

Atrogin-1/MAFbx and MuRF1 Are Downregulated in Aging-Related Loss of Skeletal Muscle

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Muscle atrophy in many conditions share a common mechanism in the upregulation of the muscle-specific ubiquitin E3-ligases atrogin-1/muscle atrophy F-box (Atrogin-1/MAFbx) and muscle ring-finger protein 1 (MuRF1). E3-ligases are part of the ubiquitin proteasome pathway utilized for protein degradation during muscle atrophy. In this study, we provide new data to show that this is not the case in age-related loss of muscle mass (sarcopenia). On the contrary, Atrogin-1/MAFbx and MuRF1 are downregulated in skeletal muscle of 30-month-old rats, and our results suggest that AKT (protein kinase B)-mediated inactivation of forkhead box O 4 (FOXO4) underlies this suppression. The data also suggest that activation of AKT is mediated through the insulin-like growth factor-1 (IGF-1) receptor, signaling via ShcA-Grb2-GAB. Using dietary restriction, we find that it impedes sarcopenia as well as the effects of aging on AKT phosphorylation, FOXO4 phosphorylation, and Atrogin-1/MAFbx and MuRF1 transcript regulation. We conclude that sarcopenia is mechanistically different from acute atrophies induced by disuse, disease, and denervation.

LOSS of skeletal muscle mass is a clinically important problem in disease as well as in normal aging (1–5). Muscle mass is under constant remodeling, and the net effect of myofibrillar protein synthesis and breakdown causes myofibers to hypertrophy or atrophy, respectively. In a variety of conditions such as cancer, diabetes, Cushing's syndrome, denervation, uremia, sepsis, disuse, and fasting, skeletal muscles atrophy through degradation of myofibrillar proteins via the ubiquitin–proteasome pathway (6–10). Recent advances assert that muscle atrophy in these conditions shares a common mechanism in the induction of the muscle-specific E3 ubiquitin ligases atrogin-1/muscle atrophy F-box (Atrogin-1/MAFbx) and muscle ring-finger protein 1 (MuRF1) (9–18). Whether this mechanism is in operation in muscle wasting during aging remains unresolved. Atrogin-1/MAFbx and/or MuRF1 messenger RNA (mRNA) levels are reportedly unchanged (human) (19,20), slightly increased (rat) (21), or decreased (rat) (22) in aged muscle. To address this issue, we used a previously established rodent model of sarcopenia, and examined transcript levels of Atrogin-1/MAFbx and MuRF1. Surprisingly, the levels of Atrogin-1/MAFbx and MuRF1 were found to be decreased. We therefore expanded the study to include analysis of molecules that can regulate the expression of these E3 ligases. Increased insulin-like growth factor-1 (IGF-1) signaling downregulates the transcription of these E3 ligases (16,23), probably through activation of phosphatidylinositol 3 kinase (PI3K) and protein kinase B (AKT/PKB), because increased activation of AKT alone is sufficient to downregulate both Atrogin-1/MAFbx and MuRF1 (13,15–17,24). IGF-1 and/or AKT suppression of the forkhead box O (FOXO) family of transcription factors (Figure 1) (13,15–17,24). AKT inactivates FOXO proteins

through ser-thr phosphorylation by which they become translocated to the cytosol, and targeted for degradation by the proteasome (25,26). Conversely, unphosphorylated FOXO proteins locate to the nucleus where they can influence E3 ligase transcription [reviewed in (27)]. IGF-1 signaling through PI3K-AKT is of particular interest in the context of muscle atrophy, because downstream targets of AKT not only depress E3 ligase transcription and block atrophy, but stimulate myofiber hypertrophy as well (3,14,17,28). The IGF-1 receptor interacts with the insulin receptor substrate (IRS-1) or the Shc-GRB2-GAB (Shc, Src homology 2 domain containing; GRB2, growth factor receptor bound protein-2; GAB, GRB2 associated binding protein) adaptor protein pathway to activate PI3K-AKT (reviewed in 28–30). Based on our previous data on increased Shc protein levels in senescent skeletal muscle (31), and the lack of evidence for IRS-1 activation in aging (32–35), we examined the role of Shc, PI3K-AKT, and FOXO signaling in regulating Atrogin-1/MAFbx and MuRF1 in sarcopenia. Dietary restriction (DR), which retards aging, was used to challenge the normal pattern of aging.

MATERIALS AND METHODS

Experimental Animals

Forty female rats (colony originated from Bkl; Harlan Sprague-Dawley [SD], Houston, TX), 10 young adult, 10 adult, and 20 aged rats (4, 12, and 30 months old, respectively), the latter of which 10 were on DR corresponding to 70% of ad libitum (AL) intake of age-matched animals, were kept under standardized conditions (with 12-hour light/dark cycle in a climate-controlled barrier unit) and used in this study. Based on in-house data on life span of female SD rats

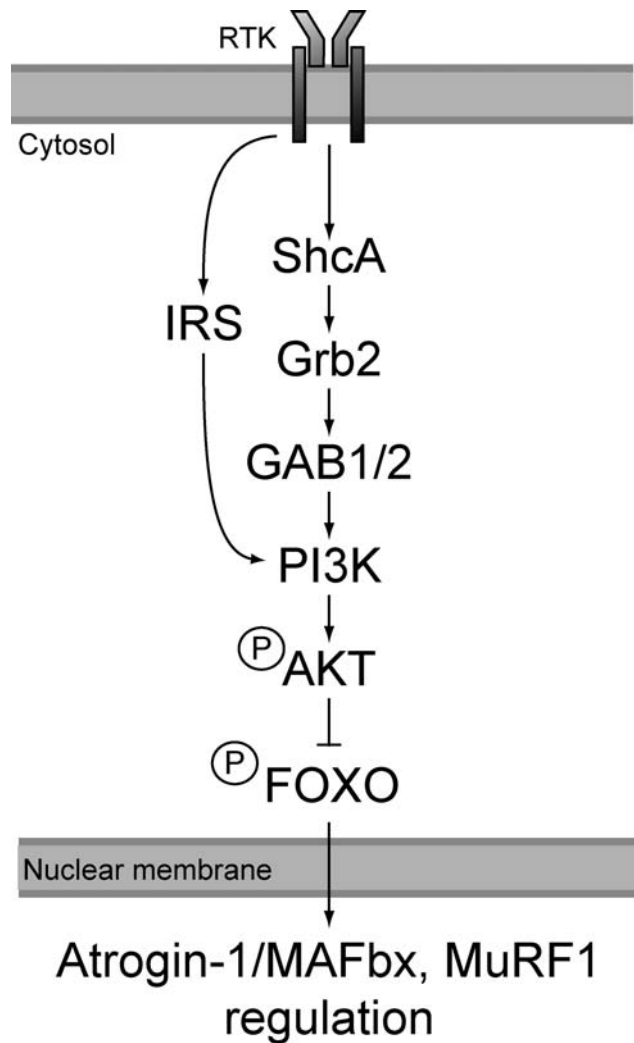


Figure 1. Schematic illustration of the signaling pathway from receptor tyrosine kinases (RTKs) to muscle-specific ubiquitin E3-ligases atrogin-1/muscle atrophy F-box (Atrogin-1/MAFbx) and muscle ring-finger protein 1 (MuRF1) expression. Encircled "P" represents phosphorylation status. AKT (protein kinase B [PKB]) inhibits the forkhead box O (FOXO)-dependent expression of Atrogin-1/MAFbx and MuRF1 through phosphorylating FOXO, thereby preventing its entry into, or initiating its exclusion from the nucleus.

(36), the 30-month-old rats were considered aged (senescent) and the 12-month-old rats were considered middle-aged. As young adults, 4-month-old rats were used because SD rats at this age have entered a slower pace of body growth (continuous until senescence) (37). All experiments were approved by the Local Ethical Committee (Stockholm's Norra Djurförsöksetiska Nämnd; project no. N54/00).

In mammals, the hind limbs become more affected by aging than do the fore limbs [for references, see (38)]. Loss of muscle mass is evident in the lower hind limb muscles, several of which, including the gastrocnemius, soleus (combined referred to as the triceps surae), extensor digitorum longus, and the plantaris, have been used to study sarcopenia in rats [e.g., (2,39,40) and references therein]. For this study, we used the gastrocnemius muscle (mixed fiber-type muscle) for mRNA and protein analysis; a ratio

between soleus muscle (predominantly slow fiber type muscle) weight (mg) and whole-body weight (g) was used to evaluate the adaptation of hind-limb muscles to their weight-bearing demands (Figure 2A and B) (37). This ratio is referred to in the text as the sarcopenia index (SI). The muscles were removed from anesthetized (chloral hydrate 300 mg/kg, i.p.) rats, frozen in liquid nitrogen, and stored at -80°C until processed.

Primers and Antibodies

Real-time polymerase chain reaction (PCR) primers (sense and antisense, 5' to 3') were: Atrogin-1/MAFbx ccatcaggagaagtggatctatgtt, gcttccccaaagtgcagta; β -actin tcgtaccactggcattgtgat, cgaagtctagggaacatagca; FOXO1 tacggccaatccagcat, tggggaggagagtcagaagt; FOXO3 cggctcattgtccagat, tcttgccagtccttcgttc; FOXO4 gtgctcgcattctactgaag, catgtcgcactccaggttct, and MuRF1 tgttctgttggagtcgtttccg, atgccggtccatgatcactt. All primary antisera were rabbit-derived and used at a dilution of 1:1000. Antibodies raised against the following targets were purchased from Cell Signaling (Beverly, MA): AKT [9272], phospho-AKT (ser473) [9271], FOXO1 [9462], phospho(ser256)-FOXO1 [9461], cross-reactive with phospho(ser193)-FOXO4 [9461], FOXO4 [9472], IGF-1R β [3027], IR β [3025]; Upstate Biotechnology (Lake Placid, NY): GAB1 (06-579), GAB2 (06-967), PI3K p85 (06-195), ShcA (06-203); or Santa Cruz Biotechnology (Santa Cruz, CA): Grb2 (sc-255). Alkaline phosphatase (AP)-conjugated antirabbit secondary antibody (1:10,000) was purchased from Amersham Biosciences (Piscataway, NJ).

RNA Isolation and Real-Time PCR

Total RNA was isolated from gastrocnemius muscle (Trizol; GibcoBRL, Life Technologies, Täby, Sweden), and the amount and purity were measured spectrophotometrically. All RNA samples were DNase treated (DNA-free; Ambion Inc., Austin, TX) resulting in OD 260/280 >1.9 , indicating a very low degree of contamination [c.f. (41)].

Tissue analysis of relative mRNA levels in gastrocnemius muscle was performed using reverse transcription (RT) and real-time PCR. Reverse transcription was performed on total RNA in a PC-960G thermal cycler (Corbett Research, Mortlake, Australia) using GeneAmp RT reagents according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). Complementary DNA, corresponding to 40 ng of total RNA, from the RT reaction was used as a template in the subsequent real-time PCR, performed in an ABI-Prism 7000 (Applied Biosystems) using SYBR-green chemistry (QuantiTect SYBR-green, Qiagen, Crawley, U.K.). [For further details, see (36).] β -actin was used as internal control to check for RNA integrity. The PCR results were not normalized because unregulated genes to be used for this purpose have yet to be identified [see also discussion in (41)].

Protein Sample Preparation and Western Blotting

Gastrocnemius muscles were homogenized in RIPA lysis buffer, pH 7.4: 50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.25% Na-deoxycholate, 1 mM NaF, 1 mM $\text{Na}_3\text{O}_4\text{V}$, 1 mM phenylmethylsulfonyl fluoride (PMSF),

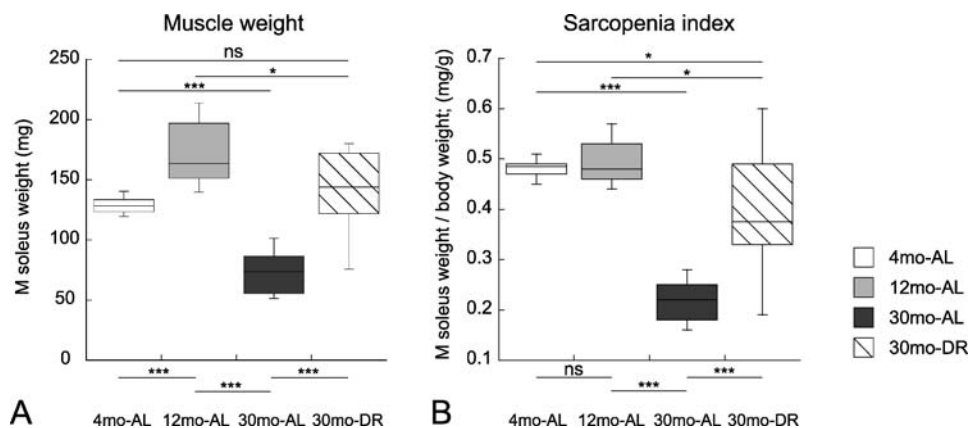


Figure 2. The boxplots show the soleus muscle weight (A) and the sarcopenia index (B) at 4 (4mo-AL; $n = 10$), 12 (12mo-AL; $n = 10$), and 30 (30mo-AL; $n = 10$ and 30mo-DR; $n = 10$) months of age. The aged animals are represented by two groups depending on feeding regimen (AL = ad libitum; DR = dietary restriction). The sarcopenia index represents the relationship between the weight of the soleus muscle (mg) and the body weight (g) of the animals. The box contains 50% of the observations and is divided by the median; error bars indicate maximum and minimum values. Statistical analysis using analysis of variance and Bonferroni's post hoc test yielded results as indicated by horizontal bars and their legends (ns = nonsignificant; $*p < .05$; $**p < .01$; $***p < .001$).

leupeptin at 10 $\mu\text{g/ml}$, and protease inhibitors (Roche Molecular Biochemicals, Indianapolis, IN). Debris was removed through centrifugation at 12,000 g . Protein concentrations were determined with the Bradford assay (Bio-Rad, Hercules, CA) using a spectrophotometer (Eppendorf, Hamburg, Germany) at 595 nm and bovine serum albumin diluted in the RIPA lysis buffer for the standard curves. All samples were rediluted to 6 $\mu\text{g}/\mu\text{l}$. Samples were denatured at 95°C for 5 minutes in a reducing loading buffer containing 5% β -mercaptoethanol. To control for protein content in the aliquots, random samples were run on gels and stained with Coomassie Blue R-250 (Sigma, St. Louis, MO). The integrated gray value for each lane was used to assess that equal amounts of protein were loaded. Equal amounts of protein (typically 60 μg) from all compared samples were separated in parallel by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto a single polyvinylidene difluoride (PVDF) membrane (Amersham Biosciences, Buckinghamshire, U.K.) and incubated (overnight at 4°C) with primary antibody diluted in 5% milk and 0.1% Tween 20. Ponceau staining (Ponceau S; Sigma) was used to confirm efficiency of transfer.

Immunodetection was managed using AP-conjugated secondary antibodies and enhanced chemifluorescence detection, according to the manufacturer's protocols (Amersham Biosciences, Piscataway, NJ). Briefly, the membranes were incubated for 90 minutes with AP-conjugated secondary antibody (1:10,000; Amersham Biosciences). Enhanced chemifluorescence detection reagents (enzyme substrate) were applied for 1–5 minutes, and the chemifluorescence was detected using a Storm fluorescence scanner (Amersham Biosciences). All Western blot experiments were repeated in triplicate to ensure reproducibility of results. In the case of ser473 phosphorylated AKT where unspecific bands were seen, stripping (Stripping buffer: 100 mM β -mercaptoethanol, 2% w/v sodium dodecyl sulfate, 62.5 mM Tris, pH 6.7; 30 minutes at 60°C) and reprobing (blocking and overnight incubation with antibody) with AKT was used to ensure analysis of specific signal (p-AKT and AKT signals

coincided on overlays). Relative protein contents were quantified from the resulting scans (TIFF images) by densitometric analysis of integrated gray levels corrected for local background, using Optimas software (Optimas Co., Bothell, WA). For illustration purposes, whole images, representative of repeated experiments, were contrast adjusted and subsequently arranged using Adobe Illustrator software (Adobe, San Jose, CA).

Statistics

All statistics were performed using Statistica 6.1 (StatSoft, Tulsa, OK). Comparisons of experimental groups were carried out with analysis of variance (ANOVA), and when significant differences were found, Bonferroni's post hoc test was used for pair-wise comparisons. Statistical significance levels were set to $*p < .05$, $**p < .01$, and $***p < .001$. The box-plot used in Figure 1 is plotted using the following definitions: box limits represent upper and lower quartile values, and are separated by the median (crossbar within box). The interquartile distance thus contains 50% of the data. Maximum and minimum values, which are not defined as outliers, are illustrated using error bars. Outliers (circles) are defined as values deviating from the quartile borders by more than 1.5 times the interquartile distance. In all other cases, error bars represent standard deviation.

RESULTS

Sarcopenia Is Associated With Decreased Expression of Atrogin-1/MAFbx and MuRF1

To address Atrogin-1/MAFbx and MuRF1 regulation in sarcopenia, we used a rodent model (37), in which the degree of hind-limb sarcopenia was characterized by the relationship between the weights of the postural hind-limb soleus muscles and the body weight (SI). As controls, 4-month-old AL adults (4mo-AL) and 12-month-old AL adults (12mo-AL), respectively, were used and were not statistically different with respect to SI (Figure 2). Aged AL animals (30mo-AL)

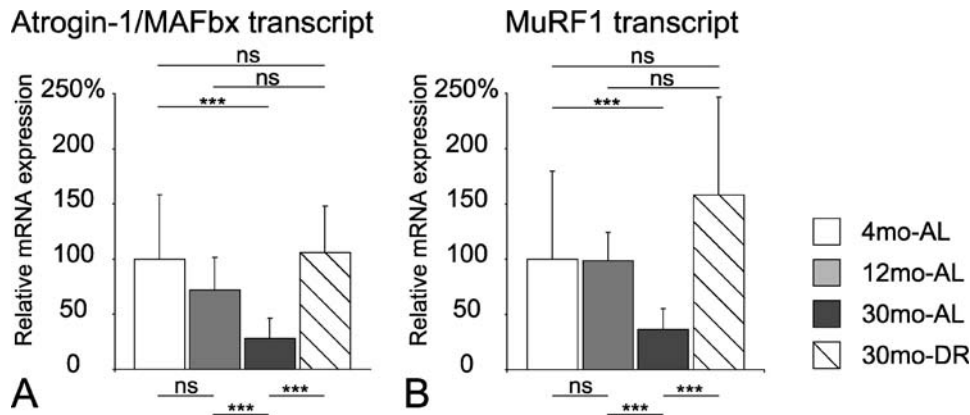


Figure 3. Relative messenger RNA (mRNA) expression levels of muscle-specific ubiquitin E3-ligases atrogin-1/muscle atrophy F-box (Atrogin-1/MAFbx) and muscle ring-finger protein 1 (MuRF1) in gastrocnemius muscle of adult 4- and 12-month-old and aged 30-month-old ad libitum-fed (AL) rodents, as well as aged 30-month-old rodents on dietary restriction (DR). Results of real-time polymerase chain reaction analysis of Atrogin-1/MAFbx (A) and MuRF1 (B) mRNA levels are plotted relative to the 4-month-old AL group (set to 100%). All statistical testing was performed using analysis of variance and Bonferroni's post hoc test; the results of the latter are indicated by horizontal bars, asterisks ($*p < .05$; $**p < .01$; $***p < .001$), or "ns" when nonsignificant. Error bars represent standard deviation.

had significantly reduced muscle weights and a lower SI compared to the adult groups (Figure 2), and aged rats on a DR (30mo-DR) showed soleus muscle weights and SI values in between those of the 30mo-AL group and the adult control groups (Figure 2).

Real-time PCR showed significantly reduced levels of Atrogin-1/MAFbx and MuRF1 mRNAs in the sarcopenic 30mo-AL group (Figure 3A and B), whereas there was no statistically significant difference in the mRNA levels of these two E3-ligases in the adult control groups and the 30mo-DR group.

Transcriptional Downregulation and ser193 Phosphorylation of FOXO4 May Suppress Expression of Atrogin-1/MAFbx and MuRF1 in Sarcopenic Muscle

To address whether the downregulation of Atrogin-1/MAFbx and MuRF1 in 30mo-AL rats was accompanied by an underlying regulation of the FOXO transcription factors, we analyzed the level of FOXO1, 3 and 4 transcripts (Figure 4A–C). Whereas all three FOXO mRNAs decreased between young adults and middle-aged adults, only FOXO4 showed a significant regulation in senescence. The association of the downregulation of FOXO4 with sarcopenia is supported by the close to normal adult FOXO4 mRNA values observed in 30mo-DR rats (Figure 4C).

The FOXO family of transcription factors is regulated on multiple levels [reviewed in (42)], and an important deactivation mechanism of the FOXO4 protein is ser193 phosphorylation [p(ser193)-FOXO] by which it is excluded from the nucleus and bound to cytosolic proteins (of the 14-3-3 family). Immunoblotting of FOXO4 in crude muscle extracts, enriched in cytosolic proteins, revealed increased levels of FOXO4 proteins in the 30mo-AL group (Figure 4D and E), and western blot analysis of p(ser139)-FOXO4 showed increased levels in 30mo-AL rats only (Figure 4F and G).

Increased PI3K and p-AKT Associates With FOXO4 Serine Phosphorylation in Senescence

Because PI3K-AKT deactivates FOXO through ser-thr phosphorylation [reviewed in (27,28)], we determined whether changes in PI3K and AKT associated with the increased levels of p(ser193)-FOXO4 in senescence. Western blot revealed that this was the case for both p85, the regulatory subunit of PI3K (Figure 5A and B), and AKT (Figure 5C and D). Analysis of levels of ser-473-phosphorylated AKT [p(ser473)-AKT] revealed increased levels of p(ser473)-AKT in aged AL animals (Figure 5E–G) and that the relationship of phosphorylated AKT to total AKT remained the same in all AL groups (ratio p-AKT/AKT was not significantly different; data not shown). It is interesting that AKT protein was increased in the 30mo-DR group; however, the level of p(ser473)-AKT relative to total AKT was lower than that in adult groups (ratio p-AKT/AKT significantly different compared to all AL groups; all p values $< .001$). Also, the levels of PI3K protein were lower in the DR group (Figure 5A–G).

IGF1R-ShcA-Grb2-GAB Pathway Is Upregulated in Sarcopenic Skeletal Muscle, a Regulatory Pattern Impeded by DR

In skeletal muscle, PI3K-AKT can be activated by insulin or IGF-1 signaling via their cognate receptors (IR and IGF-1R, respectively) and the subsequent interaction with either IRS-1 or the Shc-GRB2-GAB adaptor protein pathway (Figure 1) [reviewed in (28–30)]. Available evidence argues strongly against any increase of muscle IR and IRS-1 proteins during aging (32–35), and consistent with this we observed only a minor change in IR protein in the 30mo-AL group (Figure 6C and D). However, levels of IGF-1R protein (Figure 6A) were markedly elevated in the 30mo-AL group (Figure 6B). Extending our earlier findings of an upregulation of ShcA adapter protein in aged skeletal muscle (31), we hypothesized that the alternative pathway ShcA-Grb2-GAB was utilized to activate AKT in

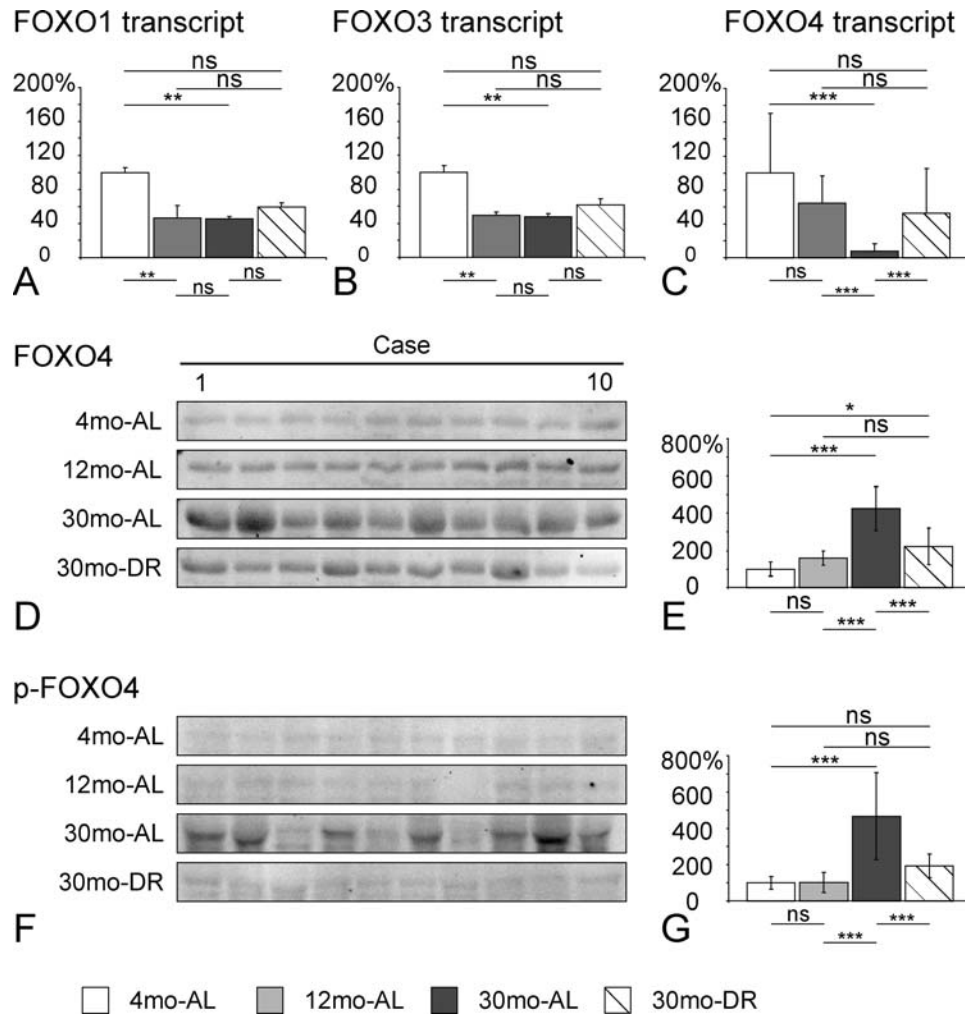


Figure 4. Results of real-time polymerase chain reaction and western blot analysis on gastrocnemius muscle of adult 4- and 12-month-old and aged 30-month-old ad libitum-fed (AL) rodents, as well as aged 30-month-old rodents on dietary restriction (DR). Relative messenger RNA (mRNA) levels of forkhead box O (FOXO) 1 (A), FOXO3 (B), and FOXO4 (C) are plotted relative to those of the 4-month-old AL group (set to 100%). Results of statistical analysis of FOXO4 (E) and ser 139 phosphorylated FOXO4 (p-FOXO4) (G) protein levels are plotted relative to those of the 4-month-old AL group (set to 100%). Panels show representative FOXO4 (D) and p-FOXO4 (F) western blot results from individual analysis of all cases from the four groups run in parallel. All statistical testing was performed using analysis of variance and Bonferroni's post hoc test; the results of the latter are indicated by horizontal bars, asterisks ($p < .05$; $^{**}p < .01$; $^{***}p < .001$), or "ns" when nonsignificant. Error bars represent standard deviation.

sarcopenia. First, we confirmed that ShcA was upregulated in the skeletal muscle (Figure 7). Second, we examined the levels of GRB2 and GAB1/GAB2 by western blot (Figure 8A, C, and D). Whereas Grb2 increased with age, being most abundant in 30mo-AL rats, high GAB1 and 2 levels were exclusively seen in 30mo-AL rats (Figure 8, A–E). The data suggest that increased signaling through IGF-1R (and possibly through other receptor tyrosine kinases [RTKs]) via the ShcA-Grb2-GAB1/2 pathway may be responsible for the increased levels of p(ser473)-AKT in skeletal muscle from aged AL rats.

In the 30mo-DR group, the levels of ShcA p46 and p52 isoforms (Figure 7, A, C, and D) were similar to those recorded in adult rats, whereas the p66 isoform was only partially normalized by DR (Figure 7A and E). It was also noted that, although significantly reduced by DR, the levels

of IGF-1R and GRB2 of old DR animals were increased relative to the 4- and 12-month-old AL groups (Figure 6C and D; Figure 8A and B). However, DR effectively counteracted the upregulations of GAB1/2, and the DR animals were indistinguishable from 4- and 12-month-old animals in this respect (Figure 8C–E).

DISCUSSION

In skeletal muscle the aging process is made obvious through the relationship between aging and loss of skeletal muscle mass. The notion of a general mechanism underlying all skeletal muscle atrophy is appealing, and recent advances in this field have identified the FOXO-regulated ubiquitin E3-ligases Atrogin-1/MAFbx and MuRF1 (9,10,12–18) as common in muscle atrophy caused by a range of etiologies.

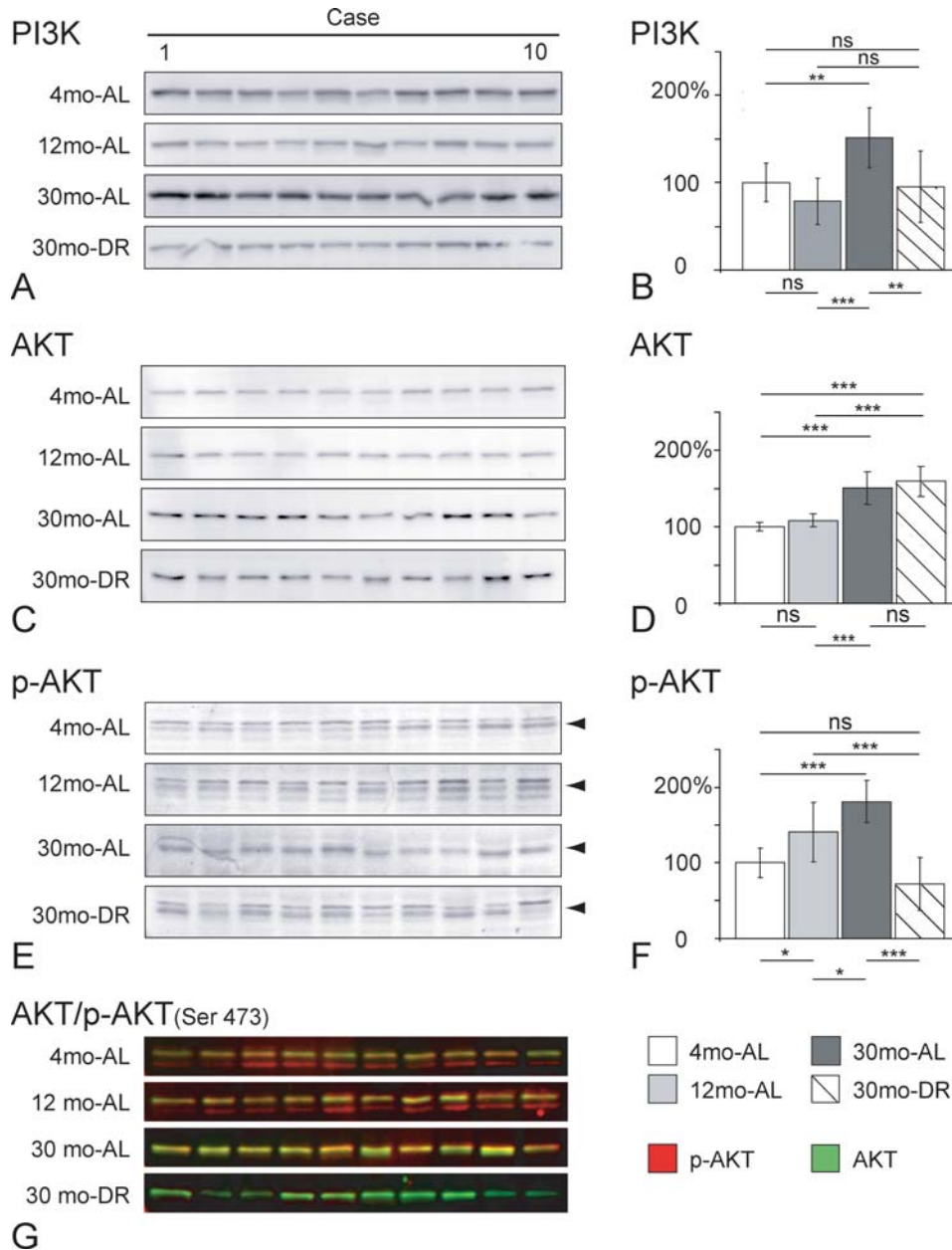


Figure 5. Relative protein levels of p85 phosphatidylinositol 3 kinase (PI3K), AKT (protein kinase B [PKB]), and ser473-phosphorylated AKT (p-AKT) in gastrocnemius muscle of adult 4- and 12-month-old and aged 30-month-old ad libitum-fed (AL) rodents, as well as aged 30-month-old rodents on dietary restriction (DR). **A, C, E, and G**, Representative western blot results from individual analysis of all cases from the four groups run in parallel. **B, D, and F**, Results of statistical analysis of protein levels are plotted relative to the 4-month-old AL group (set to 100%). **G**: To illustrate the relative phosphorylation of AKT, a color overlay was created with p-AKT and AKT in the red and green channels, respectively. To create the overlay, membranes were stripped after p-AKT detection and reprobbed with AKT antibody. The resulting scans were placed in separate channels (p-AKT, red; AKT, green) in a composite color image (RGB). The similar expression levels of p-AKT and AKT in AL animals result in color blending to produce yellow, and the lack of p-AKT in DR is seen as an unaltered green signal. The unspecific p-AKT bands, which do not align with AKT in the overlay, remain red [c.f., E]. Ratios (p-AKT/AKT) were not different between AL animals, but were significantly lower in the DR group compared to each of the AL groups ($p < .001$; data not shown). Statistical testing was performed using analysis of variance and Bonferroni's post hoc test; the results of the latter are indicated by horizontal bars, asterisks ($*p < .05$; $**p < .01$; $***p < .001$) or "ns" when nonsignificant. Error bars represent standard deviation.

In this study, we provide evidence to reject the hypothesis that sarcopenia associates with an increase of Atrogin-1/MuRF1 and MuRF1 and conclude that sarcopenia is an entity of its own. In addition, our data show that DR attenuates the progression of sarcopenia and counteracts the effect of aging on the pathways studied here.

Suppression of Atrogin-1/MuRF1 and MuRF1 in Sarcopenia

As mentioned earlier in the text, analysis of aged male m. vastus lateralis showed no change in the expression of Atrogin-1/MuRF1 or MuRF1 mRNA (19,20). However, in the rat, Scott-Pattison and colleagues (21) reported on

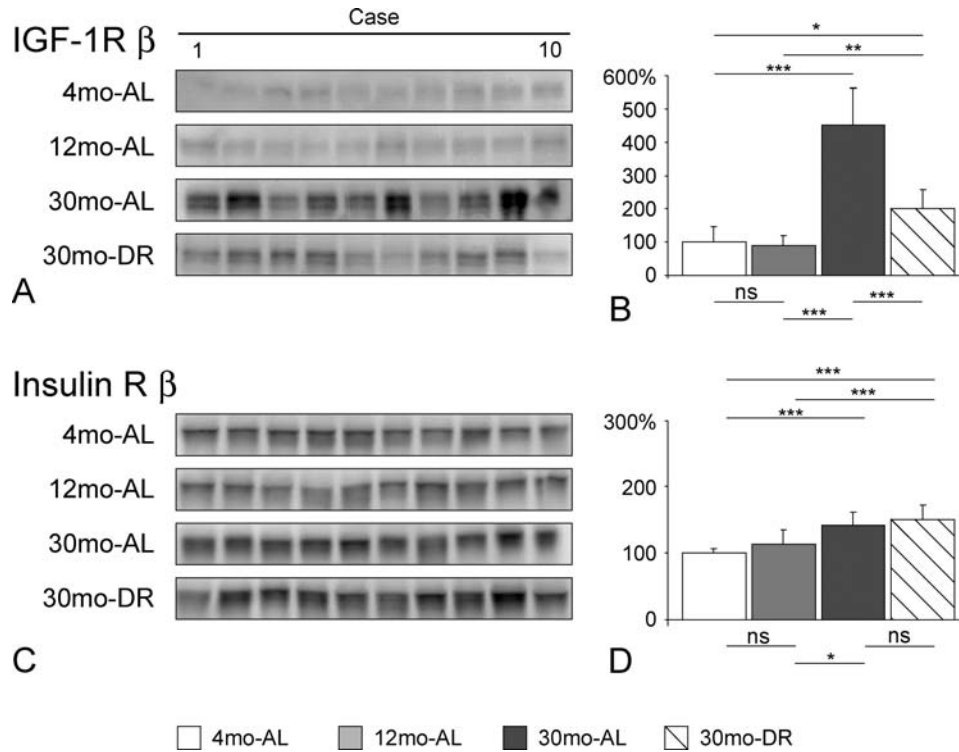


Figure 6. Relative protein levels of the insulin-like growth factor-1 receptor (IGF-1R, β -subunit) and insulin receptor (IR, β -subunit) in gastrocnemius muscle of adult 4- and 12-month-old and aged 30-month-old ad libitum-fed (AL) rodents, as well as aged 30-month-old rodents on dietary restriction (DR). **A** and **C**, Representative western blot results from individual analysis of all cases from the four groups run in parallel. **B** and **D**, Results of statistical analysis of protein levels are plotted relative to the 4-month-old AL group (set to 100%). Statistical testing was performed using analysis of variance and Bonferroni's post hoc test; the results of the latter are indicated by horizontal bars, asterisks ($*p < .05$; $**p < .01$; $***p < .001$), or "ns" when nonsignificant. Error bars represent standard deviation.

a small ($< \times 2$) but significant increase of this E3 ligase in soleus muscle of aged male (30- to 31-month-old) Fisher344 \times Brown Norway F_1 rats, whereas DeRuisseau and colleagues (22) found a significant downregulation of MuRF1 mRNA but no significant change in Atrogin-1/MAFbx mRNA in the soleus muscle of aged male (26-month-old) Fisher 344 rats. The reason for these discrepancies is unclear but may relate to differences in the muscles, as well as in the species and strains chosen for study. With respect to human muscle, it is interesting to note that Welle and colleagues (43) did not report on any increase in Atrogin-1/MAFbx mRNA in the analysis of aged female vastus lateralis, and because the present study was conducted on female SD rats, the possibility of a gender difference could be argued. However, in related work we find a similar downregulation of Atrogin-1/MAFbx and MuRF1 in sarcopenic male SD rats (Altun M, Edström E, Kessler B, Ulfhake B, unpublished observations). Furthermore, in line with the study of DeRuisseau and colleagues on male rats (22), we not only observed a downregulation of MuRF1 but also a decrease of E2_{14K} mRNA in the gastrocnemius muscle of the aged female SD rats (our unpublished observations).

The downregulation of both Atrogin-1/MAFbx and MuRF1 in sarcopenic gastrocnemius muscle found here suggests an active, possibly compensatory, regulatory mechanism in senescence. Atrogin-1/MAFbx and MuRF1 are regulated by the FOXO family of transcription factors

(13,15–17), and FOXO activity is, in turn, controlled on multiple levels [reviewed in (27,42)]. In skeletal muscle, FOXO4 is claimed to be the most abundantly expressed member of the FOXO family of transcription factors (44). Our data show that FOXO4 transcription is suppressed in sarcopenic muscles of ad libitum-fed rats whereas transcription of FOXO1 and FOXO3 do not change significantly during aging beyond 12 months of age (in gastrocnemius muscle).

FOXO proteins are also regulated by phosphorylation [reviewed in (27,42)], and inactivation occurs by phosphorylation at three sites, where ser193 seems to be the key site for exclusion of FOXO4 from the nucleus (45–47). Our findings of elevated levels of p(ser193)-FOXO4 in aged AL rats (Figure 4), in parallel with increased total protein in the cytosol-enriched lysates analyzed (Figure 4), indicate that in aged rats a larger fraction of FOXO4 is localized to the cytoplasm in an inactive phosphorylated state unable to promote transcription of Atrogin-1/MAFbx and MuRF1. Combined, our data suggest a two-level (transcription and deactivation) regulation of FOXO4 in sarcopenic muscle, a regulatory pattern which we show that DR impedes. Even modest DR (70% of ad libitum) attenuates sarcopenia, and despite considerable variation among the aged DR animals, the SI of this group was significantly improved compared to that of AL animals (Figure 2). The transcriptional downregulation of Atrogin-1/MAFbx and MuRF1 noted in the

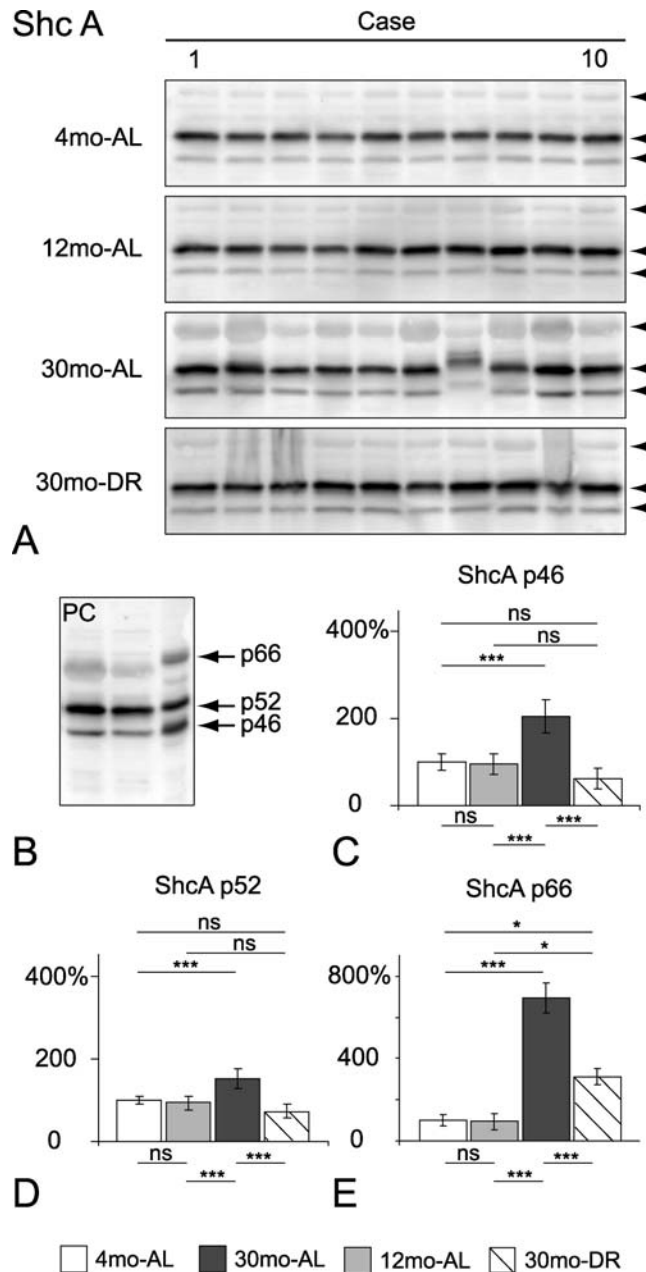


Figure 7. Relative protein levels of Src homology 2 domain containing protein A (ShcA) isoforms in gastrocnemius muscle of adult 4- and 12-month-old and aged 30-month-old ad libitum-fed (AL) rodents, as well as aged 30-month-old rodents on dietary restriction (DR). **A**, Representative western blot results from individual analysis of all cases from the four groups run in parallel. **B**, ShcA p46, p52, and p66 isoforms are all detected by the antibody: positive control lysate (PC, A431 lysate, Upstate Biotechnology) run alongside two samples. Results of statistical analysis of ShcA p46 (**C**), p52 (**D**), and p66 (**E**) protein levels are plotted relative to the 4-month-old AL group (set to 100%). All statistical testing (C–E) was performed using analysis of variance and Bonferroni's post hoc test; the results of the latter are indicated by horizontal bars, asterisks (* $p < .05$; ** $p < .01$; *** $p < .001$), or "ns" when nonsignificant. Error bars represent standard deviation.

ad libitum-fed aged animals was absent in the aged DR group (Figure 3). Combined, the data showing that DR impedes sarcopenia and abolishes the age-related down-regulation of both Atrogin-1/MAFbx and MuRF1 underscores the specificity of these regulatory changes in senescence; consistent with a role for FOXO4 in these regulatory changes, phosphorylation-inactivated FOXO4 was significantly lower in DR than in aged AL animals.

Is IGF-1R/p-AKT Underlying ser193 Phosphorylation of FOXO4 in Senescence?

PI3K-AKT is a major component responsible for deactivation of FOXO through ser-thr phosphorylation [reviewed in (3,27,28)], and in this study changes in PI3K and AKT were associated with the increased levels of p(ser193)-FOXO4 in senescence. PI3K mediates phosphorylation of AKT (48,49), and in AL animals levels of

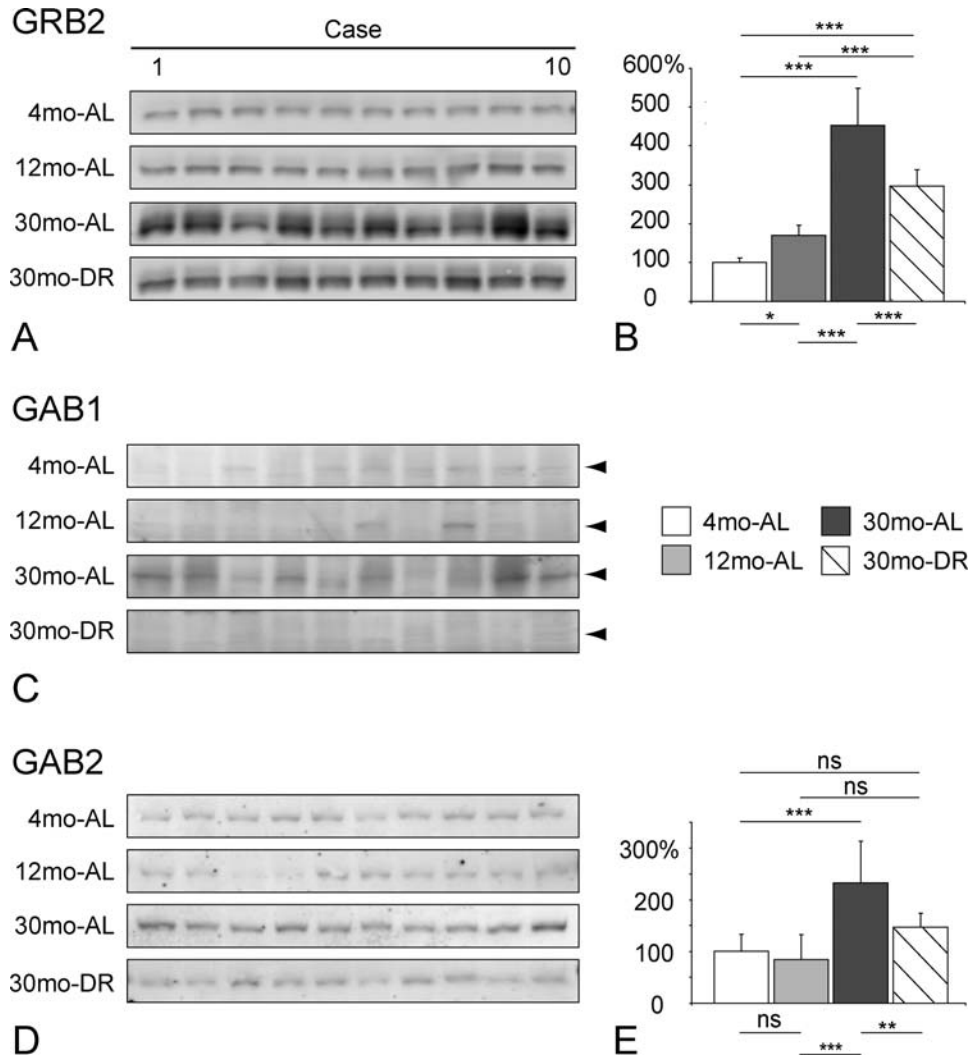


Figure 8. Relative protein levels of growth factor receptor bound protein-2 (GRB2), and GRB2-associated binding proteins 1 and 2 (GAB1 and GAB2) in gastrocnemius muscle of adult 4- and 12-month-old and aged 30-month-old ad libitum-fed (AL) rodents, as well as aged 30-month-old rodents on dietary restriction (DR). **A**, **C**, and **D**, Representative western blot results from individual analysis of all cases from the four groups run in parallel. **B** and **E**, Results of statistical analysis of protein levels are plotted relative to those of the 4-month-old AL group (set to 100%). Statistical testing was performed using analysis of variance and Bonferroni's post hoc test; the results of the latter are indicated by horizontal bars, asterisks ($*p < .05$; $**p < .01$; $***p < .001$), or "ns" when nonsignificant. Error bars represent standard deviation.

p(ser473)-AKT increased in proportion to total AKT with aging (Figure 5E–G).

A significant route by which PI3K-AKT is activated in skeletal muscle is via insulin or IGF-1, signaling through their RTKs, and their subsequent interaction with either IRS-1 or the Shc-GRB2-GAB adaptor protein pathways (Figure 1) [reviewed in (28–30)]. Available evidence argues strongly for unchanged or decreased IR activation or RTK-mediated phosphorylation of IRS-1 in aging (32–35). In this study, a small age-related increase in IR protein was found. Because the increase was evident already in the 12-month-old, as well as the DR animals, we do not associate this regulation with sarcopenia. In contrast, the IGF-1R was dramatically increased in aged AL animals, suggesting a relationship to sarcopenia (Figure 6A and B). IGF-1R activation of PI3K-AKT is of particular interest in the

balance between skeletal muscle anabolism and catabolism because AKT can promote muscle hypertrophy via other downstream targets such as the mammalian target of Rapamycin (mTOR) and glycogen synthase kinase 3 (GSK3); thus, this pathway can both decrease protein degradation and promote protein synthesis (3,14,17,28). However, there is no evidence for increased protein synthesis in senescence [recently reviewed in (5)], and considering the number of potential AKT targets, the activity of AKT may well be context dependent [reviewed in (50–52)].

Over the past few years focus has shifted from circulating IGF-1 to locally produced (autocrine or paracrine) IGF-1, because transgenic models show that liver-derived IGF-1 is dispensable (53), whereas local overproduction of IGF-1 promotes muscle anabolism and counteracts myofiber atrophy [reviewed in (28); see also (54,55)]. Independent

experiments corroborate that increased levels of IGF-1, PI3K, or AKT can suppress muscle atrophy in a variety of experimental paradigms mimicking clinical conditions, through decreased Atrogin-1/MAFbx and MuRF1 expression (13,15,16). In agreement with these findings, local overexpression of IGF-1 suppresses muscle atrophy via inactivation of FOXO4 and downregulation of the muscle-specific E3 ligases (24). Levels of IGF-1 and IGF-1R transcripts in skeletal muscle are unaltered, or elevated, during aging (37,56); here we provide evidence that suggests increased IGF-1R signaling by showing that aged sarcopenic muscle contains higher levels of IGF-1R protein. Although other RTKs cannot be excluded, the data suggest that the IGF-1R may be responsible for the increased levels of p(ser473)-AKT in skeletal muscle from aged AL rats. As discussed above, IRS-1 probably does not contribute to an increased IGF-1 signaling in senescence; however, receptor autophosphorylation and downstream signaling may be conveyed, and even amplified, via the ShcA-GRB2-GAB1/2 adapter protein pathway (29,30). It may be speculated that the existence of multiple signaling pathways, as well as different isoforms of intercalated adapter proteins (see below), may play a role in the specification of the downstream signaling in the case of a pleiotrophic cytokine such as IGF-1 [which promotes progenitor cell activation and proliferation, precursor cell differentiation and maturation from the replicative state, as well as anabolism of the fully differentiated myofiber [see, e.g., (3,57)].

Although the findings obtained here with increased expression of IGF-1R and ShcA-GRB2-GAB in sarcopenic muscle may explain the phosphorylation state of AKT, the mechanism sustaining increased AKT levels remains unclear. Available data support a role for increased AKT phosphorylation not only in muscle hypertrophy (58), but also in recovery after catabolic or atrophic conditions (59). Skeletal muscle recovery involves regenerative programs, which include the activation, proliferation, and differentiation of satellite cells (57), and sarcopenic muscle also shows many of the hallmarks of muscle regeneration [(37), and references therein]. In skeletal muscle, the transcription factor MyoD seems to interact with AKT in a positive feedback loop during progenitor cell differentiation (60,61). Because high expression levels of MyoD are seen in sarcopenic skeletal muscle (36), the parallel increases of AKT and p(ser473)-AKT may reflect a prolonged regenerative activity in sarcopenia (36). In certain situations, evidence indicates that AKT can switch cell phenotype from a mitogenic to a differentiating by inactivating Raf signaling (62,63).

ShcA Adaptor Proteins Show a Specific Age-Related Regulation, Which Is Only Partially Reversed by DR

Extending our previous data on ShcA regulation in aging (31), we here show that DR completely reverses the increase of the p46 and p52 isoforms of ShcA but not ShcA p66 isoform in senescence. Based on the association of ShcA p66 to cellular oxidative stress responsiveness (64,65), the marked upregulation of this protein during aging has been considered to reflect oxidative stress (31). Given that the most widely recognized benefit of DR is that it reduces

oxidative stress (66), the finding of an upregulation of ShcA p66 but not p46/p52 in DR animals was unexpected. However, Natalicchio and colleagues [(63), see also (64)] recently showed that the ShcA p66 isoform, working in opposition to the p46/p52 isoforms, may play an important role in modulating signaling downstream of the IGF-1R; inhibition of ShcA p66 expression caused cell differentiation or maturation disturbances *in vitro*. Thus, the expression of ShcA p66 may reflect myofiber differentiation in the regenerative response to sarcopenia [see also (37)]. Certainly, more work is needed to resolve the precise role of the different ShcA isoforms in tyrosine kinase signaling and the possible switches in the signaling downstream of the IGF-1R.

Conclusion

Given that loss of muscle mass is one of the most prevalent signs of mammalian aging and, still, sarcopenic muscle shows signs of regeneration and suppression of the signaling pathways mechanistic in disease- and disuse-induced muscle atrophy, we may ask what mechanism causes the net loss of muscle tissue in senescence. The finding of Atrogin-1/MAFbx and MuRF1 downregulations raises the question of the contribution of proteolysis to sarcopenia. Available data on the expression and catalytic activity of the ubiquitin–proteasome pathway in aged skeletal muscle are conflicting [reviewed in (5,69)] but suggest unaltered or decreased activities (70–73), and evidence for increased proteolytic activity in the calcium-dependent calpain or the lysosomal cathepsin pathways are missing [reviewed in (5)]. A net loss of muscle mass will be the result of a negative balance in the rate of synthesis and the rate of degradation of myofibrillar proteins. There is some evidence indicating that the efficiency of myoprotein synthesis decreases in senescence [reviewed in (5)], however, more work is needed to bring this issue to a close. It is interesting that there is evidence indicating that whereas muscle atrophy induced by glucocorticoid treatment (dexamethasone [DEX]) occurs through increased proteolysis in young adult rats, muscle atrophy in response to DEX in aged rats progresses through decreased synthesis (71). In line with this, we find in related work that DEX treatment does not induce transcription of Atrogin-1/MAFbx or MuRF1 mRNAs in senescent male SD rats (Altun M, Edström E, Kessler B, Ulfhake B, unpublished results). Combined, these observations underscore that loss of muscle mass during aging is mechanistically different from acute muscle atrophy occurring in adults in a variety of clinical situations.

Several lines of evidence suggest that sarcopenia is driven by an imbalance between fiber loss and renewal rather than by myofiber atrophy *per se* (20,37). Evidence in support of increased regenerative activity is found in the increased expression of myogenic regulatory factors (37,75), increased nuclear content, and centrally located nuclei in myofibers (37,39), as well as in recent findings of fine caliber myofibers expressing embryonic myosin in sarcopenic muscle (37). Thus, the increased number of small diameter fibers in aged sarcopenic muscle (2,76) may in part represent an accumulation of regenerating fibers, which fail to compensate for the loss of muscle fibers. Thus, regeneration

itself may be failing or outmatched by a loss of fibers more extensive than hitherto appreciated.

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