeletal muscle regeneration. EMBO workshop - Molecular mechanisms of muscle growth and wasting in health and disease. Ascona, Switzerland. September 20-25th, 2015.

Hana Sustova, Simone Reano, **Elia Angelino,** Emanuela Agosti, Michele Ferrara, Sara Clerici, Andrea Graziani, Nicoletta Filigheddu. Positive effects of unacylated ghrelin on dystrophic muscle in vivo. Brno, Czech Republic. The Biomania Student Scientific Meeting 2015. September 22th, 2015

Elia Angelino, Simone Reano, Michele Ferrara, Hana Sustova, Emanuela Agosti, Sara Clerici, Andrea Graziani, Nicoletta Filigheddu. Unacylated ghrelin enhances satellite cells activity and promotes skeletal muscle regeneration. International spring research day. Lugano-Vezia, Switzerland. June 19th, 2015.

Simone Reano, **Elia Angelino**, Michele Ferrara, Hana Sustova, Emanuela Agosti, Andrea Graziani, Nicoletta Filigheddu. Exploring the therapeutics potential of unacylated ghrelin in muscle dystrophy. MDA scientific conference, Washington DC, USA. March 11-14th, 2015.

Emanuela Agosti, **Elia Angelino,** Simone Reano, Hana Sustova, Michele Ferrara, Sara Clerici, Andrea Graziani, Nicoletta Filigheddu. Protective effects of unacylated ghrelin in aging mice. Brallo-Pavia, Italy. June 8th-12th, 2015.

Hana Sustova, Simone Reano, **Elia Angelino**, Emanuela Agosti, Michele Ferrara, Sara Clerici, Andrea Graziani, Nicoletta Filigheddu. Positive effects of unacylated ghrelin on dystrophic muscle in vivo. Biochemical PhD students meeting. Brallo-Pavia, Italy. June 8th-12th, 2015.

Elia Angelino, Simone Reano, Michele Ferrara, Omar Sabry, Andrea Graziani, Nicoletta Filigheddu. Ghrelina deacilata promuove la rigenerazione del muscolo scheletrico. Giornata del dottorato. Alessandria, Italy. September 26, 2014.

Ferrara M, Sabry O, Reano S, **Angelino E**, Ciraolo E, Coletti D, Hirsch E, Costelli P, Filigheddu N, Graziani A. A GHSR1a-independent signaling contributes to ghrelin anti-cachectic activity in skeletal muscle and is reciprocally regulated by PI3-kinase beta and gamma isoforms. 2nd cancer cachexia conference. Montreal, Canada. September 26-28, 2014.

Reano S, **Angelino E**, Sabry O, Ferrara M, Graziani A, Filigheddu N. Ghrelin promotes skeletal muscle regeneration. 7th Cachexia Conference. Kobe, Japan. December 9-11, 2013.

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Filigheddu N, Reano S, **Angelino E**, Ferrara M, Sabry O, Graziani A. Role of ghrelin in skeletal muscle regeneration. Regenerative medicine: From biology to therapy. Cambridge, England. October 30 – november 1, 2013.

Reano S, Ferrara M, **Angelino E**, Sabry O, Coletti D, Filigheddu N, Graziani A (2013). Role of acylated and unacylated ghrelin in skeletal muscle regeneration. EMBO Workshop – Molecular mechanisms of muscle growth and wasting in health and disease, Ascona, Switzerland. September 15 – 20, 2013.

Simone Reano, **Elia Angelino**, Michele Ferrara, Omar Sabry, Andrea Graziani, Nicoletta Filigheddu. Role of ghrelin in skeletal muscle regeneration. Gordon Research Conference, Lucca, Italy. July 7 – 12, 2013.

Nicoletta Filigheddu, Simone Reano, Michele Ferrara, Paolo Ettore Porporato, **Elia Angelino**, Viola Gnocchi, Yuxiang Sun, Andrea Graziani. Acylated and Unacylated Ghrelin impair skeletal muscle atrophy without inducing hypertrophy and indipendently of GHSR1, the only known ghrelin receptor. Society for muscle biology, New York, USA. June 4 – 8, 2012.

Nicoletta Filigheddu, Simone Reano, Michele Ferrara, Paolo Ettore Porporato, **Elia Angelino**, Giulia Ronchi, Stefano Geuna, Yuxiang Sun, Andrea Graziani. Unacylated ghrelin and ghrelin counteract skeletal muscle atrophy. ABCD meeting 2011, Ravenna, Italy. Semptember 8 – 10, 2011.

CONGRESS

International spring research day. Lugano-Vezia, Switzerland. June 19th, 2015.

SEMINARS

- 06/11/2014 Dysregulated antigen receptor signaling: molecular lessons from two congenital lymphoproliferative disorders, Snow (University of the Health Sciences Bethesda, USA)
- 14/11/2014 Tissue engineering: the state of the art, Boccafoschi (University of Piemonte Orientale, Novara)

- 17/11/2014 Optical coherence tomography from bench to bedside shening the light during percutaneous vascular intervention, Gabrio (University of Piemonte Orientale, Novara)
- 21/11/2014 Regenerative medicine, Prat (University of Piemonte Orientale, Novara)
- 25/11/2014 La scoperta del bosone di Higgs, Arcidiacono and Ruspa (University of Piemonte Orientale, Novara)
- 28/11/2014 Humoral responses to HCV infection and clinical outcomes, Patel (University of Glasgow, UK)
- 04/12/2014 Uncovering the role β-HPV in field cancerization: a collaboration in progress, Patel (Cardiff, UK)
- 05/12/2014 Focus on deliver: from basics of NAFLD to hot topics in HBV & HCV infections, Safadi
- 16/12/2014 From the legend of Prometheus to regenerative medicine, Musaro (Rome)
- 17/12/2014 Microglia microvescicles: messengers from the diseased brain, Furlan (San Raffaele University, Milan)
- 19/01/2015 Anticancer strategy Targeting cancer cell metabolism in ovarian cancer, Song College of Medicine Seoul National University)
- 20/01/2015 Different molecular mechanisms regulate hepatocyte differentiation during the transitions between epithelial and mesenchymal states, Alonzi (Istituto Nazionale per le Malattie Infettive “L. Spallanzani” IRCCS, Rome)
- 21/01/2015 Targeting the liver to cure myocarditis: a lesson from a model of STAT3-dependent autoimmune myocarditis, Poli (University of Turin)
- 27/01/2015 Myeloid cells as therapeutic target in cancer, Sica (University of Piemonte Orientale, Novara)
- 11/03/2015 Proof of principle for cell therapy: from autologous transplantation of tissue specific progenitors to gene corrected patient specific injured pluripotent stem cells, Bosnakovski (University "Goce Delcev" Stip, Macedonia)
- 09/04/2015 Signal control in iNKT cell development and function, Zhong (Duke University, North Carolina, USA)
- 21/04/2015 Actin-based mechanisms in the control of gene expression and cell fate, Percipalle (Karolinska Institutet, Sweden)
- 07/05/2015 An integrated approach to the diagnosis and treatment of ovarian cancer, McDonald (Georgia Tech University, Atlanta, USA)
- 14/05/2015 Conflicting interests and scientific communication, Ruff (Ottawa, Canada)
- 25/05/2015 Ribosomopathies, Ellis (University of Lousville, USA)
- 05/06/2015 Recent developments in (cutaneous) Human Polyomavirus research, Feltkamp (Leiden University Medical Center, The Netherlands)
- 26/06/2015 Satellite cells and muscle regeneration, Guttridge (Ohio State University, USA)
- 28/07/2015 Stem kidney cells, Cantaluppi
- 03/09/2015 Cell based models for studying molecular mechanisms of Facioscapulohumeral Muscolar Distrophy (FSHD); Toward animal model for Facioscapulohumeral Muscolar Distrophy (FSHD), Bosnakovski (University "Goce Delcev" Stip, Macedonia)