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Research paper

Isolation, expression analysis and characterization of NEFA-interacting nuclear protein 30 and RING finger and SPRY domain containing 1 in skeletal muscle

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Muscle atrophy results from a range of physiological conditions, including immobilization, spinal cord damage, inflammation and aging. In this study we describe two genes, NEFA-interacting nuclear protein 30 (*Nip30*) and RING Finger and SPRY domain containing 1 (*Rspry1*), which have not previously been characterized or shown to be expressed in skeletal muscle. Furthermore, *Nip30* and *Rspry1* were transcriptionally induced in response to neurogenic muscle wasting in mice and were also found to be expressed endogenously at the RNA and protein level in C_2C_{12} mouse muscle cells. Interestingly, during analysis of *Nip30* and *Rspry1* it was observed that these genes share a 230 base pair common regulatory region that contains several putative transcription regulatory elements. In order to assess the transcriptional activity of the *Nip30* and *Rspry1* regulatory regions, a fragment of the promoter of each gene was cloned, fused to a reporter gene, and transfected into cells. The *Nip30* and *Rspry1* reporters were both found to have significant transcriptional activity in cultured cells. Furthermore, the Nip30-Rspry1 common regulatory region contains a conserved E-box enhancer, which is an element bound by myogenic regulatory factors that function in the regulation of muscle-specific gene expression. Therefore, in order to determine if the predicted E-box was functional, *Nip30* and *Rspry1* reporters were transfected into cells ectopically expressing the myogenic regulatory factor, MyoD1, resulting in significant induction of both reporter genes. In addition, mutation of the conserved E-box element eliminated MyoD1 activation of the *Nip30* and *Rspry1* reporters. Finally, GFP-tagged Nip30 was found to localize to the nucleus, while GFP-tagged Rspry1 was found to localize to the cytoplasm of muscle cells.

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1. Introduction

Skeletal muscle shows amazing plasticity and has the capacity to continuously regulate its size in response to external cues such as mechanical load, neural activity, hormones, growth factors, stress and nutritional status. The maintenance of muscle mass is controlled by a balance between protein synthesis and protein degradation pathways, a balance which shifts toward protein degradation during atrophy (Mitch and Goldberg, 1996; Jagoe and Goldberg, 2001). Although protein degradation systems have been extensively studied, work to characterize the molecular genetic underpinnings of atrophy has historically lagged behind. Therefore, in order to address this deficiency, several studies designed to identify genes that are differentially expressed in response to a range of atrophy-promoting stimuli, including hind limb unweighting, corticosteroid exposure, immobilization, denervation and inflammation have been conducted (Bodine et al., 2001; Gomes et al., 2001). These studies provided a wealth of information and identified several important atrophy-induced genes, including the ubiquitin E3 ligases, MuRF1 and MAFbx, which are expressed predominantly in skeletal and cardiac muscle and are up-regulated during virtually all atrophy conditions suggesting that these gene products are key regulators of muscle wasting (Bodine et al., 2001; Gomes et al., 2001).

Abbreviations: BioGRID, Biological General Repository for Interaction Datasets; bp, base pair; bHLH, basic helix-loop-helix; CDD, Conserved Domain Database; DMEM, Dulbecco's Modified Eagle Medium; FBS, Fetal Bovine Serum; Fam192a, family with sequence similarity 192, member a; GEO, Gene Expression Omnibus; GFP, Green Fluorescent Protein; MAFbx, Muscle Atrophy F-box; M-MLV, Moloney Murine Leukemia Virus; MuRF1, Muscle RING Finger 1; MyHC, myosin heavy chain; NCBI, National Center for Biotechnology Information; Nip30, NEFA-interacting nuclear protein 30; NEFA, nonesterified fatty acid; ONPG, ortho-nitrophenyl-β-D-galactopyranoside; PVDF, polyvinylidene difluoride; Rspry1, RING Finger and SPRY domain containing 1; RING, really-interesting-new-gene; RLU, Relative Light Units; SD, Standard Deviation; SEAP, secreted alkaline phosphatase; SMART, Simple Modular Architecture Research Tool; SPRY, Sp1A and ryanodine receptor; STRING, Search Tool for the Retrieval of Interacting Genes/Proteins; TS, triceps surae; UPS, Ubiquitin Proteasome System.

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Table 1
List of primers used in this study.

Primers Sequence (5′-3′)	
Gene Cloning	
Rspry1-F	GCAAGCTTATGATTGTCTTTGGTTGGGCTGTG
Rspry1-R	GGATCCGGTCCATGATATCTCTTCACATGTG
Nip30-F	GCAAGCTTACCATTATGGATGGAGAGGACG
Nip30-R	GCGGATCCGTGGTAATATCAGGAGCTAGGGTGC
Promoter Cloning	
Rspry1-Pro500-F	GCACGCGTGCTTGGAGTTCACCCTCTCCACGGCCG
Rspry1-Pro500-R	GCCTCGAGCCCTCCCTCAGTCCCCTAGGAAACGC
Nip30-Pro500-F	GCACGCGTCTCCGACCCAGGCTTCAGCGGCTGTCC
Nip30-Pro500-R	GCCTCGAGAACAGCTCTCTCCCTCCCCGAGCGCC
Site-directed Mutagenesis	
Rspry1-Ebox-Mut-F	CCGACCCGCGAGATCGCGCGACACAAATCTTGA
Rspry1-Ebox-Mut-R	GTCAAGATTTGTGTCGCGCGATCTCGCGGGTCGG
Nip30-Ebox-Mut-F	GTCAAGATTTGTGTCGCGCGATCTCGCGGGTCGG
Nip30-Ebox-Mut-R	CCGACCCGCGAGATCGCGCGACACAAATCTTGA
Sequencing	
Rspry1-Seq-F	CTAGACGAATGTCCATTGCC
Rspry1-Seq-R	CAGCACGGGTGCACATGAGG

The identification of MuRF1 and MAFbx ushered in an era of muscle atrophy research that focused on elucidating potential targets of these E3 ligases, however; to date only a few direct MuRF1 and MAFbx targets have been characterized (Kedar et al., 2004; Clarke et al., 2007; Cohen et al., 2009; Csibi et al., 2009; Jogo et al., 2009; Lagirand-Cantaloube et al., 2009; Bodine and Baehr, 2014). Interestingly, of the few targets identified, MyoD1 and myogenin, both myogenic regulatory factors that are involved in muscle differentiation and transcriptional regulation of muscle-specific genes, have been shown to be ubiquitinated and targeted for degradation by MAFbx (Jogo et al., 2009; Lagirand-Cantaloube et al., 2009). The surprisingly small number of target proteins identified for these canonical markers of muscle wasting suggests that the molecular mechanisms of skeletal muscle atrophy remain incomplete. Furthermore, while it is assumed that MuRF1 and MAFbx function in some manner to regulate protein degradation, since they are expressed early in the atrophy process and reach peak expression levels during the height of protein degradation and muscle loss, the fact that there have been so few targets identified for these E3 ligases, indicates that the atrophy cascade is likely far more complicated than originally imagined (Foletta et al., 2011; Bodine and Baehr, 2014).

In order to provide a more complete picture of the molecular genetic events that lead to skeletal muscle atrophy and to identify other potential modulators of muscle wasting, a microarray study was performed using skeletal muscle isolated from mice undergoing neurogenic atrophy in response to denervation and corticosteroid-induced atrophy in response to dexamethasone exposure (Furlow et al., 2013). The data generated from this study identified several hundred annotated and predicted genes that displayed differential expression in response to muscle wasting (Furlow et al., 2013). The fact that the majority of published work to date has focused on only a small subset of the genes that comprise the mammalian genome makes the identification and characterization of the remaining pool of uncharacterized genes an important and fertile area for further investigation. Therefore, by undertaking a deeper analysis of the microarray data from the Furlow et al. study, we identified and characterized two genes, *Nip30* and *Rspry1*, which have not previously been shown to be expressed in skeletal muscle. Furthermore, *Nip30* and *Rspry1* were induced in response to neurogenic

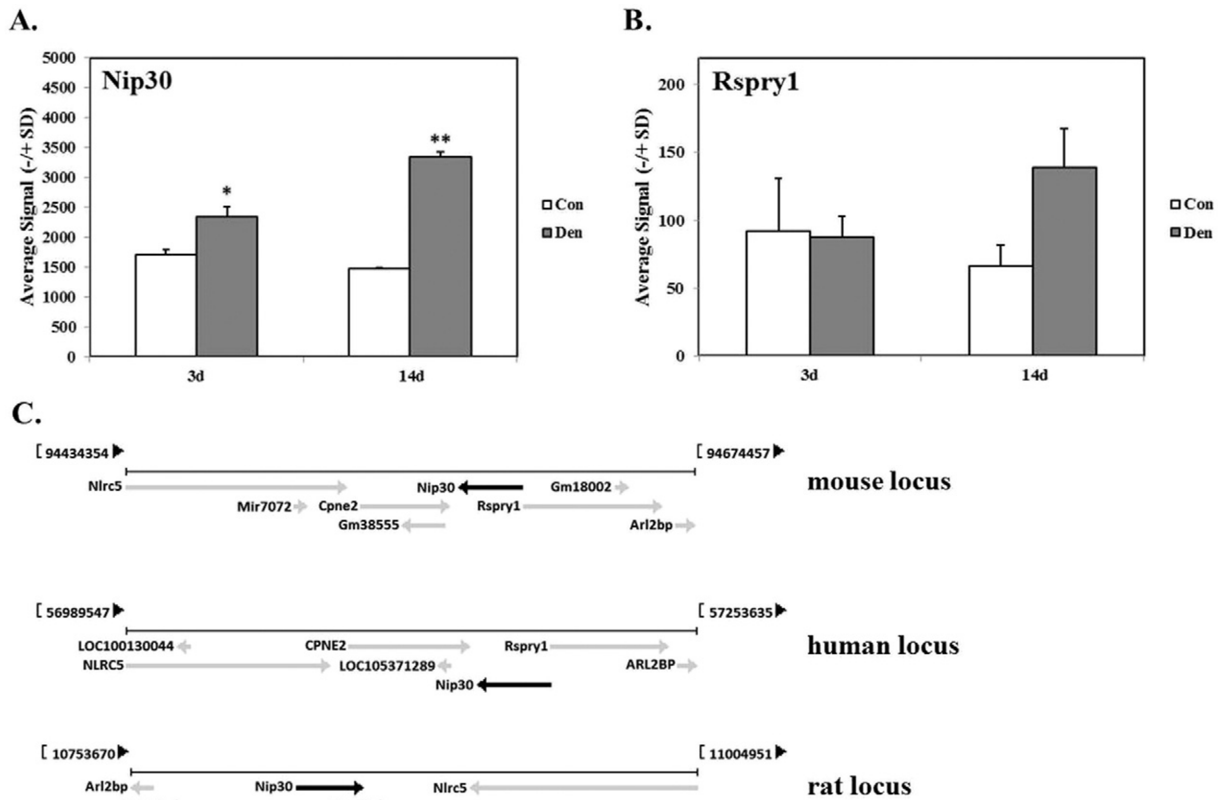

Fig. 1. *Nip30* and *Rspry1* are induced during skeletal muscle atrophy. Whole genome expression analysis was conducted on triceps surae muscle from wild-type control (CON) mice after 3 days (3D) and 14 days (14D) of denervation (DEN). (A) *Nip30* expression increased significantly at both 3 and 14 days of denervation. (B) *Rspry1* expression only showed increased expression at 14 days of denervation, but was not statistically significant. Each condition represents the average expression from three animals and error bars represent ± SD. White bars, controls; gray bars, DEN. Significant difference between denervated mice and control mice in the same group, (*: $P < 0.05$, **: $P < 0.01$). (C) Schematics of the conserved *Nip30* and *Rspry1* loci in mouse, human and rat. The schematic images were downloaded from the Gene database available on the NCBI webpage (http://www.ncbi.nlm.nih.gov/gene).

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