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Short communication

# UBE2D2 is not involved in MuRF1-dependent muscle wasting during hindlimb suspension

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ABSTRACT

The Ubiquitin Proteasome System (UPS) is mainly responsible for the increased protein breakdown observed in muscle wasting. The E3 ligase MuRF1 is so far the only enzyme known to direct the main contractile proteins for degradation (*i.e.* troponin I, myosin heavy chains and actin). However, MuRF1 does not possess any catalytic activity and thus depends on the presence of a dedicated E2 for catalyzing the covalent binding of polyubiquitin (polyUb) chains on the substrates. The E2 enzymes belonging to the UBE2D family are commonly used for *in vitro* ubiquitination assays but no experimental data suggesting their physiological role as *bona fide* MuRF1-interacting E2 enzymes are available. In this work, we first found that the mRNA levels of critical E3 enzymes implicated in the atrophying program (MuRF1, MAFbx, Nedd4 and to a lesser extent Mdm2) are tightly and rapidly controlled during the atrophy (up regulation) and recovery (down regulation) phases in the soleus muscle from hindlimb suspended rats. By contrast, E3 ligases (Ozz, ASB2β and E4b) implicated in other processes (muscle development or regeneration) poorly responded to atrophy and recovery. UBE2B, an E2 enzyme systematically up regulated in various catabolic situations, was controlled at the mRNA levels like the E3s implicated in the atrophying process. By contrast, UBE2D2 was progressively repressed during atrophy and recovery, which makes it a poor candidate for a role during muscle atrophy. In addition, UBE2D2 did not exhibit any affinity with MuRF1 using either yeast two-hybrid or Surface Plasmon Resonance (SPR) approaches. Finally, UBE2D2 was unable to promote the degradation of the MuRF1 substrate α-actin in HEK293T cells, suggesting that no functional interaction exists between these enzymes within a cellular context. Altogether, our data strongly suggest that UBE2D2 is not the cognate ubiquitinating enzyme for MuRF1 and that peculiar properties of UBE2D enzymes may have biased *in vitro* ubiquitination assays.



## 1. Introduction

Ubiquitin (Ub) is a widespread protein (8.5 kDa) used for creating several signals (monoUb, Ub chains, *etc.*) that posttranslationally modifies proteins for regulating cell metabolism within eukaryotes (Ravid and Hochstrasser, 2008). Ub chains can be catalyzed using different internal lysines like K48, which are *bona fide* degradation signals targeting protein substrates to the 26S proteasome. Protein ubiquitination may involve other Ub lysine residues (K6, K11, K27, K29, K33, or K63), but targeting also includes forked (*e.g.* K6-K11), heterologous (Ub-SUMO) or simply monoUb (Polge et al., 2013b; Ciechanover and Stanhill, 2014). K48 and K11 chains generally target proteins for degradation through the 26S proteasome while other Ub-modified proteins are directed towards different fates, thus highlighting the complexity of Ub signaling in eukaryotic cells.

Protein ubiquitination is achieved through an enzymatic cascade implicating three classes of enzymes. A single E1 (Ub

*Abbreviations:* UPS, ubiquitin proteasome system; HS, hindlimb suspension; MuRF1, muscle ring finger protein-1; UBE2D2, ubiquitin-conjugating enzyme E2 D2; UBE2B, ubiquitin-conjugating enzyme E2 B; MHC, myosin heavy chain; SPR, surface plasmon resonance; Y2H, yeast two-hybrid.

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activating enzyme) activates Ub and transfers high energy Ub to one of the 37 mammalian E2s (Ub conjugating enzymes). Then, E2 enzymes transfer Ub on target proteins in combination with E3 ubiquitin ligases (>600). An E2 can cooperate with different E3s and *vice versa*, which enables the specific targeting of virtually any cellular protein. The recognition specificity of the target protein belongs to E3 enzymes but most E3s lack enzymatic activity so that only the E2-E3 couple is functionally relevant. Moreover, recent studies proved that E2 enzymes largely influence the fate of the substrate as they determine the type of chain built and its localization on the substrate, which means E2s are central players in the ubiquitination machinery (Ye and Rape, 2009; David et al., 2010; Van Wijk and Timmers, 2010). Thus, the identification of the ubiquitinating enzymes targeting muscle proteins for breakdown is of considerable interest for future development of new therapeutical strategies.

The UPS largely controls muscle mass in nearly any catabolic situations but also during recovery processes (Taillandier et al., 1996; Voisin et al., 1996; Lecker et al., 1999; Taillandier et al., 2003; Lin et al., 2005; Mcfarlane et al., 2006; Polge et al., 2011; Polge et al., 2013a; Sandri, 2013). The muscle-specific E3 ligase MuRF1 is involved in the targeting of major contractile proteins like actin, myosin heavy chains and troponin I. Indeed, MuRF1 is one of the so-called atrogenes that are systematically up or down regulated in atrophying conditions (Lecker et al., 2004). However, the identity of the E2(s) catalyzing Ub transfer in conjunction with MuRF1 remains elusive, as only *in vitro* ubiquitination assays have been performed (see (Polge et al., 2015) for a recent review). In these works, the UBE2D family has been extensively used with MuRF1, and actin-$Ub_n$ (UBE2D2), MHCII-$Ub_n$ (UBE2D1, D2 and D3) and Troponin I-$Ub_n$ (UBE2D3) were successfully catalyzed (Kedar et al., 2004; Clarke et al., 2007; Fielitz et al., 2007; Polge et al., 2011). The UBE2D enzymes are highly homologous (90–93% identity in humans) and were equally efficient when used *in vitro*. However, no data are available about MuRF1-UBE2D interactions and UBE2D enzymes are generally not up regulated in catabolic muscles except upon glucocorticoid treatment (dexamethasone) (Chrysis and Underwood, 1999; Lecker et al., 2004; Polge et al., 2016). This is in striking contrast with MuRF1 that is up regulated in any catabolic situation.

In this work, (1) we followed the regulation of MuRF1 and UBE2D2 gene expression in atrophying muscles from hindlimb suspended rats (unweighting) and subsequent recovery in reloaded animals and (2) we aimed at defining whether MuRF1 and UBE2D2 are able to physically interact for clarifying the potential involvement of UBE2D2 in muscle atrophy.

## 2. Materials and methods

### 2.1. Animals and muscle treatments

The experiments were conducted in accordance with the National Research Council *Guide for the Care and Use of Laboratory Animals*. Male Wistar rats (Iffa-Credo, L'Arbresle, France) of an average body weight of 120 g were randomly assigned to the following groups: hindlimb suspended (HS), recovery from hindlimb suspension (R-HS1, R-HS4 and R-HS7) and their corresponding controls (CT, R-CT1, R-CT4, R-CT7) (n = 8 per group). After 4 days of standard housing, unweighted rats were suspended by the tail as previously described (Taillandier et al., 1996; Taillandier et al., 2003). All animals were maintained in a temperature-controlled room (22 ± 1 °C) with a 12:12 h light:dark cycle. After 9 days of hindlimb suspension, R-HS animals were reloaded and had a standard cage activity for 18 h, 4 or 7 days of recovery (R-HS1, R-HS4 and R-HS7, respectively). Rats were anesthetized and killed by exsanguination and compared to their respective age-matched controls (CT, R-CT1, R-CT4 and R-CT7).

Soleus muscles from control and reloaded rats were rapidly excised, frozen in liquid nitrogen and stored at −80 °C. Total RNA was extracted as described by Chomczynski and Sacchi, 1987.

### 2.2. qRT-PCR

mRNA levels of E3 ligases (MuRF1, MAFbx, Nedd4, Mdm2, ASB2ß, Ozz and E4b) and E2 enzymes UBE2D2 and UBE2B were determined by quantitative RT-PCR. The primers were designed using PrimerBlast (http://www.ncbi. nlm.nih.gov/tools/primer-blast/) and were from Sigma-Aldrich. Reverse transcription of total RNA into DNA was performed using the QuantiTect® Reverse Transcription kit (Qiagen®). qPCR was performed using the FastStart DNA Master SYBR Green I kit (Roche), according to the manufacturer's instructions using a CFX96 thermocycler (BIORAD). Calculations were made using the comparative ΔCt method with YWHAZ, HPRT1 and 36B4 housekeeping genes (see Supplementary Table S1 for primers).

### 2.3. Yeast two-hybrid experiments

We used the "Matchmaker™ Gold Yeast Two-Hybrid System" (Y2H) from Clontech, based on the reconstitution of the GAL4 transcription factor. Y2HGold clones containing pGBKT7 constructs (MuRF1 or p53) were mated against Y187 clones containing pGADT7 constructs (MuRF3 or UBE2D2) on YPDA medium for a period of 12 h. Diploids were then selected after replication on a selective medium lacking leucine and tryptophan. Interactions were assayed by the activation of HIS3 and AUR1-C reporter genes. Diploids obtained were then further replicated on medium lacking leucine, tryptophan and histidine and supplemented with 20 mM Aureobasidin A. 2.5 mM 3-Amino-1,2,4-triazole (3-AT, a competitive inhibitor of the product of the HIS3 gene) were added to avoid non-specific interactions. Growth on these selective plates was followed over a period of 21 days, p53 (from Clontech) being used as a negative control. MuRF1 and MuRF3 oligomerization was used as a positive control.

### 2.4. Protein expression and surface plasmon resonance (SPR) analyses

Untagged recombinant protein UBE2D2 was from Enzo Life Sciences. GST and GST-MuRF1 were expressed and purified using sepharose 4B affinity matrix (GE Healthcare) as previously described (Polge et al., 2011). Surface Plasmon Resonance (SPR) experiments were performed with a BIAcore T200 instrument (GE Healthcare), at 25 °C. GST-MuRF1 and GST were covalently immobilized on a CM5 sensor chip by standard amine coupling generating multiple orientations of the fixed protein on the surface. Interaction evaluation was performed in running buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 0.05% (v/v) surfactant P20) at a flow rate of 30 μL/min. For interaction assays, UBE2D2 protein was diluted to 500 nM and 1 μM, and GST-MuRF1 to 100 nM and injected onto the GST reference and GST-MuRF1 surfaces for 70 s at 30 μL/min.

### 2.5. Cell culture and transfection

MuRF1, α-actin and UBE2D2 coding sequence were sub-cloned in pcDNA3.1. HEK293T cells were cultured in DMEM. Cells were plated in 6-well dishes and transfected by the calcium phosphate co-precipitation method. Cells were transfected or cotransfected with plasmid(s) encoding for GFP (Mock), actin, MuRF1 and UBE2D2, and were harvested after 48 h of transfection. Cells were lyzed and soluble proteins were obtained as previously described