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Homer 2 antagonizes protein degradation in slow-twitch skeletal muscles

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ABSTRACT

Homer represents a new and diversified family of proteins made up of several isoforms. The presence of Homer isoforms, referable to 1b/c and 2a/b, was investigated in fast- and slow-twitch skeletal muscles from both rat and mouse. Homer 1b/c was identical irrespective of the muscle, Homer 2a/b was instead characteristic of the slow-twitch phenotype. Transition in Homer isoform composition was studied in two established experimental models of atrophy, i.e., denervation and disuse of slow-twitch skeletal muscles of the rat. No change of Homer 1b/c was observed up to 14 days after denervation, whereas Homer 2a/b was found to be significantly decreased at 7 and 14 days after denervation by 70% and 90%, respectively, and in parallel to reduction of muscle mass; 3 days after denervation, relative mRNA was reduced by 90% and remained low thereafter. Seven-day hind-limb suspension decreased Homer 2a/b protein by 70%. Reconstitution of Homer 2 complement by *in vivo* transfection of denervated soleus, allowed partial rescue of the atrophic phenotype, as far as muscle mass, muscle fiber size and ubiquitinazion is concerned. The counteracting effects of exogenous Homer 2 were mediated by down-regulation of MuRF1, Atrogin and Myogenin, i.e., all genes known to be up-regulated at the onset of atrophy. On the other hand, slow-to-fast transition of denervated soleus, another landmark of denervation atrophy, was not rescued by Homer 2 replacement. The present data show that: a) down-regulation of Homer 2 is an early event of atrophy, and b) Homer 2 participates in the control of ubiquitinization and ensuing proteolysis via transcriptional down-regulation of MuRF1, Atrogin and Myogenin. Homers are key players of skeletal muscle plasticity and Homer 2 is required for trophic homeostasis of slow-twitch skeletal muscles.

Homer; denervation; disuse; atrophy, protein degradation; slow-twitch skeletal muscles

INTRODUCTION

A novel activity-dependent control of multi-protein signalling complexes has been shown in neuronal synapses and is mediated by a family of proteins termed Homers. Homers assemble proteins such as metabotropic glutamate receptors (mGluRs), transient receptor potential channels (TRPCs), intracellular Ca^{2+} channels, e.g., ryanodine receptor (RyR), inositol (1,4,5)-trisphosphate receptor (IP_3R), scaffolding proteins, e.g., Shank, small GTPases, transcription factors and cytoskeletal proteins (see for a review 10). Homer 1b/c, 2a/b, 3a/b, known as constitutive isoforms, have been reported to cross-link multimers via the coiled-coil (CC) domain (17, 52), whereas Homer 1a has been considered an immediate early gene (IEG) product (4) able to antagonize CC-Homers. Although doubts have been cast upon the unequivocal role of Homer 1a (48), Homers can be regarded as regulators of multimeric complexes involved in signal transduction.

In skeletal muscle, Homers have been detected at the protein (38, 39, 43, 44, 51) and mRNA levels (40, 44, 51). The regulatory equilibrium between constitutive Homers and up-regulated Homers could be particularly relevant not only in resting conditions, but also during muscle adaptation to exercise and environmental stress. It is worth noting that mechanical (low-voltage electrical stimulation or low-frequency fatigue), metabolic (hypoxia) and hormonal stimuli, all appear to be associated with induction of a number of IEGs, such as *c-fos, c-jun*, *egr-1* and HSPs (13). Post-natal differentiation of striated muscles and attainment of fiber-type specificity - fast-*versus* slow-twitch skeletal muscles - entail complex genetic programs. Adaptive muscle changes entailing structural rearrangement and morphological remodeling, e.g., development, hypertrophy and regeneration, are processes coupled to up-regulation of several, distinct IEGs (14, 24), and, at least in the case of skeletal muscle regeneration, we recently reported a switch in Homer isoform composition, Homer 1a and Homer 3 being selectively up-regulated (3).

Homer 2 enhances transcription of several slow-twitch muscle-specific genes, including myoglobin and slow troponin I promoter, via RyR/NFAT activation, according to Stiber et al. (46).

Expression and accumulation of Homer 2 at the NMJ of human skeletal muscle fibers are modulated by neuronal activity, according to Salanova et al. **(**39***).*** Thus, the rationale of the present paper was to ascertain whether Homer 2 plays a causative role in the atrophy process and in fiber type specialization of slow-twitch skeletal muscles, using *in vivo* models of skeletal muscle atrophy, i.e., denervation and disuse.

Here, we report that: a) Homer 2 is present only in slow-twitch skeletal muscle fibers, b) denervation and disuse determine almost complete disappearance of Homer 2 at the protein and mRNA level, c) Homer 2a/b expression is motor nerve-dependent, and d) *in vivo,* acute re-introduction of Homer 2, via cDNA electrotransfer, partially rescues the denervation atrophy phenotype, as judged by effects on muscle mass, fiber size and ubiquitinazion, but does not revert the slow-to-fast transition. Based on present and recent data, it appears that transition in Homer composition is relevant in skeletal muscle adaptation. In particular, Homer 2 is causally involved in atrophy of slow-twitch skeletal muscles, transcriptionally down-regulates key atrophy genes and exercises a negative feedback control on protein degradation.

MATERIALS and METHODS

Tissue sources

All experiments were carried out on adult male Wistar rats (~ 250 g of body weight) and CD1 mice (~ 45 g of body weight) except those for disuse atrophy (see below). For denervation and regeneration experiments, rats were anaesthetized with intra-peritoneal injection of ketamine (1.5 mg/100 g), subjected to appropriate surgical procedures, and killed by cervical dislocation at specified time points. Experimental protocols have been approved by institutional review boards. Soleus, a representative, predominantly slow-twitch skeletal muscle, and *Extensor Digitorum Longus* (EDL) and *Tibilais Anterior* (TA), representative predominantly fast-twitch skeletal muscles, were used. For comparative purposes, in some experiments, we also used soleus and *Adductor Magnus* from New Zealand white rabbits; by conventional criteria such muscles are deemed pure slow-twitch and pure fast-twitch skeletal muscles.

Antibodies and cDNAs

The following primary antibodies were used for Western Blots: polyclonal antibodies specific for either Homer 1b/c from Chemicon, Homer 1a or Homer 2a/b from Santa Cruz Biotechnology; monoclonal antibodies specific for ubiquitinylated conjugates and anti-V5 from Enzo Life Sciences and from Invitrogen, respectively. Goat anti-rat IgG (Sigma), rabbit anti-goat IgG (Sigma) and goat anti-mouse IgG (Sigma) conjugated to alkaline phosphatase were used as secondary antibodies.

cDNA coding for V5-tagged Homer 2 was generated as described in Stiber et al. (46).

Denervation of hind-limb skeletal muscles of the rat

The sciatic nerve was cut unilaterally at the level of trochanter. About 0.5-1 cm of the peripheral nerve stump was removed in order to obtain a permanent denervation of the lower hind limb. At 1, 7 and 14 days after denervation, rats were sacrificed. Soleus, EDL and TA muscles from the

denervated limb were dissected out, frozen in liquid nitrogen and utilized for further studies, the control muscles being the contralateral, innervated muscles.

Disuse atrophy

The hind limb-unloading experimental protocol was performed following the recommendations provided by the European Convention for the protection of Vertebrate Animals used for Experimental and Scientific purposes (Council of Europe number 123, Strasbourg, 1985) and authorized by the Animal Ethics Committee of the University of Padova and the Italian Health Ministry (103/2007B). Six-week old female Wistar rats (140-160g of body weight) were caged individually. Hind limb muscles were unloaded using the tail-suspension model (7, 16). Each animal was weighted before and after the suspension period. Tail-suspended rats were sacrificed after 7 days of unloading. Soleus muscles were excised, weighted and frozen in liquid nitrogen.

In vivo transfection

Male adult Wistar rats (~ 180 g of body weight) were used. The right soleus muscles were exposed and injected with 0.06 ml of a saline solution containing 50 μg of plasmid cDNAs. Electrodes were applied on both sides of the leg. Electroporation was carried out with a BTX ECM 830 square wave pulse generator using the protocol described by Nori et al. (29), i.e., pulse stimulation by field electrodes (220 V/linear cm) with six 20-msec pulses at 200 ms intervals. The damage evoked by the overall procedure was estimated in 4 distinct soleus muscles, 10 days after experimentation, by counting central nuclei whose presence is index of damage and of ensuing regeneration. Several transverse sections of each soleus were examined and the number of central nuclei/100fibers ranged from 0.02 to 0.12. Thus, the extent of damage was deemed to be irrelevant.

Denervation, where applicable, was carried out immediately after electroporation.

Homogenates from soleus and EDL skeletal muscles of rat and mouse

Homogenates of skeletal muscles were obtained as follows: frozen tissues were triturated in a mortar, homogenized with Polytron for 10 sec at 20,000 RPM in 10 volumes of 3% SDS, 1 mM EGTA, 20 μm phenylmethanesulphonyl fluoride, 0.8 mM benzamidine, boiled for 5 minutes and centrifuged at 18,000 g for 30 min in order to remove debris. Homogenates were kept at -20° C until use. Protein concentration was determined according to Lowry et al. (20).

SDS-Polyacrylamide Gel Electrophoresis (PAGE), Western Blot and quantitative densitometry

SDS-PAGE on either 7.5% (in the case of Homer 1 b/c), 12.5% (in the case of Homer 1a) or 5-10% linear gradient gels (in the case of Homer 2 a/b), transfer to nitrocellulose and Western blot were carried out essentially as previously described (29).

Homers were quantified by densitometry of immunoblots of homogenates. Densitometric analysis was performed with Scion Image for Windows, version Beta 4.0.2 (Scion Corp., Frederick, MD; www.scioncorp.com). Each value for Homer isoforms is given as ratio to that of the corresponding contralateral sample.

RNA extraction and quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from either muscles or thin-sections using TRIzol® (Invitrogen) extraction method or RNeasy Micro Kit (Qiagen) according to the manufacturer's instructions. RNA was eluted in RNase-free water and stored at -80°C until use. The concentration and quality of each sample were measured by NanoDrop (Thermo Scientific). 400 ng of RNA were converted to cDNA by using random hexamers and SuperScript II RT (Invitrogen) following the manufacturer's instructions. RT was performed in a thermal cycler (Applied Biosystems, Foster City, CA): 25°C for 10 min, 42°C for 60 min, 95°C for 5 min, and 4°C for 5 min. All RNA samples were simultaneously converted to cDNA in order to minimize technical variability. Negative controls (no RNA or no reverse transcriptase enzyme) were simultaneously run for RNA and genomic DNA contamination.

Primers for qPCR were already published or designed (*) using Primer3 software (http://frodo.wi.mit.edu/, Whitehead Institute for Biomedical Research) and their thermodynamic specificity was determined using BLAST sequence alignment (NCBI) and vector NTI® (Invitrogen) software. All primers were purchased from Eurofins MWG Operon. Primer sequences were as follows:

HOMER1*	Fw	GCACATATGAGTAAGACCTTCCTGAC
	Rv	AGCTACAGTAGGCACACAACC
HOMER2*	Fw	TCTTGCTTCTCTGGCTTTGT
	Rv	CTGCGTAAACGGCTAAGGTA
ATROGIN (Fbxo32)	Fw	GCAAACACTGCCACATTCTCTC
(23)	Rv	CTTGGGGTGAAAGTGAGACG
MuRF1 (Trim63)*	Fw	ACCTGCTGGTGGAGAACATC
	Rv	CTTCGTGTTCCTTGCACATC
MYOGENIN*	Fw	GTACCCAGTGAATGCAACTCC
	Rv	ACGATGGACGTAAGGGAGTG
MYOSIN Myh2* (fast 2A)	Fw	TGAAGAGTAAGGCAGCTCTG
	Rv	GAATCACATGGGGACATGAC
MYOSIN Myh7 (β slow)	Fw:	TATGCTGGAGCTGATGCAC
(37)	Rv:	GGACACGGTCTGAAAGGATG
MYOSIN MyH1* (2x)	Fw	CAGGGTCCGTGAACTTGAAGGA
	Rv	GGTCCTGGAGCCTGAGAACG
PGC1 α (23)	Fw	GGAATGCACCGTAAATCTGC
	Rv	TTCTCAAGAGCAGCGAAAGC
TBP1 (37)	Fw	TCAAACCCAGAATTGTTCTCC
	Rv	AACTATGTGGTCTTCCTGAATCC

199 PPIA, Peptidylprolyl isomerase A, (41)

200 Fw AGCATGTGGTCTTTGGGAAGGTG

201 Rv CTTCTTGCTGGTCTTGCCATTCC

202

203 qPCR was performed in duplicate in a 96-well IQ5 Thermal Cycler (Bio-Rad) using SYBR
204 Green chemistry. PPIA, TBP1 and GAPDH were tested as putative reference genes, being PPIA
205 and TBP1 the most stable genes to normalize C_T values. All samples were run simultaneously with
206 RNA- and RT-negative controls. The efficiency of each run was determined by a standard curve.
207 Normalization was performed by delta C_T method. Data are expressed as means ± SE. Comparisons
208 were made by using t-test.

209

210 *Dual luciferase assay on rat soleus*

211 Promoter activity of the slow MHC was measured using the Dual Luciferase reporter assay
212 system (Promega) and luciferase activity was determined with an analytical luminometer. Relative
213 luciferase units were calculated by determining the ratio of the intensity of the light produced by the
214 firefly luciferase reporter plasmid to that produced by the *Renilla* luciferase pRL-TK plasmid.

215 Soleus muscles were injected with 60 µl of 0.9% NaCl, containing 50 µg of firefly luciferase
216 reporter construct, and 5 µg of pRL-TK vector, and electroporated as described above. SOL
217 muscles were homogenized in 0.5 ml of passive lysis buffer (Dual Luciferase reporter assay system;
218 Promega) and luciferase activity was determined.

219

220 *Confocal immunofluorescence*

221 For soleus muscles, 6 µm longitudinal and transverse sections were obtained and incubated with
222 primary antibodies at room temperature for 60 min as described (29). After extensive washing,
223 muscle sections were incubated for 30 min with either Cy2-conjugated anti-mouse (Chemicon) or

rhodamine isothiocyanate anti-rabbit antibodies (Dako). Analysis was carried out in a Leica W5 microscope.

Statistical analysis

One-way ANOVA was used to evaluate the changes over time for both relevant proteins and muscles wet weight. All values are means ± SE. Post hoc comparison were performed with Fisher's protected least significance difference test. Dunnet test was applied to compare each time point value with the post-denervation day 1 value. Correlations between changes in Homer isoforms and type 1 MHC content were assessed by linear regression analysis. Individual means were compared with a paired 2-tailed *t*-test. Differences were considered statistically significant at the 0.05 level of confidence.

RESULTS

Expression of Homer 1 and 2 isoforms in skeletal muscles

Representative slow-twitch (soleus) and fast-twitch (EDL) skeletal muscles from both adult rat and mouse were analysed for their content in Homer 1b/c and Homer 2a/b (Fig. 1A). Rat, mouse, and, in a few cases, rabbit muscles were used because conflicting results have been reported depending upon species (compare Ref. 51, for rat, with Ref. 43, for mouse).

Western blot analysis was carried out with antibodies specific for Homer 1b/c. The apparent molecular weight of the immunostained proteins (~ 47 kDa) was consistent with the calculated mass of Homer 1b/c (4, 17). Single, unique bands were detected in all lanes and the amount of Homer 1b/c was virtually identical in muscles samples irrespective of tissue (slow and fast muscles) and species (rat and mouse).

Comparison of homogenates from soleus and EDL probed with anti-Homer 2a/b antibodies, indicates that soleus muscles displayed by far the largest amount of Homer 2a/b; in EDL samples, from either rat or mouse, Homer 2a/b was barely detectable. Densitometric analysis of immunostained proteins yields a ratio of about 10:1 for soleus and EDL muscles, respectively (see also legend to Fig. 1A).

Since slow-twitch skeletal muscles contained much larger amounts of Homer 2a/b than fast-twitch skeletal muscles, Homer 2a/b might be referable, in predominantly fast-twitch muscles, to the presence of slow-twitch fibers, i.e., 5-6 % and 4% in EDL of rat (26, 35) and mouse (28), respectively. In fact, when rabbit skeletal muscles were analysed, e.g., soleus and *Adductor Magnus* as pure slow-twitch and pure fast-twitch skeletal muscles, respectively, no detectable Homer 2a/b was found in rabbit *Adductor* (data not shown).

In summary, distribution of Homer 2a/b in skeletal muscles appears to be species-independent and fiber type-dependent. Published discrepancies (43, 51) might be accounted for by

differences in antibody specificity, animal age and type of skeletal muscle, e.g., it was not specified whether fast-twitch or slow-twitch muscles were used (compare Refs. 43, 51).

Expression of Homer 1 and 2 isoforms in denervated soleus of the rat (denervation atrophy)

We next asked whether there are Homer isoforms changes associated to skeletal muscle atrophy triggered by motor denervation. Soleus muscles were collected at 1, 7 and 14 days after denervation (see Materials and Methods for details) and the content of Homer 1a, Homer 1b/c (Fig. 1B) and Homer 2a/b (Fig. 1C) was determined by Western blot of muscle homogenates, and compared to that of innervated, contralateral soleus muscles. Relative levels of Homer 1a/b and Homer 1a, as judged by densitometry, were rather constant over time. Thus, denervation did not significantly affect expression of Homer 1 isoforms (Fig. 1B). On the other hand, expression of Homer 2a/b sharply decreased after 1d, the decrease being significant after 7d; after 14 d, Homer 2a/b was barely detectable (note both Western blot and densitometric data in Fig. 1C). Therefore, denervation virtually abolished fiber type-specific expression of Homer 2a/b. Fig. 1D shows, on the same time-scale, muscle mass decrease, i.e., the extent of muscle atrophy caused by denervation. Application of paired t-test indicates that decrease of either muscle mass or Homer 2a/b was not significant 1 d after denervation. However, slope of Homer 2a/b reduction was steeper, suggesting that Homer 2a/b down-regulation is as an early event in muscle atrophy.

Parallel studies were carried out by RT-PCR analysis. Levels of Homer 1 mRNA were unchanged upon denervation (Fig. 2), whereas Homer 2a/b transcription was quickly turned off. In fact, Homer 2a/b mRNA levels decreased sharply by about 40% within 24 hours (not shown), attained a residual 10% by post-denervation day 3 and remained constant and low until post-denervation day 14 (Fig. 2).

Sharp decrease of Homer 2a/b in rat soleus following hind-limb suspension (*disuse atrophy)*

Soleus muscles were obtained after 7-day hind-limb suspension of the rat, an established model of disuse inducing atrophy and slow-to-fast transition of postural muscles. A significant reduction of Homer 2a/b was observed (Fig. 3), in agreement with mRNA data obtained in similar experimental models (45).

Functional role of Homer 2 in skeletal muscle atrophy: gain-of-function studies

Based on present results and previous studies on different models of atrophy (12, 30-32, 39), it appears that Homers are expressed as part of the myogenic differentiation program, that Homer 2 expression is tissue-specific and that Homer 2 transcription is down-regulated in atrophy. The functional role of Homer in atrophy and skeletal muscle adaptation is unknown and was, thus, directly investigated. The specific issue was whether re-introduction of exogenous Homer 2 could reverse some of the adaptive changes induced by denervation, i.e., whether Homer 2 could rescue, at least in part, the denervation phenotype. To this effect, we relied upon an established model of *in vivo* transient transfection of rat soleus via electroporation (9, 29).

First of all, qualitative expression and sub-cellular localization of recombinant Homer 2-V5 was assessed (Fig.4), upon transient transfection with plasmid cDNAs coding for epitope tagged V5-Homer 2 (pH2-V5). As judged by a series of Western blots of soleus homogenates (Fig. 4A), electroporation of innervated soleus with pH2-V5 resulted in the expression of an exogenous protein recognized by anti-V5 antibodies (lane a) and having an apparent mol wt of about 45 kDa. Innervated soleus electroporated with the empty vector (vector) was negative for V5 reactivity antibodies (lane b). In denervated soleus, only transfection with Homer 2-V5 resulted in reconstitution of an exogenous Homer 2 complement (compare lane c with lane d). Immunofluorescence of longitudinal (Fig. 4B) and transverse (Fig. 4C) sections of rat soleus was carried out with antibodies specific for the V5 tag since no commercially available antibody for Homer 2 is adequate for immunofluorescence. As shown in Fig. 4B, recombinant Homer 2 displayed a regular sarcomeric pattern and localized at the Z line level (panels b and c), away from

the A-I band where E-C coupling takes place. This finding is in perfect agreement with the sarcomeric distribution of endogenous Homer in rat skeletal muscle previously reported by Stiber et al. (46) and Salanova et al. (38, 39). Finally, Fig. 4C is a representative picture of the extent of transfection and shows several transfected fibers. Thus, the electroporation procedure appears to be effective as far as expression and sub-cellular localization of recombinant Homer 2 is concerned.

The next question was whether Homer 2 replacement interferes with muscle mass reduction, a typical, macroscopic landmark of denervation atrophy. Fig. 5A,B shows that exogenous Homer 2 partially counteracted the muscle mass reduction by about 20%. Panel 5A presents data normalized to the animal weight, whereas panel B compares the relative weight of denervated soleus in the absence and presence of exogenous Homer 2.

The subsequent question was whether Homer 2 replacement decreases muscle mass reduction by acting on fiber size. Denervation induced drastic decrease of cross-sectional area (CSA) in both not-trasfected (negative) fibers and transfected (positive) fibers, as shown in Fig. 5C and Fig. 5D, respectively. Homer 2 replacement was able to partially reverse CSA decrease, as shown in Fig. 5D (compare fourth column with third column). Denervated and Homer 2-transfected (EP-Den H2) fibers were on average bigger than denervated and mock-transfected fibers (Fig. 5D) as well as contiguous, not-transfected fibers of the very same muscle (Fig. 5E) by about 30%.

Atrophy is mainly accomplished via activation of pathways for protein degradation. In this framework, the question was whether Homer 2 replacement antagonizes any protein breakdown pathways. As expected and as shown in Fig. 6A, the ubiquitin pathway is activated in denervated soleus. Electroporation *per se* did not change the ubiquitinization pattern, whereas exogenous Homer 2 inhibited ubiquitination by about 60% (Fig. 6B). Fig. 6C shows, in four pairs, that exogenous expression of Homer 2-V5 was comparable in denervated soleus (d) and contralateral non-denervated soleus (c). Thus, Homer 2 appears to exert its effects by counteracting activation of proteolysis.

353 *Homer 2 and transcriptional control in slow-twitch skeletal muscles during atrophy*

354 Ubiquitin-ligases MuRF1, Atrogin (ATR) and Myogenin (MyoG) are genes postulated to be
355 causally linked to initiation of muscle atrophy (21, 42). There are several studies addressing
356 transcriptional control in fast-twitch skeletal muscles, yet incomplete analysis is available for
357 denervated rat soleus (see for review 48). In denervated mouse soleus, MyoG has been recently
358 implied in early events of atrophy via control of Atrogin-1 and MuRF-1 genes (21).

359 Thus, a thorough RT-PCR analysis was carried out to ascertain expression profiles of MuRF1,
360 ATR and MyoG in soleus and TA, a representative fast-twitch skeletal muscle, following
361 denervation (Fig. 7A-C). As expected, MuRF1 was up-regulated in both denervated TA (6) and
362 denervated soleus, although about a 10-fold induction and 2-fold induction were observed at d3 in
363 TA and soleus, respectively (Fig. 7A). ATR and MyoG expression profiles were temporally and
364 qualitatively similar in TA and soleus, although denervation-induced up-regulation was less marked
365 in soleus (Fig. 7B and 7C).

366 Within this framework, the crucial question was whether Homer 2 controls transcription of either
367 MuRF1, ATR or MyoG, and, thus, we investigated the effects of Homer 2 replacement on
368 denervation-induced up-regulation of either MuRF1, ATR or MyoG (Fig. 7D-F). In denervated
369 soleus, at d7 and even more at d14, Homer 2 replacement partially counteracted up-regulation of all
370 three genes causally involved in skeletal muscle atrophy: attenuation was estimated to be about 40%
371 for MURF, 40% for ATR and 30% for MyoG. Since MuRF1 is deemed to be largely responsible for
372 the ubiquitination increase after denervation (6), these findings nicely explain the lower
373 ubiquitination level in Homer 2-transfected denervated soleus (compare Fig. 6).

374

375 *Denervation-induced slow-to-fast transition in soleus: Homer 2 is not involved*

376 Given the proposed role of Homer 2 in human skeletal muscles (39), the effects of Homer 2 were
377 investigated on two peculiar features of denervation-induced slow-to-fast transition in soleus,
378 namely up-regulation of type 2X MHC and down-regulation of mithocondrial biogenesis.

qRT-PCR analysis was carried out in rat soleus and mRNAs referable to either fast 2A, β-slow or 2X MHCs were measured 14 days after denervation in rat soleus. As expected, MHC isoform transition was detected, i.e., fast 2A and β-slow MHCs were drastically down-regulated whereas 2X MHC was sharply up-regulated (Fig. 8A). Upon replacement of Homer 2 via transfection, no significant effect was observed on the expression of MHC isoforms (Fig. 8B). An additional insight was gathered by Dual Luciferase Assay in order to measure directly the promoter activity of the slow MHC (Fig. 8C): as expected, the slow MHC promoter was down-regulated upon denervation; on the other hand, either over-expression of Homer 2 (in control soleus) or Homer 2 replacement (in denervated soleus) did not influence the promoter activity of slow MHC.

PGC1-α, the master gene of mithocondrial biogenesis, is known to be down-regulated in denervation. Homer 2 effects on regulation of mithocondrial biogenesis during denervation of soleus, were monitored by qRT-PCR. Fig. 8D shows that PGC1-α was halved at post-denervation day 14 and that Homer 2 replacement did not counteract such a decrease. Thus, it appears that Homer 2 was not involved in mithocondrial biogenesis. Under the prevailing experimental conditions, Homer 2 does not appear to be involved in the characteristic slow-to-fast transition occurring upon denervation of soleus. On the other hand, the lack of effects of Homer 2 replacement on denervation-dependent slow-to-fast transition, underscores the specific and clear-cut role of Homer 2 in counteracting protein breakdown and muscle atrophy.

DISCUSSION

Homer 2 in slow-twitch skeletal muscle fibers

Tissue differentiation, commitment and maintenance of phenotypic specialization as well as plasticity are long-lasting and intriguing themes of skeletal muscle research. Motor innervation is one of the key factors controlling gene expression in skeletal muscle and the low frequency firing pattern is the paramount determinant for promoting and maintaining the slow-twitch phenotype (13). Ca^{2+} plays a pivotal role as an intracellular messenger: it modulates the expression of specific muscle genes by transducing the tonic motor nerve activity into a program of gene expression specific of slow-twitch fibers (5) and is instrumental in hypertrophy/atrophy of both skeletal and cardiac muscles (5, 27). Homers might be involved in such pathways by acting on either RyR and IP_3R Ca^{2+} release channels (11, 46, 49, 50) or TPRC (53) and, thus, dynamically affecting intracellular Ca^{2+} homeostasis.

Consistent with Rosenberg and Williams (36), who mentioned a higher content of Homer 2a/b in slow oxidative myofibers, the present data clearly show that Homer 2a/b is expressed only in fully differentiated slow-twitch skeletal muscle fibers. Should Homer 1 and Homer 2 modulate intracellular Ca^{2+} homeostasis via gating of both RyR and IP_3R and through the assembly of macromolecular complexes also including TPRC, regulatory mechanisms must be different depending upon the skeletal muscle phenotype. Differential expression of Homer isoforms, as shown by the present communication, would be coherent to this variable regulatory network.

Homer 2 in denervation and disuse atrophy of slow-twitch skeletal muscle fibers

Skeletal muscle atrophy is caused by different aetiological factors, e.g., disuse, denervation, microgravity, starvation, aging, as well as by diseases (cancer cachexia, AIDS, uremia) associated with inflammation (34). Interestingly, a small subset of genes is involved in all atrophy models whereas a larger number of genes is differentially expressed and, thus, specific for each type of

atrophy (18). Denervation provokes not only muscle atrophy and loss of contractile force but also fiber type transition. In denervated rat soleus, both slow-twitch and fast-twitch fibers undergo rapid atrophy -muscle mass reduction- (8) accompanied by slow-to-fast phenotype transition (15, 19). Several biochemical, metabolic and functional features are modified, each with a different time course: full-fledged phenotype transformation occurs over a much longer time scale than atrophy, since it requires several weeks compared to a few days.

In the general context of phenotype determination, plasticity and adaptive responses, the role of Homer isoforms is being unravelled (3, 46), in particular the molecular mechanisms and intracellular pathways are under investigation. The specific biological question addressed by the present work is whether Homer 2 down-regulation is causally involved in denervation-induced atrophy and fiber type specification.

Fourteen days after denervation of rat soleus, Homer 2a/b was found to be almost completely turned off. Sciatic nerve section and withdrawal of the tonic firing pattern of innervating motor neurons bring about, among other functional, biochemical and molecular changes, the rapid and specific disappearance of Homer 2a/b. Since denervation-induced slow-to-fast-twitch transition is well established (15, 30), down-regulation of Homer 2a/b is congruous and unambiguously supports the notion that Homer 2a/b is marker of the slow-twitch phenotype. Under the prevailing experimental conditions, lack of down-regulation of both Homer 1a and Homer 1b/c would indicate that Homer 1 is not a slow-twitch specific marker.

Homer 2a/b decrease might be due, at least in part, to the reduction of the number of the slow-twitch fibers characteristic of denervated soleus: however, such an interpretation does not hold since decrease of Homer 2a/b by far precedes that of type 1 muscle fibers taking place at least 3 weeks after denervation (25, 30). Likewise, it does not appear plausible that Homer 2a/b reduction is due to acceleration of proteolytic processes since Homer 2a/b does not differ from other CC-Homer family members with regard to proteolytic sensitivity (1) and relative content of Homer 1 isoforms was not affected throughout the experimental time course of denervation.

457 The interesting issue is whether and how down-regulation of Homer 2a/b is causally involved in
458 denervation atrophy. The observation that neither Homer 1b/c nor Homer 1a were affected in
459 denervation atrophy and similar findings in age-induced (31) and disuse (32, 39) atrophy, i.e., no
460 other Homer isoform was decreased, rule out the possibility that disappearance of Homer 2a/b is a
461 non-specific epiphenomenon of atrophy. On the other hand, the present data and previous findings
462 by Pattison et al. (32), who reported down-regulation of Homer 2 mRNA in disuse atrophy of rat
463 soleus -about 86 % decrease after 10 days of immobilization-, and by Pattison et al. (31), who
464 reported 40% reduction of Homer 2a/b mRNA in rat soleus muscles derived from aged rats where
465 muscle atrophy is mainly but nor exclusively due to peripheral denervation (2, 33), indicate that
466 neurological and non-neurological atrophic phenomena display selective Homer 2 down-regulation.
467 Both muscle activity and neuro-trophic factors influence molecular, biochemical and mechanical
468 properties of the muscle fiber (13); however, since Homer 2 is down-regulated in atrophic muscle in
469 the presence of intact neuromuscular junctions (31, 39 and Fig. 3), it seems that Homer 2 down-
470 regulation is also due to inactivity *per se*. The search for putative muscle-derived trophic factors
471 controlling post-synaptic Homer 2 expression is worth future investigation.
472
473 *Homer 2 contributes to the trophic control of protein turnover*
474 Gain-of-function studies, implemented *in vivo* by electroporation of plasmid cDNA coding
475 for recombinant Homer 2 (Figs. 4-8), show that Homer 2 replacement in denervated soleus partially
476 rescues the phenotype: in fact, exogenous Homer 2 counteracted decrease of muscle mass, of fiber
477 size and of ubiquitination via down-regulation of specific genes which are known to be up-regulated
478 and to ignite atrophy, e.g., MuRF1, ATR and MyoG. Since MyoG induction in denervated muscle
479 contributes to the development of muscle atrophy via regulated expression of ubiquitin ligases ATR
480 and MURF1 (21), it appears that Homer 2 plays a two-level, negative feedback control in
481 atrophying rat soleus. Mitigation of MuRF1, ATR and MyoG and attenuation of ubiquitination are
482 more pronounced than measured effects on muscle mass and fiber size: the variable extent of

483 denervation phenotype rescue was probably accounted for by differences in parameters being
484 analysed, some of them simple (e.g., mRNA levels), others complex (e.g., muscle mass).

485 Homer 2 is causally involved only in this specific feature of phenotype adaptation, i.e.,
486 muscle trophysm, since the slow-to-fast transition, as judged by the same experimental approach,
487 was not affected (Fig. 8). This finding indirectly confirms the notion that atrophy and fiber type
488 transition are two independent processes occurring during both denervation and disuse.

489 Based on previous findings [i.e., Homer 2 expression during soleus regeneration (3)], it
490 appears that Homer 2 is one of the molecular transducers for trophic control pathways in slow-
491 twitch skeletal muscles. In the overall balance between protein synthesis and proteolysis, it appears
492 that Homer 2 selectively interferes with the latter pathway. As shown here for the first time in
493 denervation atrophy, Homer 2 antagonizes protein degradation via negative transcriptional control
494 of MuRF1 and ATR. Although MuRF1 and ATR also control fiber size in fast-twitch fibers, the
495 present findings indicate that Homer 2-dependent regulation might be restricted, given the fiber
496 type-specific expression, only to slow-twitch fibers.

497 According to recent studies in disuse atrophy, muscle mass reduction is biphasic and due to
498 an early and transient rise in protein breakdown that is followed by a sustained and conspicuous
499 reduction in protein synthesis (22, 34). In this respect, it can be argued that Homer 2 disappearance,
500 as shown here, might determine the shift in balance between protein synthesis and proteolysis in
501 early phases, when protein breakdown is triggered.

502 How does Homer 2 exert its control on either MuRF1, ATR or MyoG thus participating in
503 regulation of slow-twitch muscle trophysm? Does Homer 2 act directly or, more likely, via changes
504 of intracellular Ca^{2+} concentration? According to Pattison et al. (31), down-regulation of Homer 2
505 mRNA in disuse atrophy, may be part of a general adaptive response to "lower Ca^{2+} regulatory
506 mechanisms" in skeletal muscle. Thus, established and putative networks of regulatory mechanisms
507 and intracellular pathways in which interplay between Ca^{2+} and Homer might occur, should be
508 investigated in future work.

In summary, the present paper shows that Homer 2 is a marker of slow-twitch skeletal muscles and antagonizes protein breakdown. Homer 2 plays a role in muscle plasticity and transition between trophic states with up-regulation of Homer 2 favouring growth and down-regulation of Homer 2 favouring atrophy.

GRANTS

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673 **LEGENDS TO FIGURES**

674

675 **Fig. 1. Presence of Homer isoforms in soleus and EDL (F) skeletal muscles (panel A) and**
676 **rapid and conspicuous decrease of Homer 2a/b and of muscle mass in rat denervated soleus**
677 **(model of denervation atrophy).**

678 Panel A: Western blots were carried out with antibodies specific for either Homer 1b/c or Homer 2
679 a/b, on homogenates (200 μg/lane) of predominantly slow-twitch and fast-twitch muscles from both
680 rat and mouse (2 experiments for each muscle). Relative content of Homer 2a/b in EDL and soleus
681 from rat and mouse was determined by densitometry of immunoblots performed with specific
682 antibodies for Homer 2 a/b.

683 Panels B-D: Homogenates were obtained from soleus at specified, post-denervation days, as
684 indicated on the abscissa. Data of Homer 1a and Homer 1a/b content (Panel B) and of Homer 2a/b
685 content (Panel C) were obtained from densitometry of Western blots performed with specific
686 antibodies for each Homer isoform. *Inset* in Panel C: Western blots of soleus homogenates (200 μg)
687 were decorated with antibodies specific for Homer 2a/b; key to lanes: d. denervated, c. contra-
688 lateral control muscle obtained at 1 (1d), 7 (7d) and 14 (14d) post-denervation days. Panel D: Data
689 of muscle mass (wet weight). In panels B-D, data are presented as ratios of denervated muscles over
690 contralateral control muscles, and values are means ± SE; n = 4. **P< 0.01, significantly different
691 from day 1 values. *P < 0.05, denervated vs. contralateral muscles.

692

693 **Fig. 2. Time course of Homer 1 and Homer 2a/b transcripts of rat denervated soleus by qRT-**
694 **PCR.**

695 qRT-PCR was carried out on muscles obtained at post-denervation days 3, 5, 7 and 14 and on
696 contralateral control muscles. Data are normalized to those of the reference gene TBP1 (see
697 Materials and Methods for details) and are given as means ± SE; n=4, *P<0.05.

698

Fig. 3. Rapid decrease of Homer 2a/b in rat soleus after 7-day of hind-limb suspension (model of disuse atrophy).

Western blot of homogenates of soleus (200 μg) were decorated with antibodies for Homer 2a/b. Control soleus muscles were obtained from rats freely housed in cages. Densitometric data are presented as absolute OD values and are means ± SE; n = 4, **P<0.01.

Fig. 4. Re-introduction of Homer 2 in control and denervated soleus by *in vivo* transfection with plasmidic cDNA coding for epitope-tagged Homer 2-V5.

Panel A: Electroporation (EP) and *in vivo* transfection of soleus muscles, with (den) or without sciatic nerve transection, were carried out with either pHomer 2-V5 (H2-V5) or pcDNA3 (vector). Key to lanes: a, electoporated with pHomer 2-V5; b, electroporated with pcDNA3; d, electroporated with pHomer 2-V5 and denervated; e, electroporated with pcDNA3 and denervated. Homogenates (200 μg) were analyzed by Western blots and decorated with antibodies specific for V5. Ponceau red staining of nitrocellulose membranes shows equal protein loading in each lane (loaded). Panel B: Immunolocalization of exogenous Homer 2-V5 following electroporation of rat soleus with pHomer 2-V5. Asterisks denote not transfected fibers. *Inset* from Panel B: a, immunofluorescence, b, phase contrast, c, merge image. Arrows point to the Z-line. Panel C: Transverse sections of soleus transfected with pHomer 2-V5. Bar, 10 μm.

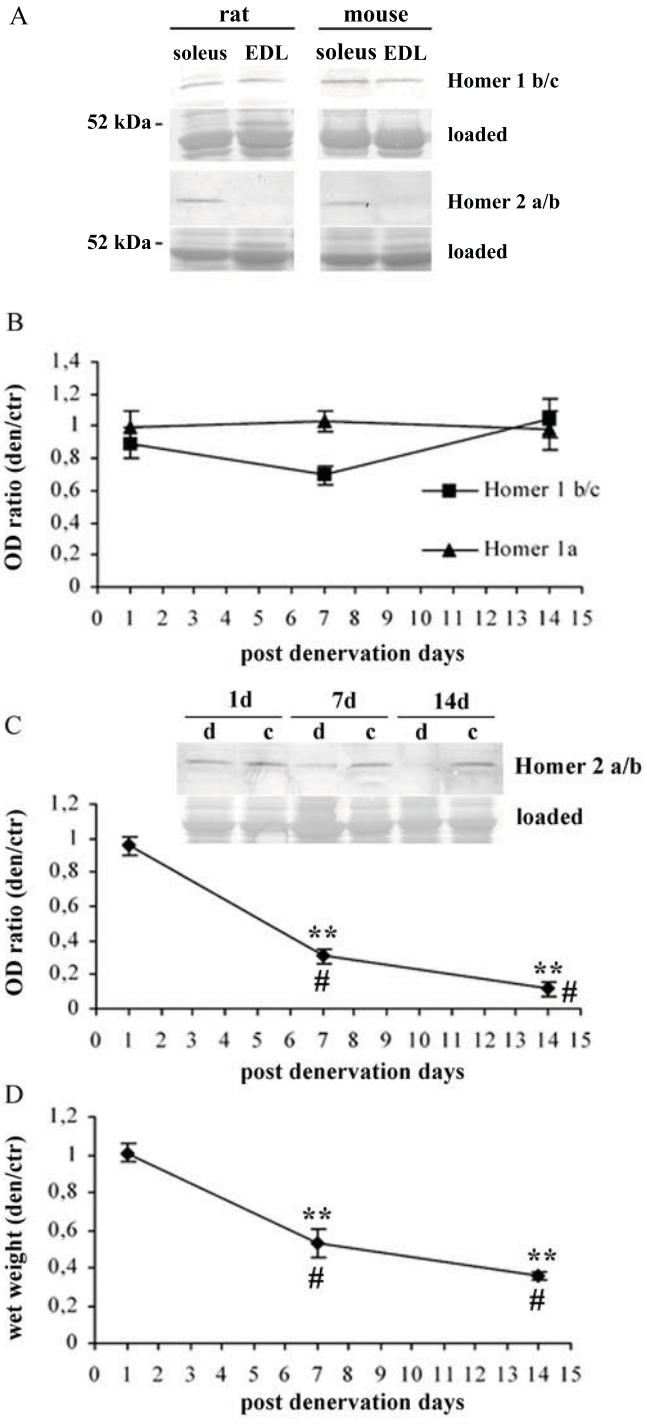
Fig. 5. Effect of Homer 2 re-introduction on muscle mass (panels A, B) and fiber CSA (panels C-E) of denervated soleus following *in vivo* transfection. Panel A: The weight of each soleus muscle was normalized to that of the rat. Bars on the left hand side represent measurements from normal soleus muscles electroporated with either pcDNA3 (empty vector, v) or pHomer 2-V5 (H2); Bars on the right hand side represent CSA measurements from denervated soleus muscles electroporated with either pcDNA3 (v) or pHomer 2-V5 (H2). Data are given as means ± SE; n = 4. ***P< 0.001, *P< 0.05. Panel B: The weight of each soleus was expressed as percentage of control.

Data are given as means ± SE; n = 4, *P< 0.05. Panels C-E: CSA was determined by morphometry, as described in Materials and Methods. CSA was measured in not transfected -also referred to as negative or not fluorescent- fibers (Panel C) and transfected -also referred to as positive, fluorescent- fibers (Panel D). Bars on the left hand side represent CSA measurements from normal soleus muscles electroporated with either pcDNA3 (v) or pHomer 2-V5 (H2); Bars on the right hand side represent CSA measurements from denervated soleus muscles electroporated with either pcDNA3 (v) or pHomer 2-V5 (H2). Data are given as means ± SE; n = 4, ***P< 0.001. In Panel E, CSA was measured in positive and negative fibers of the same soleus. Data are given as means ± SE; n = 4, *P< 0.05.

Fig. 6. Effect of Homer 2 re-introduction on ubiquitinization pathways of denervated soleus following *in vivo* transfection. Panel A: ubiquitination in control (c/Ctr) and denervated (d/Den) soleus. Panel B: ubiquitination in mock-transfected (vector) and transfected (Homer 2) denervated soleus. Histograms on the right-hand side represent densitometric values in arbitrary OD units and are given as means ± SE; n = 4. **P< 0.01. Panel C: exogenous expression of Homer 2-V5 was comparable in denervated soleus (d) and contralateral non-denervated soleus (c).

Fig. 7. Up-regulation of MuRF1 (panel A), ATR (panel B) and MyoG (panel C) following denervation of TA and soleus, and down-regulation of MuRF1, ATR and MyoG of denervated SOL upon re-introduction of Homer 2 (panels D-F). qRT-PCR was carried out, as described in Materials and Methods, on muscle samples from either TA or soleus (panels A-C) and on muscle samples from either mock-transfected and denervated SOL (vector) or transfected and denervated soleus (Homer 2), obtained at specific time points, following denervation (d3, d5, d7, d14), as indicated on the abscissa. ctr values were obtained on muscles that were not denervated. ctr values are different since it is known that “electric pulse associated with plasmid electroporation … significantly increased atrogin-1 and MuRF-1 mRNA levels, whereas plasmid injection alone had

751 no effect on gene expression" (52). Data are normalized to those of the reference gene TBP (see
752 Materials and Methods for details) and are given as means ± SE; n=4-8, *P<0.05.
753
754 **Fig. 8. Effect of Homer 2 re-introduction on slow-to-fast transition of denervated soleus.** qPCR
755 was carried out as previously described and data were normalized to those of the reference gene
756 PPIA (see Materials and Methods for details). Panel A: 14 days after denervation (Den), MHC type
757 2A and β-slow were decreased whereas MHC type 2X was increased, as compared to control (Ctr).
758 Panel B: In mock-transfected, denervated soleus (Ep-Den v) and in transfected, denervated soleus
759 (Ep-DenH2), no significant change was detected in either MHC type 2A, β-slow or 2X. Panel C:
760 Promoter activity of slow MHC was monitored in a Luciferase assay, as described in Materials and
761 Methods. Denervation decreased, as expected, slow MHC promoter activity, but Homer 2
762 replacement did not change this activity, as outlined in the graph on the left-hand side. Panel D:
763 Activity of the PGC1α gene was measured by qRT-PCR, as described in Materials and Methods.
764 Denervation, as expected, decreased PGC1α activity (Den and Ep-Den v), but Homer 2 replacement
765 (Ep-DenH2) was without any effect. qPCR data are given as means ± SE; n=4 for control and
766 denervated muscles, n=9,13 for electroporated muscles. *P<0.05, **P<0.01, ***P<0.001.
767


A
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EDL
Homer 1 b/c
52 kDa -
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Homer 2 a/b
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OD ratio (den/ctr)
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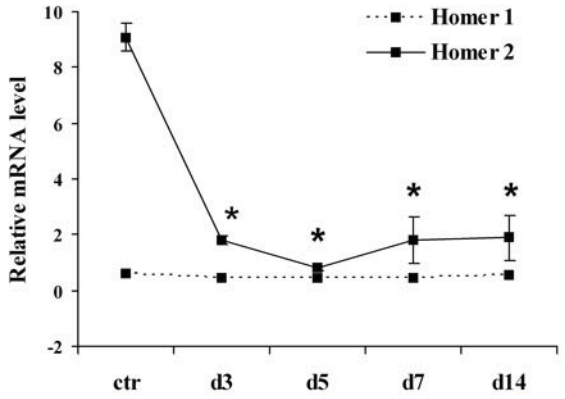
Homer 1
Homer 2
Relative mRNA level
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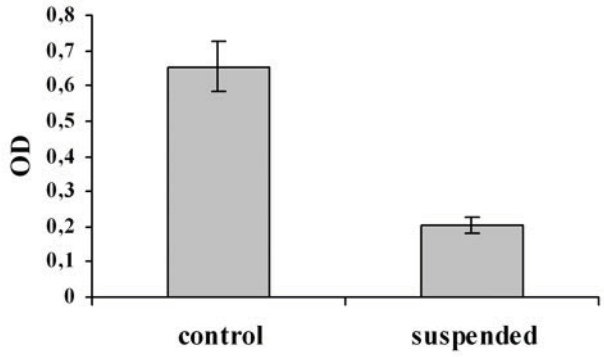
8
6
4
2
0
-2
*
*
*
*
ctr
d3
d5
d7
d14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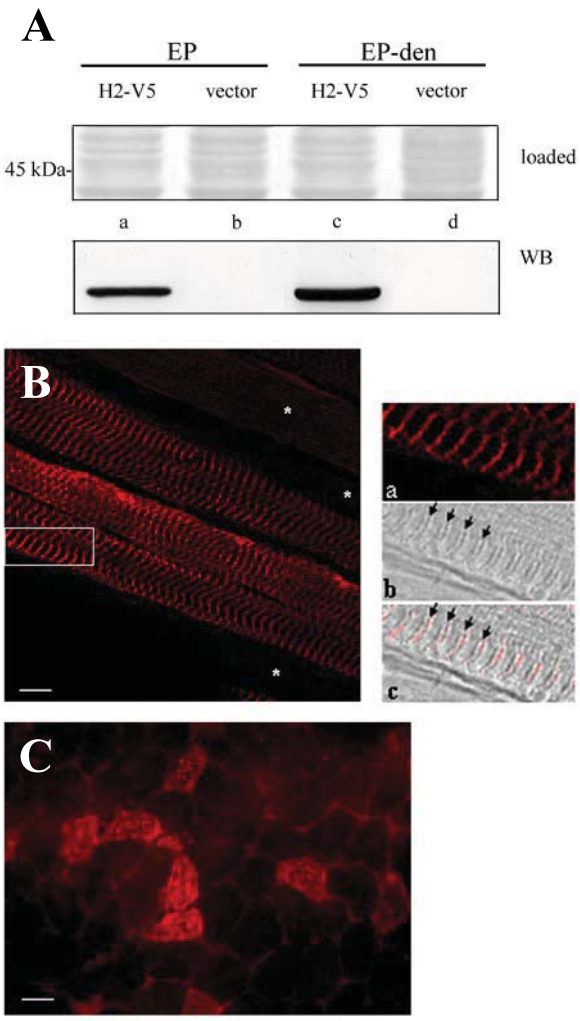
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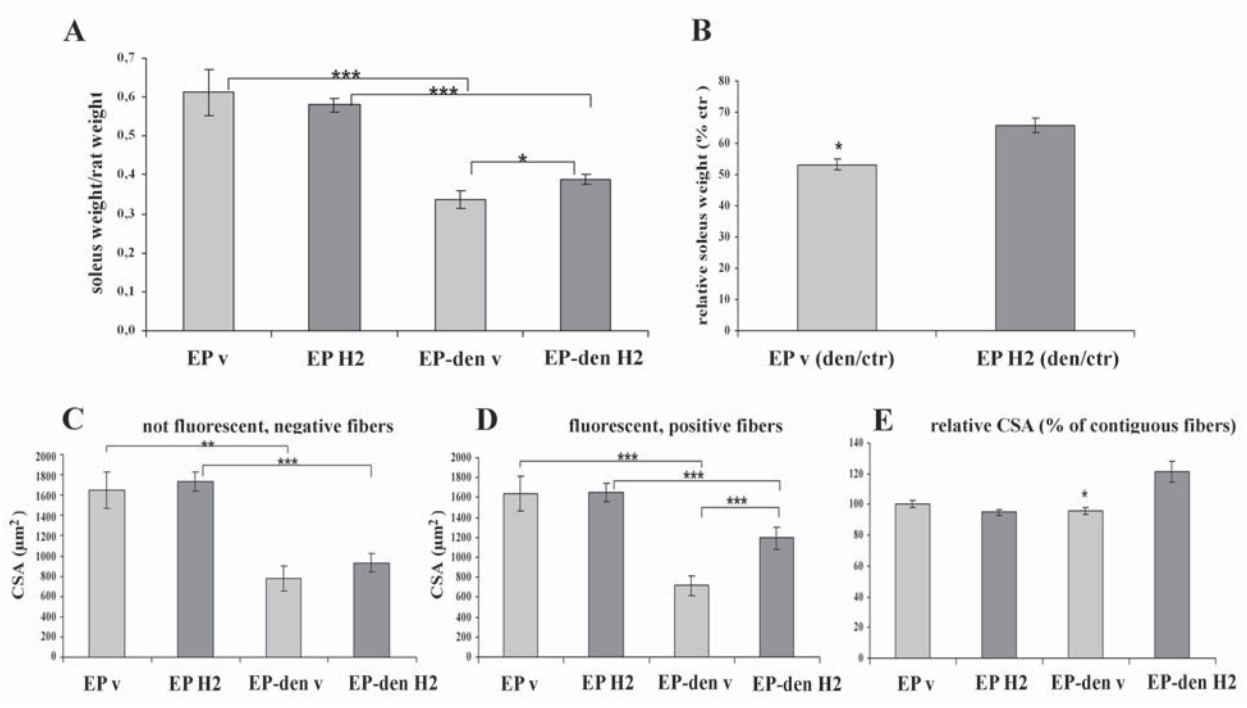
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soleus weight/rat weight
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0,6
0,5
0,4
0,3
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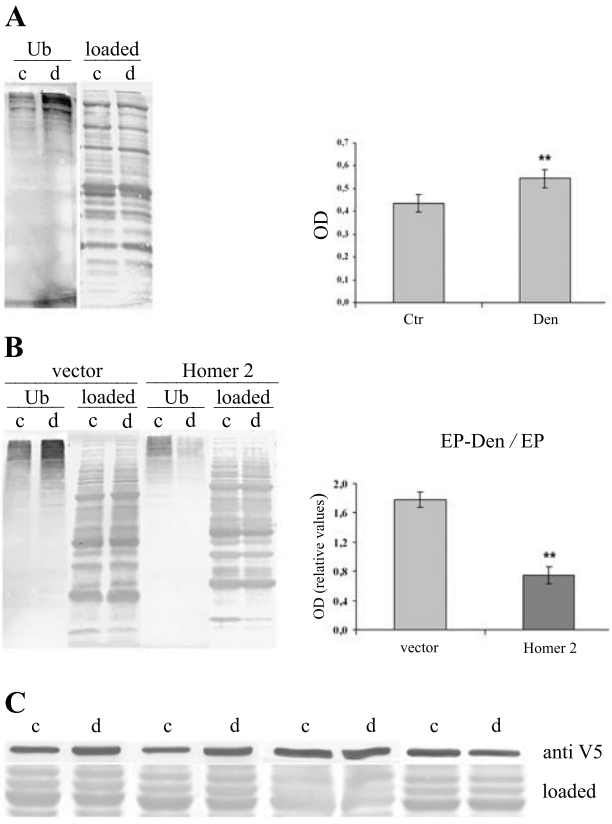
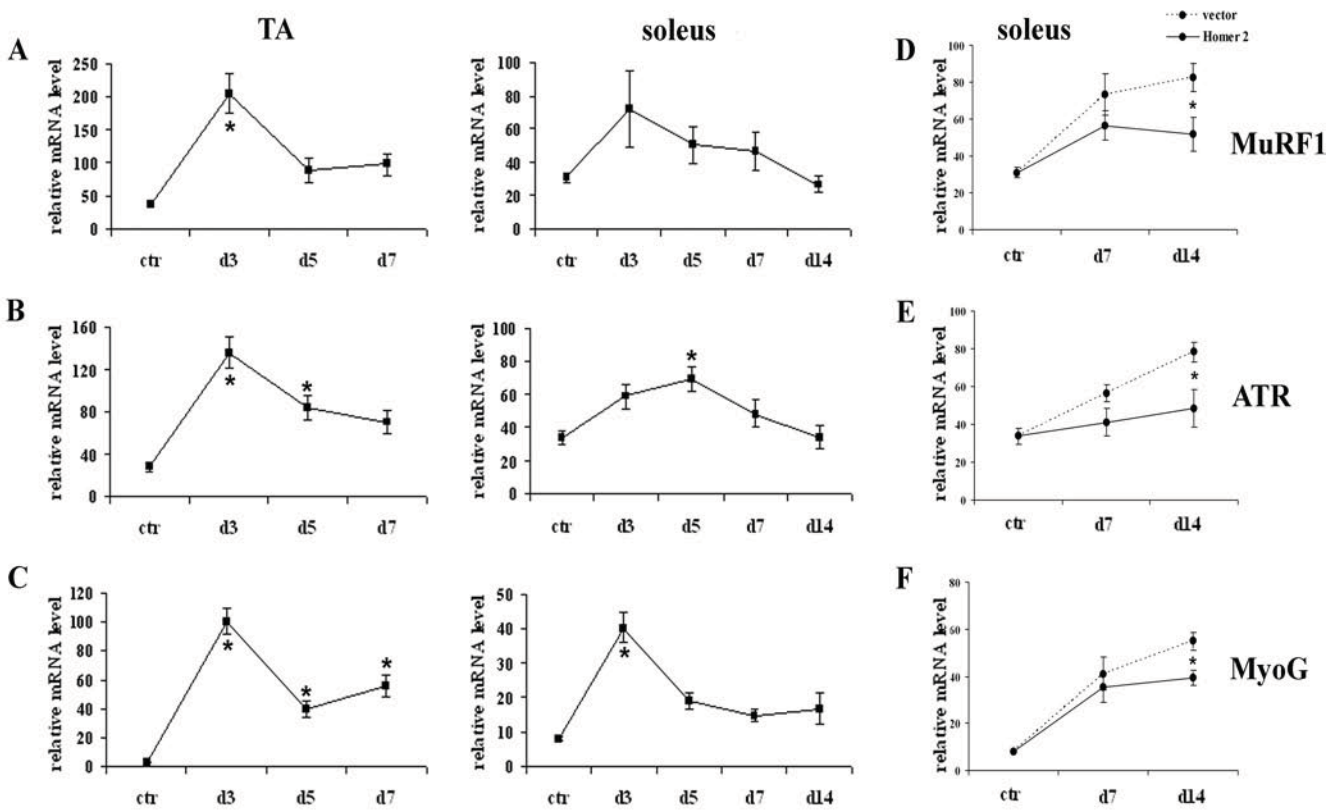
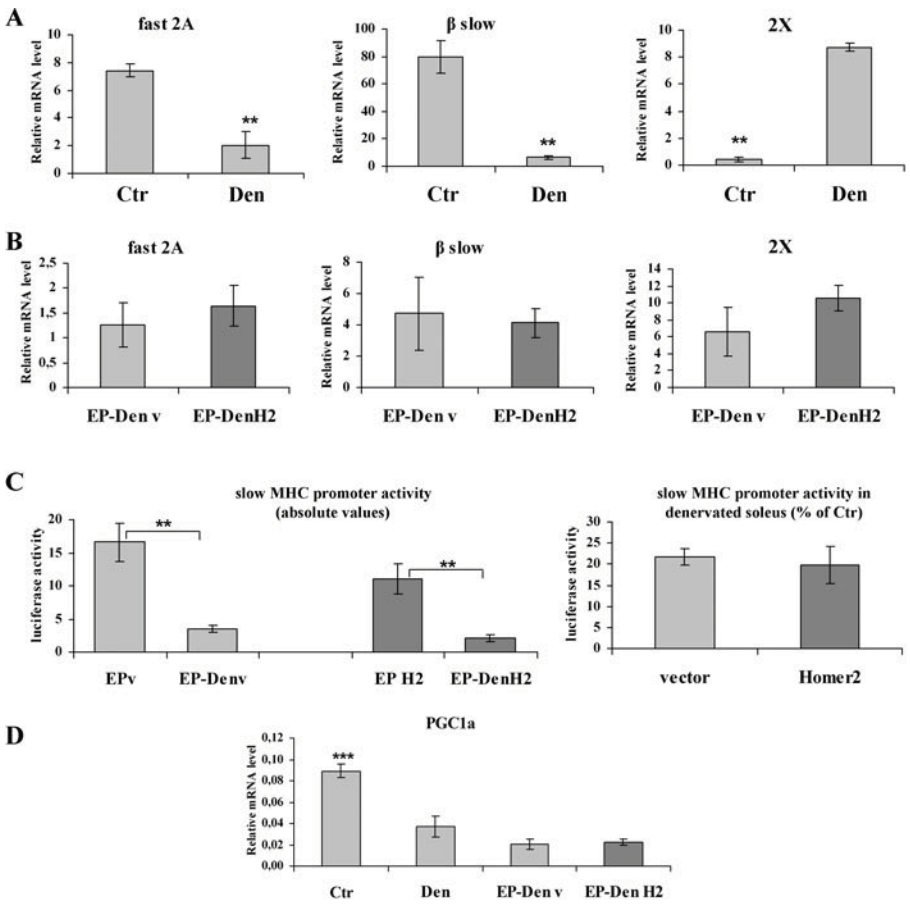
*
EP v
EP H2
EP-den v
EP-den H2
B
relative soleus weight (% ctr)
80
70
60
50
40
30
20
10
0
*
EP v (den/ctr)
EP H2 (den/ctr)
C
not fluorescent, negative fibers
CSA (µm²)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
**
***
EP v
EP H2
EP-den v
EP-den H2
D
fluorescent, positive fibers
CSA (µm²)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
***
***
***
EP v
EP H2
EP-den v
EP-den H2
E
relative CSA (% of contiguous fibers)
140
120
100
80
60
40
20
0
*
EP v
EP H2
EP-den v
EP-den H2


A
Ub
loaded
c d c d
OD
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0,0
**
Ctr
Den
B
vector
Homer 2
Ub
loaded
Ub
loaded
c d c d c d c d
EP-Den / EP
OD (relative values)
2,0
1,6
1,2
0,8
0,4
0,0
**
vector
Homer 2
C
c d c d c d c d
anti V5
loaded


A
TA
soleus
D
soleus
vector
Homer 2
relative mRNA level
ctr
d3
d5
d7
d14
MuRF1
B
ATR
C
MyoG
E
F


A
fast 2A
β slow
2X
Relative mRNA level
Ctr
Den
B
fast 2A
β slow
2X
EP-Den v
EP-DenH2
C
slow MHC promoter activity
(absolute values)
luciferase activity
EPv
EP-Denv
EP H2
EP-DenH2
slow MHC promoter activity in
denervated soleus (% of Ctr)
vector
Homer2
D
PGC1a
EP-Den H2