

A novel ubiquitin-binding protein ZNF216 functioning in muscle atrophy

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The ubiquitin–proteasome system (UPS) is critical for specific degradation of cellular proteins and plays a pivotal role on protein breakdown in muscle atrophy. Here, we show that ZNF216 directly binds polyubiquitin chains through its N-terminal A20-type zinc-finger domain and associates with the 26S proteasome. ZNF216 was colocalized with the aggresome, which contains ubiquitinated proteins and other UPS components. Expression of *Znf216* was increased in both denervation- and fasting-induced muscle atrophy and upregulated by expression of constitutively active FOXO, a master regulator of muscle atrophy. Mice deficient in *Znf216* exhibited resistance to denervation-induced atrophy, and ubiquitinated proteins markedly accumulated in neurectomized muscle compared to wild-type mice. These data suggest that ZNF216 functions in protein degradation via the UPS and plays a crucial role in muscle atrophy.

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Introduction

The ubiquitin–proteasome system (UPS) is one of the major protein degradation pathways in eukaryotic cells. The UPS plays key regulatory roles in many cellular processes, including cell cycle control, the regulation of transcription and protein quality control (Hershko and Ciechanover, 1998; Pickart and Cohen, 2004). Aberrations of this system lead to many forms of pathogenesis, such as malignancies, neurodegenerative disease and inflammatory response (Glickman and Ciechanover, 2002). The UPS includes sequential, multistep reactions: ubiquitin-conjugation of target proteins by E1, E2 and E3 enzymes, recognition of ubiquitinated proteins by ubiquitin-binding proteins

or 19S subunits of proteasome and proteolysis in the proteasome.

Many catabolic conditions, such as low-insulin state, hyperthyroidism, sepsis and cancer cachexia lead to enhancement of protein breakdown in skeletal muscle known as muscle atrophy (Mitch and Goldberg, 1996; Lecker *et al*, 1999). In muscle atrophy, the UPS plays a pivotal role in protein breakdown (Price *et al*, 1996; Tawa *et al*, 1997). Several studies indicate that mRNAs encoding UPS components are increased in atrophying muscle (Medina *et al*, 1991; Wing and Goldberg, 1993; Bailey *et al*, 1996; Price *et al*, 1996; Jagoe *et al*, 2002). In particular, the E3 ubiquitin ligases MAFbx/Atrogin-1 and MuRF-1 (muscle RING finger 1) are known to be markers of muscle atrophy (Bodine *et al*, 2001; Gomes *et al*, 2001). Both are induced in multiple models of muscle atrophy including immobilization, denervation and hindlimb suspension, and mice deficient in either gene are resistant to denervation-induced muscle atrophy (Bodine *et al*, 2001). Goldberg and co-workers proposed that atrophy-related genes, whose expression is induced in multiple types of muscle atrophy, are called ‘atrogenes’ (Sandri *et al*, 2004). Recently, it was demonstrated that the IGF-I/PI3K/Akt pathway is an important regulator of muscle mass in muscle hypertrophy and atrophy (Sacheck *et al*, 2004; Sandri *et al*, 2004; Stitt *et al*, 2004). In that case, the transcription factor FOXO plays a pivotal role in activating atrogenes such as MAFbx/Atrogin-1 (Gomes *et al*, 2001).

Although many UPS players such as E3 ligases have been characterized, the mechanism of how ubiquitinated proteins are delivered to the proteasome have not been fully elucidated. A component of 19S proteasome, Rpn10/S5a, recognizes the ubiquitinated proteins (Young *et al*, 1998; Wilkinson *et al*, 2000). It has been shown that yeast proteins, Rad23p and Dsk2p, bind to ubiquitinated substrates and to the 26S proteasome through their UBA and Ubl domains, respectively, thereby functioning as shuttle proteins that present polyubiquitinated proteins to the proteasome (Chen *et al*, 2001; Funakoshi *et al*, 2002; Elsasser and Finley, 2005). Loss-of-function of shuttle proteins results in abnormal accumulation of polyubiquitinated proteins (Lambertson *et al*, 1999; Saeki *et al*, 2002). However, yeast can survive when both *RAD23* and *DSK2* genes are mutated, suggesting that other mechanisms or molecule(s) possessing a shuttle function exist (Saeki *et al*, 2002). Here, we show that ZNF216, a novel ubiquitin-binding protein containing an A20-type zinc-finger, is such a factor. *Znf216* expression is upregulated in skeletal muscle in experimental models of muscle atrophy, and *Znf216*-deficient mice exhibit resistance to muscle atrophy accompanied by abnormal accumulation of polyubiquitinated proteins in skeletal muscle. Our findings suggest that ZNF216, with its potential function of anchoring ubiquitinated proteins to the proteasome, plays a critical role in degrading muscle proteins.

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Results

ZNF216 directly binds to polyubiquitin

We have identified a gene, *Znf216* (*Za20d2*, Mouse Genome Informatics), encoding an A20 zinc-finger (Znf-A20) motif-containing protein, as a RANKL-induced gene upregulated upon osteoclast formation using a microarray technique (Hishiya *et al*, 2005). *Znf216* was originally identified as a candidate gene for hearing loss and is expressed in cochlear and skeletal muscle (Scott *et al*, 1998; Huang *et al*, 2004). To determine the function of ZNF216, we searched for molecules that associate with ZNF216 using yeast two-hybrid screening and isolated several clones encoding a gene for polyubiquitin C. To determine whether ZNF216 interacts with ubiquitin in mammalian cells, we transfected HEK293 cells with an expression vector for FLAG-tagged ZNF216 and HA-tagged ubiquitin and performed co-immunoprecipitation experiments. ZNF216 possesses A20-type (amino acids 11–35) and AN1-type (amino acids 154–191) zinc-finger domains at its N- and C-termini, respectively (Figure 1A). Endogenous ubiquitylated proteins, which appear as smears, were co-immunoprecipitated with FLAG-tagged ZNF216 (Figure 1B). Notably, N-terminal deletion (Δ N; amino acids 36–213) or point mutants (M1 and M3) of the A20-type zinc-finger (ZnF-A20) domain abolished ubiquitin-binding ability of ZNF216, indicating that the ZnF-A20 domain is indispensable for binding to ubiquitin (Figures 1A and B). Whereas in non-denaturing conditions, ubiquitylated molecules were present with FLAG-tagged ZNF216, these molecules completely disappear from immunoprecipitates following heat denaturation, which abolishes noncovalent protein–protein interactions (Figure 1C), suggesting that ZNF216 associates with ubiquitylated proteins rather than being ubiquitylated itself. Next, to determine whether ZNF216 binds to ubiquitin directly, we performed GST pull-down assays using GST-ZNF216 fusion proteins (Figure 1D) and purified polyubiquitin. As shown in Figure 1E, GST-ZNF216 but not GST bound to polyubiquitin chains. As expected, binding of ZNF216 to polyubiquitin chains was completely abolished by a point mutation in the ZnF-A20 domain (M1, Figure 1E). Furthermore, a GST fusion protein containing only the ZnF-A20 domain (amino acids 2–60) could bind to polyubiquitin chains, suggesting that ZNF216 directly binds to polyubiquitin chains, and that the ZnF-A20 domain is required for binding to polyubiquitin. As for other ZnF-A20 containing proteins, AWP1 (ZA20D3) also possessed polyubiquitin-binding activity but the ZnF-A20 domain(s) of Rabex-5 (Horiuchi *et al*, 1997) and A20/TNFAIP3 (Opipari *et al*, 1990) proteins did not (Supplementary Figure S1).

ZNF216 associates with the 26S proteasome

We also identified molecules associating with ZNF216 by proteomic analysis of complexes formed with FLAG-tagged ZNF216. Molecules expressed in HEK293 cells and that co-immunoprecipitated with FLAG-tagged ZNF216 were analyzed by tandem mass spectrometry. By this analysis, every subunit of the 26S proteasome complex was identified as associating with FLAG-tagged ZNF216 (data not shown). To identify the region of ZNF216 required for association with the 26S proteasome, lysates of cells expressing either FLAG-tagged ZNF216 or its mutants were immunoprecipitated with anti-FLAG antibody. Co-precipitation of proteasomal compo-

nents was monitored by immunoblotting using an antibody against Rpn7p (S10a), a non-ATPase subunit of the 19S regulatory subunit. As shown in Figure 2A, this protein efficiently co-precipitated with FLAG-tagged ZNF216. The interaction was also observed with truncated or point mutants of ZnF-A20 (Δ N or M1), indicating that ubiquitin-binding ability is dispensable for association with the 26S proteasome. To determine whether endogenous ZNF216 proteins are also associated with the 26S proteasome, we performed a GST pull-down assay using the ubiquitin-like (Ubl) domain of hHR23B, a human homologue of Rad23, which is known to bind to the 26S proteasome. As shown in Figure 2B, GST-Ubl but not GST was pulled down with the endogenous 26S proteasome. Endogenous ZNF216 was also detected in the GST-Ubl/26S proteasome complex (upper panels, Figure 2B). Furthermore, purified recombinant ZNF216 did not bind to GST-Ubl (lower panel, Figure 2B), suggesting that endogenous ZNF216 is not directly bound to the Ubl domain but associates with the 26S proteasome.

Colocalization with the aggresome

Next, we determined the subcellular localization of ZNF216. Indirect immunofluorescence of ZNF216 expressed in COS-7 cells showed that the protein was largely cytoplasmic but was seen to a lesser extent in the nucleus (Figure 3A). Aggresomes, which are insoluble aggregates of ubiquitylated proteins complexed with the proteasome and induced by treatment with proteasome inhibitors, are known to mimic inclusions seen in pathogenic UPS disorders (Johnston *et al*, 1998; Kopito, 2000; Lelouard *et al*, 2002). As shown in Figures 3D–H, ZNF216 proteins were colocalized with aggresomes induced by treatment with the proteasome inhibitor MG132. ZNF216 itself was not ubiquitylated as shown in Figure 1C.

Induction of ZNF216 expression upon muscle atrophy

Biochemical and cell biological evidence presented here strongly suggests that ZNF216 functions in the UPS. In skeletal muscle, it is generally accepted that the UPS plays a critical role in muscular atrophy, and expression of atrophy-related genes including those encoding UPS components is induced in atrophying muscle (Jagoe *et al*, 2002; Lecker *et al*, 2004). As *Znf216* was predominantly expressed in brain and skeletal muscle (Scott *et al*, 1998), we investigated the relationship between ZNF216 and muscle atrophy. To determine whether *Znf216* expression is induced during muscle atrophy, an *in vitro* model of muscle atrophy was utilized. It has been reported that addition of dexamethasone to cultures of differentiated C2C12 myotubes causes formation of myotubes exhibiting signs of atrophy, including a reduction in myotube diameter (Stitt *et al*, 2004). Such treatment dramatically induced expression of *Znf216* (Figure 4A).

Next, expression of *Znf216* was determined in *in vivo* experimental models of muscle atrophy. Mice that undergo fasting for 2 days show significant decreases in body weight, as well as in the mass of the gastrocnemius muscles (data not shown). In this model, fasting for 2 days results in dramatic increases in *Znf216* mRNA (Figure 4B) and protein (Supplementary Figure S3) in muscle. Although there were differences in induction patterns of two differently sized transcripts of *Znf216* by atrophy-inducing stimuli, both transcripts encode the same protein (Supplementary Figures

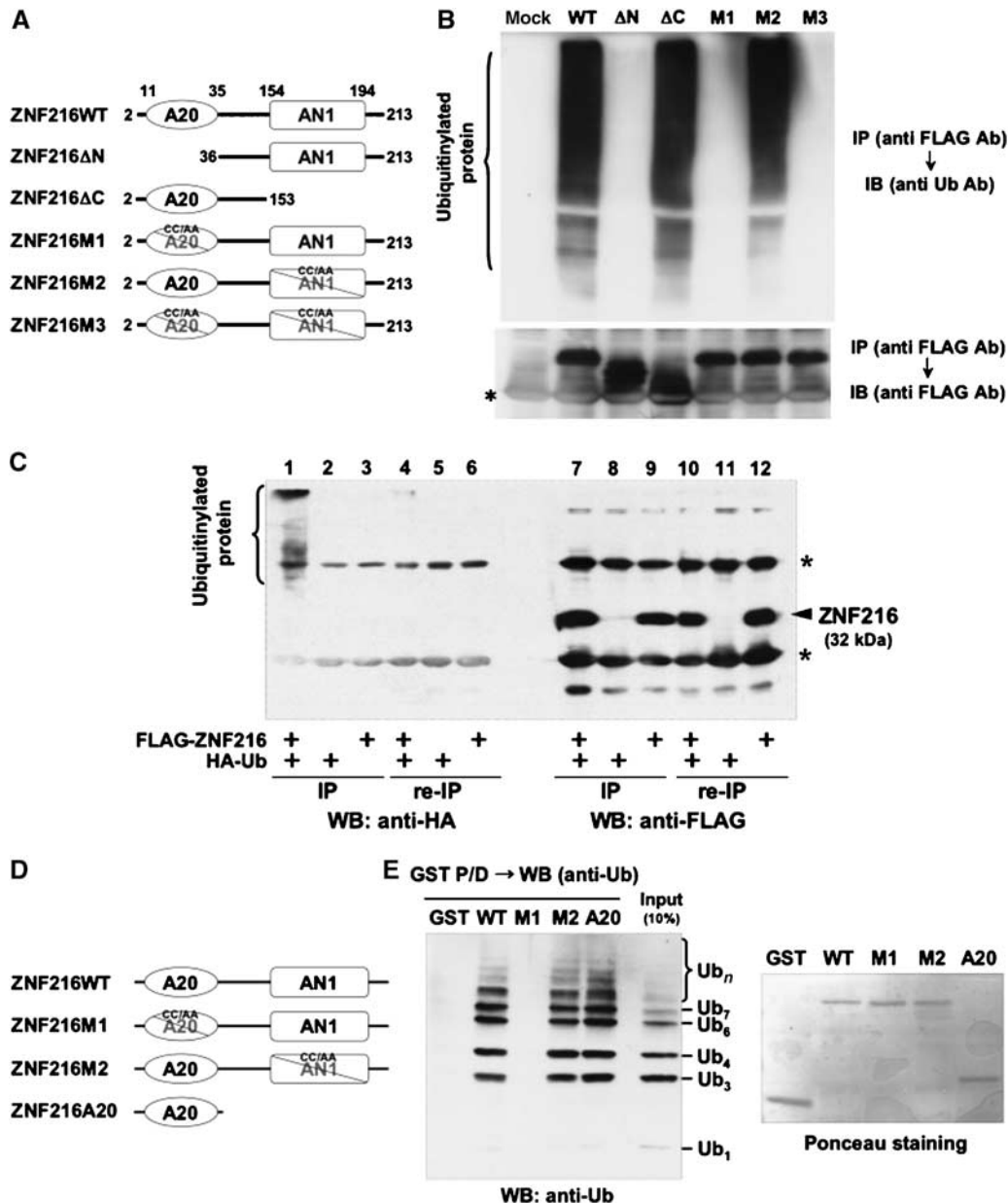


Figure 1 ZNF216 binds polyubiquitin directly through the ZnF-A20 domain. (A) Schematic representation of the primary structure of wild-type ZNF216 and its mutants. ZNF216ΔN (aa 36–213) and ZNF216ΔC (aa 2–153) constructs lack the ZnF-A20 (aa 11–35) and ZnF-AN1 (aa 154–194) domains, respectively. Cysteine residues at positions 30 and 33 within the ZnF-A20 were substituted with alanines (C30A/C33A) in ZNF216M1, and both cysteines 170 and 175 within the ZnF-AN1 were substituted with alanines (C170A/C175A) in ZNF216M2. Both ZnF-A20 and ZnF-AN1 domains were mutated in ZNF216M3. (B) Co-precipitation of ubiquitinated proteins and ZNF216. FLAG-tagged ZNF216 or mutants were expressed in HEK293 cells, and cell extracts were immunoprecipitated with anti-FLAG antibody. Ubiquitinated proteins detected with anti-ubiquitin antibody were precipitated with FLAG-tagged ZNF216 but not with ZnF-A20 mutants. Expression levels of FLAG-tagged ZNF216 constructs are shown at the bottom. Bands corresponding to immunoglobulin chains are marked by an asterisk. (C) ZNF216 is minimally ubiquitinated. HEK293 cells expressing FLAG-tagged ZNF216 or HA-tagged ubiquitin were lysed and immunoprecipitation was performed using anti-FLAG antibody. Aliquots of precipitated beads were boiled and immunoprecipitated again (re-IP). Each sample was separated on gels and probed with anti-HA (left) or anti-FLAG antibody (right). Bands for immunoglobulin chains are marked by asterisks. (D) Constructs used for *in vitro* binding assay. ZNF216WT, ZNF216M1 and ZNF216 M2 were as indicated in (A). ZNF216A20 possesses only the A20 domain (aa 2–60). All constructs were produced as GST fusion proteins. (E) *In vitro* ubiquitin binding assay. Left panel: GST protein fused to the constructs indicated in (D) was incubated with purified K48-linked polyubiquitin chains, followed by precipitation with GSH beads. In all, 10% of purified polyubiquitin chains was separated without pull-down to evaluate protein amount (10% input). Right panel: the membrane was stained with ponceau to evaluate levels of GST fusion protein.

S2 and S3). Expression of MuRF-1 (Figure 4B) and MAFbx (Gomes *et al*, 2001) was also induced in fasting. Upregulation of *Znf216* was also observed in a model of denervation-induced muscle atrophy. Neurectomy promotes significant reduction (~20%) in the weight of gastrocnemius muscles

within the first 7 days postsurgery. As expected, expression of *Znf216* and MuRF-1 was induced in gastrocnemius muscles by denervation-induced muscle atrophy (Figure 4C). These results suggest that *Znf216* expression is associated with atrophy in skeletal muscles.

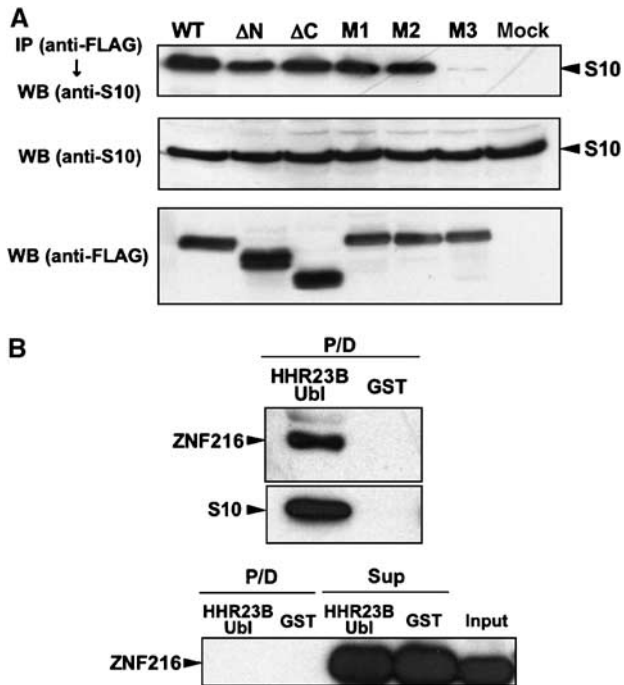


Figure 2 Interaction of ZNF216 with the 26S proteasome in mammalian cells. **(A)** Co-precipitation of the 26S proteasome and ZNF216. Co-precipitated proteins with FLAG-ZNF216 were resolved by SDS-PAGE and detected by immunoblotting using anti-S10a/Rpn7p antibody (anti-S10) or anti-FLAG antibody. Aliquots of cellular extracts were immunoblotted without immunoprecipitation to evaluate protein expression in the bottom panels. **(B)** ZNF216 was detected in the 26S proteasome fraction. Upper panel, cell lysates were incubated with a GST fusion of HHR23B Ubl (HHR23B Ubl) to isolate the 26S proteasome. Precipitated proteins (P/D) were separated and probed with anti-S10 or anti-ZNF216 antibody. Lower panel: purified recombinant ZNF216 was incubated with a GST fusion of HHR23B Ubl or GST protein. Precipitated (P/D) or not precipitated (Sup) proteins were probed with anti-ZNF216 antibody. No direct binding of ZNF216 to the Ubl domain of HHR23B was detected.

The transcription factor FOXO has been reported to play a critical role in muscular atrophy by inducing atrophy-related genes, including MAFbx/Atrogin-1 (Sandri *et al*, 2004; Stitt *et al*, 2004). Therefore, we asked whether FOXO activation upregulated *Znf216* expression. To do so, we employed a Cre-loxP system (Furukawa-Hibi *et al*, 2002) in which constitutively active FOXO4 (AFX-TM) created by mutation of the three Akt phosphorylation sites, T32A, S253A and S315A (Brunet *et al*, 1999), was expressed in C2C12-AFX-TM cells following infection by Cre recombinase-expressing adenovirus (Cre) (Figure 4D). Both AFX-TM mRNA and protein were induced 24 h after infection with Cre but not with control adenovirus (Furukawa-Hibi *et al*, 2002). ZNF216 mRNA was markedly increased in C2C12-AFX-TM cells as a result of infection with Cre but not following infection with control virus (Figure 4E). These results suggest that ZNF216 may function as a downstream effector of FOXO in muscle atrophy.

Generation of mice lacking ZNF216

To investigate the *in vivo* function of ZNF216, mice deficient for ZNF216 (*Znf216*^{lex/lex}) were generated by gene trapping at Omnibank of Lexicon Genetics (Zambrowicz *et al*, 1998). The structure of the predicted trapped gene is shown in Figure 5A. The trapping vector, VICTR48, was inserted 3.3 kbp upstream of exon 3, which encodes the first methionine of mouse *Znf216* (Figure 5A). *Znf216*^{lex/lex} mice were born from interbred heterozygous *Znf216*^{+/lex} mice in Mendelian ratios, indicating that ZNF216 is dispensable for embryogenesis or fetal development. No ZNF216 mRNA or protein was detected in *Znf216*^{lex/lex} mice by Northern or immunoblot analyses, respectively (Figures 5B and C), indicating that the mice are ZNF216 nulls. Expression levels of ZNF216 in *Znf216*^{+/lex} heterozygotes were nearly one-half those of wild-type mice. *Znf216*^{lex/lex} mice were viable and fertile, without gross abnormalities or apparent pathological alteration, but they weighed less than sex- and age-matched controls (Figure 5D). At 45 weeks, the average weights of *Znf216*^{+/+} and

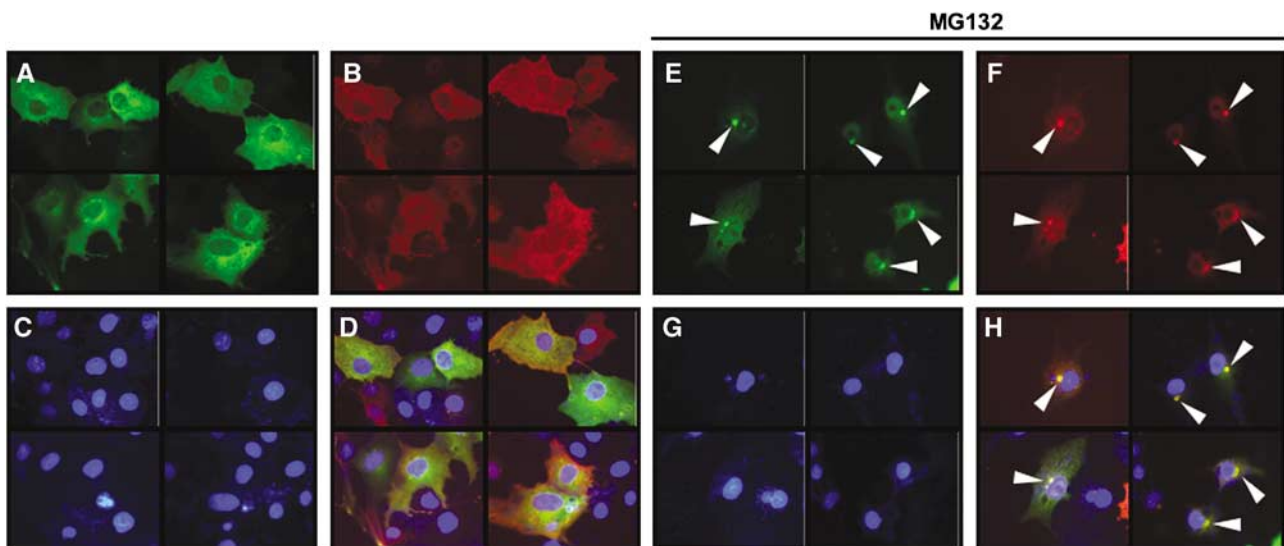


Figure 3 ZNF216 is localized in 'aggresomes' with ubiquitinated proteins. **(A-H)** COS cells were transfected with expression vectors for FLAG-tagged ZNF216 and HA-tagged ubiquitin. Fixed cells were subjected to indirect immunofluorescence using **(A, E)** anti-FLAG (with AlexaFluor 488 anti-mouse IgG, green) and **(B, F)** anti-HA (with AlexaFluor 546 anti-rat IgG antibodies, red) antibodies. **(C, G)** Nuclei were stained with DAPI in the same fields of each panel. **(E-H)** Transfected COS cells were treated with the proteasome inhibitor, MG132 (0.5 μM). Aggresomes formed are indicated by arrowheads. The merged images were shown in **(D and H)**.

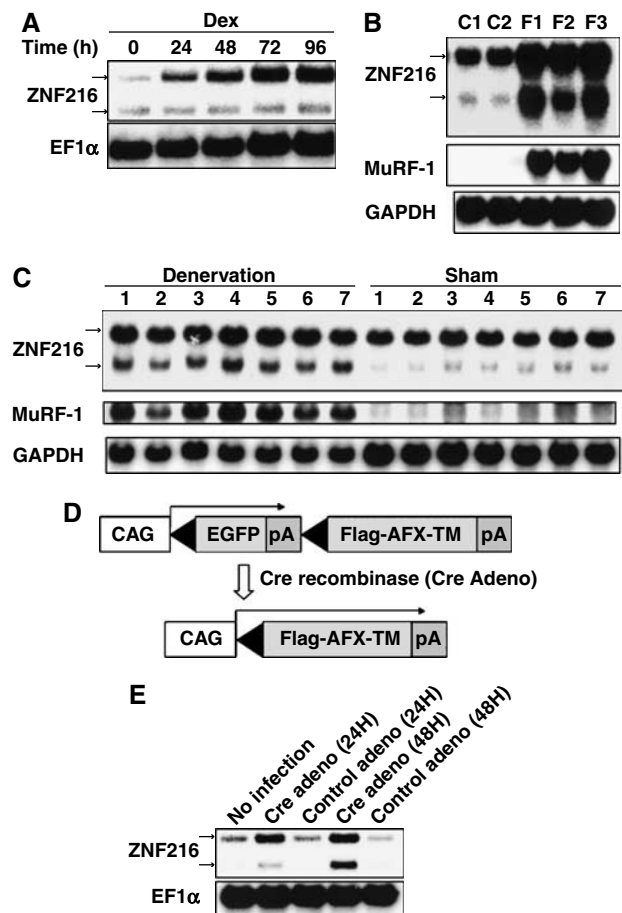


Figure 4 Expression of ZNF216 is induced by muscle atrophy. (A) C2C12 myoblast cells were differentiated into myotubes, and treated with 100 μ M Dex for the indicated times. Northern blotting was performed to reveal the effect of Dex on ZNF216 expression. The entire coding region of ZNF216 was used as a probe, which recognized 2.4 and 1.5 kb mRNA species arising from alternative splicing and polyadenylation. The loading control was elongation factor α (EF1 α). (B) Fasting-induced muscle atrophy. Three mice were fasted (F1 ~ F3), and two mice (C1, C2) were fed freely. After 2 days, RNA was purified from gastrocnemius muscle, and Northern blotting was performed to determine ZNF216 expression. The membrane was re-probed with MuRF-1 and GAPDH. (C) Denervation-induced muscle atrophy was induced by cutting the sciatic nerve of the hindlimb of seven mice (1 ~ 7). The opposite limb was sham operated as the control. At 7 days after surgery, total RNA was purified from gastrocnemius muscles, and Northern blotting was performed to detect ZNF216 expression. The membrane was re-probed with MuRF-1 and GAPDH. (D) Cre-loxP-mediated, constitutively active FOXO expression system. cDNA encoding FLAG-tagged constitutively active FOXO4 (AFX-TM) is separated from the CAG promoter of an expression vector by a loxP-flanked EGFP-poly(A) cassette. Infection with adenovirus expressing Cre recombinase (Cre) results in excision of the DNA fragment located between the two loxP sequences and expression of FLAG-tagged AFX-TM. (E) ZNF216 is downstream of FOXO. Total RNAs were prepared from C2C12-AFX-TM cells at the indicated times after infection with adenovirus expressing Cre (Cre) or lacZ (control) and probed by *Znf216* or EF1 α . A marked increase in expression of *Znf216* was observed only in Cre-infected cells.

Znf216^{lex/lex} male mice were 42.66 \pm 7.06 g (*n* = 14) and 33.16 \pm 4.44 g (*n* = 9), respectively. The average weights of female *Znf216*^{+/+} and *Znf216*^{lex/lex} mice were 34.46 \pm 4.21 g (*n* = 14) and 26.85 \pm 5.38 g (*n* = 11), respectively. After 30 weeks, both female and male *Znf216*^{lex/lex} mice showed no or subtle increases in weight, whereas *Znf216*^{+/+} or

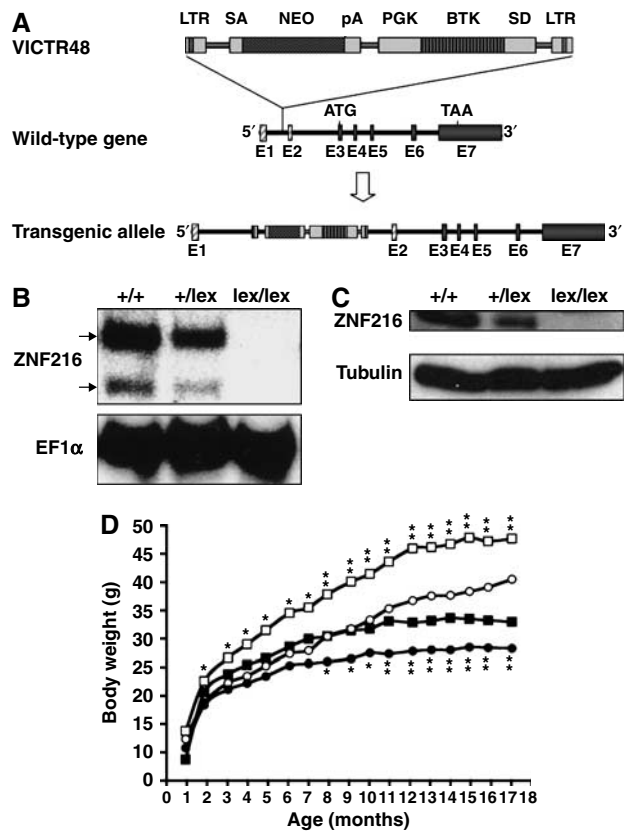


Figure 5 Disruption of *Znf216* gene in mice. (A) Gene trap strategy of *Znf216* gene. The structure of the trapping vector, VICTR48, is shown in the upper line. The wild-type allele and the trapped, transgenic allele follow the vector. The retroviral vector, VICTR48, was integrated between exons 1 and 2 of the *Znf216* gene and transcription of downstream exons encoding ZNF216 was diminished. Exons are depicted by striped (noncoding exons) or shadowed boxes (protein-coding exons) and numbered (E1 and E2). LTR, long terminal repeat; SA, splice acceptor site; SD, splice donor site; pA, polyadenylation signal; PGK, PGK promoter. (B) Northern blot analysis. Total RNA was prepared from brains of *Znf216*^{+/+}, *Znf216*^{+/-lex} or *Znf216*^{lex/lex} mice. Full-length mouse ZNF216 cDNA was used as a probe. The membrane was re-probed using an EF1 α probe. (C) Immunoblot analysis. Extracts from brain of *Znf216*^{+/+}, *Znf216*^{+/-lex} or *Znf216*^{lex/lex} mice were immunoblotted with antibody against ZNF216. The membrane was re-probed using anti-tubulin antibody. (D) Growth curve of *Znf216*^{lex/lex} mice. Body weights at each time point of *Znf216*^{+/+} and *Znf216*^{lex/lex} mice were indicated as open square boxes (males) or circles (females) and closed square boxes (males) or circles (females), respectively. **P* < 0.05; ***P* < 0.005.

Znf216^{+/-lex} mice gained weight as they aged (Figure 5D). The size of most organs in *Znf216*^{lex/lex} mice was reduced in proportion with body weight. However, the fat volume of aged (> 30 weeks of age) *Znf216*^{lex/lex} mice was significantly decreased, suggesting that the marked difference in body weight between wild-type and aged *Znf216*^{lex/lex} mice is mainly caused by decreased fat mass seen in *Znf216*^{lex/lex} mice (not shown). Detailed phenotypic characterization of aged mutant mice will be provided elsewhere.

***Znf216*^{lex/lex} mice exhibit partial resistance to denervation-induced muscle atrophy**

To further explore the involvement of ZNF216 in muscle atrophy, neurectomy of sciatic nerve was undertaken in wild-type and *Znf216*^{lex/lex} mice. As shown in Figure 6A, 7

days after denervation, significant muscle weight loss and reduction in fiber sizes of the gastrocnemius muscle were observed in wild-type mice. By contrast, such decreases in muscle weight were significantly attenuated in *Znf216^{lex/lex}* mice (Figure 6A). Sections of gastrocnemius muscle also showed larger fibers in muscle from neurectomized *Znf216^{lex/lex}* mice than in control muscle (Figure 6B). However, there was no significant difference in fiber area between sham-operated wild-type and *Znf216^{lex/lex}* mice (wild type + sham operated, $1988 \pm 530 \mu\text{m}^2$; wild type + denervation, $1379 \pm 345 \mu\text{m}^2$; lex/lex + sham operated, $1776 \pm 484 \mu\text{m}^2$; lex/lex + denervation, $1393 \pm 344 \mu\text{m}^2$). As shown in Figure 6C, the reduction in fiber area was also less apparent in *Znf216^{lex/lex}* mice compared to wild-type mice. These results suggest that ZNF216 plays a crucial role in reduction of muscle mass on denervation-induced muscle atrophy.

Abnormal accumulation of ubiquitinated proteins in muscle from *Znf216^{lex/lex}* mice

To investigate what abnormalities occur during denervation-induced muscle atrophy in *Znf216^{lex/lex}* mice, we examined

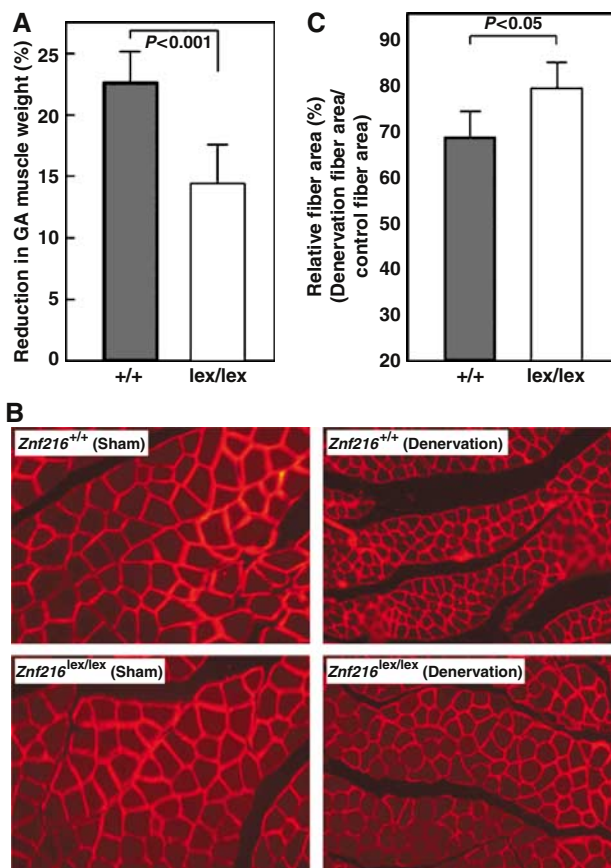


Figure 6 Denervation induced muscular atrophy was attenuated in *ZNF216^{lex/lex}* mice. (A) Reduction of GA muscle weight upon neurectomy. Percent decreases in muscle weights are shown as a percent of control, calculated as the left/right muscle weights. (B) Cross-sections from gastrocnemius muscle were stained by indirect immunofluorescence with anti-laminin. The reduction in size was also significant in muscle fibers of control mice but less in *Znf216^{lex/lex}*. (C) Muscle fiber cross-sectional areas were measured in transverse tissue section (B). Percent relative fiber area of denervated muscle to control fiber area (sham-operated) are shown.

expression levels of factors involved in muscle atrophy. As expected, expression of MAFbx/Atrogin-1 and MuRF-1 was dramatically induced by denervation-induced muscle atrophy in gastrocnemius muscle from wild-type mice (Figure 6A). In *Znf216^{lex/lex}* mice, expression of MAFbx/Atrogin-1 and MuRF-1 was also induced at levels comparable to those seen in wild-type mice. Induction of *Pmsa1* and *Pmsd11*, genes encoding the 26S proteasome subunits $\alpha 6$ and Rpn6, respectively, was also indistinguishable between *Znf216^{lex/lex}* and wild-type mice (Figure 7A). Furthermore, proteasome activities in gastrocnemius muscles were comparable between wild-type and *Znf216^{lex/lex}* mice (Figure 7B). Thus, induction of relevant ubiquitin ligases or proteasome components was not affected in *Znf216^{lex/lex}* mice. It is known that ubiquitinated proteins accumulate during muscle atrophy (Medina *et al*, 1991; Wing *et al*, 1995). As shown in Figure 7C, following denervation, ubiquitinated proteins accumulated in the gastrocnemius muscle of wild-type mice, but higher levels of ubiquitinated proteins accumulated in muscle derived from *Znf216^{lex/lex}* mice (~2-fold: $P < 0.001$ in neurectomized *Znf216^{lex/lex}* versus wild-type muscle). Similar results were obtained by fasting-induced muscle atrophy, although no difference in the levels of ubiquitinated proteins from controls (sham-operated or fed) was observed between genotypes (Figure 7C). These results indicate that ZNF216 is a critical regulator of muscle atrophy, most likely functioning to regulate degradation of muscle proteins without altering expression of proteasomal components or known E3 ligases.

Effect of ZNF216 on UPS-mediated protein degradation

Accumulation of ubiquitinated protein under any circumstance might be because of loss of inhibition of ubiquitination and/or deubiquitination (DUB). However, no inhibition or DUB activity was observed (Supplementary Figures S4 and S5). As shown in Figure 7D, association of ZNF216 protein to the proteasome was significantly increased when atrophy was induced, suggesting that ZNF216 may be involved in association of ubiquitinated proteins and the proteasome. The biochemical activity of ZNF216 is similar to that of the UPS proteins, hHR23 and hPLIC, both of which have a shuttle function and are known to bind to both polyubiquitinated proteins and the 26S proteasome (Hartmann-Petersen and Gordon, 2004; Elsasser and Finley, 2005). Interestingly, overexpression of hHR23 and hPLIC results in stabilization of unstable proteins such as p53 (Kleijnen *et al*, 2000; Glockzin *et al*, 2003). To determine if ZNF216 functioned similarly, we employed a degradation system using unstable GFP (Bence *et al*, 2001). In this system, the CL1 peptide, which functions as a degron, is fused to EGFP (EGFP-CL1). Degradation by conjugation with the degron is mediated by the UPS (Bence *et al*, 2001). EGFP-CL1, constitutively expressed in HEK293 cells, is unstable and the estimated half-life ($t_{1/2}$) of EGFP-CL1 in this system is about 11 min. Ubiquitinated EGFP-CL1 protein stabilized by treatment with a proteasome inhibitor was associated with ZNF216 but EGFP itself was not (not shown). As shown in Figure 8A, protein degradation was markedly retarded in the presence of ectopic ZNF216 ($t_{1/2} > 30$ min) compared to cells transfected with the loss of function mutant ZNF216M3 or mock-transfected cells. Rapid turnover of EGFP-CL1 protein was inhibited by treatment with the

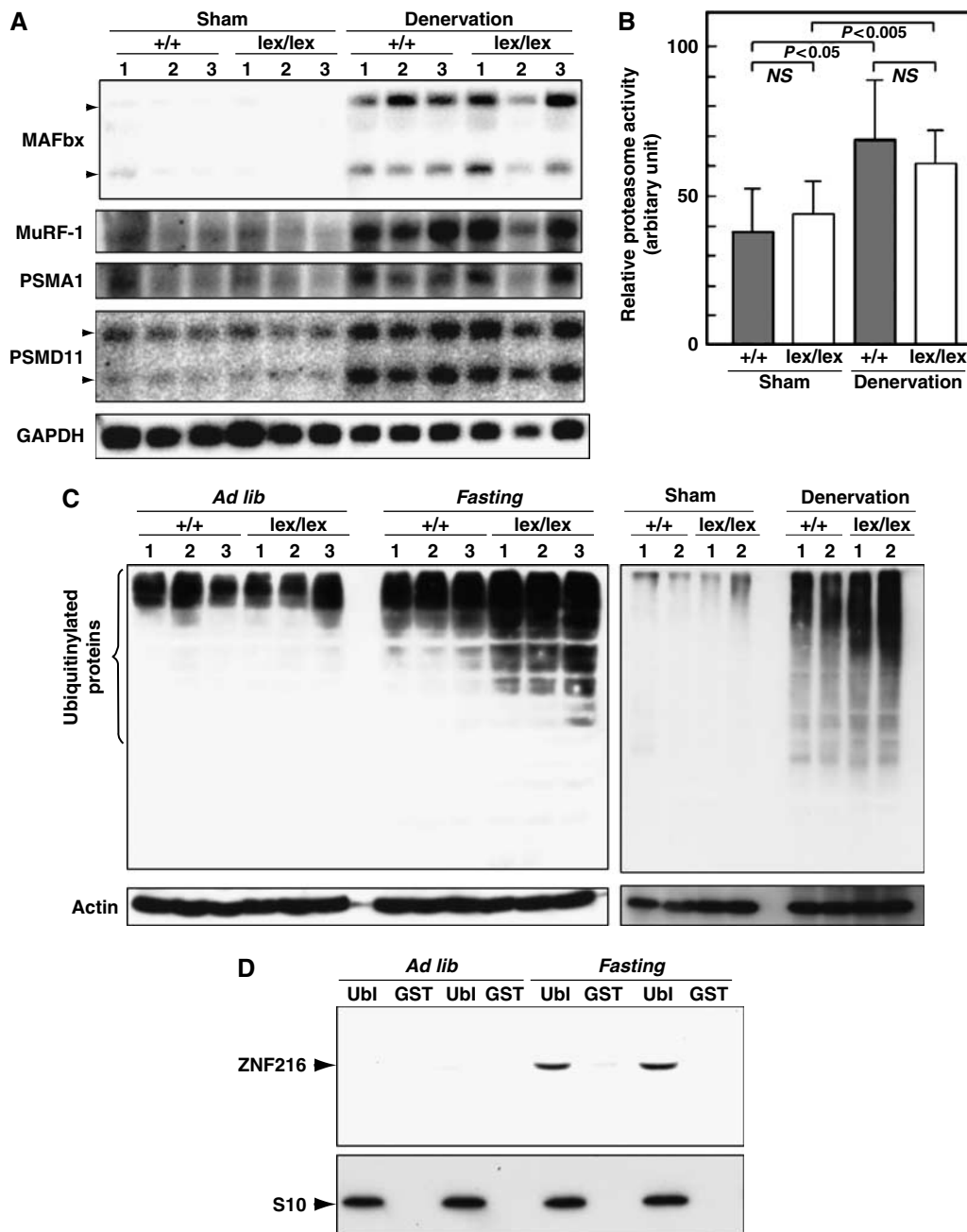


Figure 7 Changes in UPS upon muscular atrophy. (A) Expression of UPS components in denervation-induced muscular atrophy. Total RNAs were purified from gastrocnemius muscle, and Northern blotting was performed using indicated probes. Expression of genes for ubiquitin-ligases, such as MAFbx or MuRF-1, and proteasome subunits PSMA1 and PSMD11 was induced by muscle atrophy at comparable levels between wild-type and *ZNF216^{lex/lex}* mice. (B) Proteasome activity. Proteasome activities in muscle extracts from wild-type or *ZNF216^{lex/lex}* mice were measured and are shown as arbitrary units. No significant difference in proteasome activity between wild-type and *ZNF216^{lex/lex}* was observed. (C) High levels of ubiquitinated proteins accumulated in muscles from *ZNF216^{lex/lex}* mice than in muscles from wild-type mice. Muscle extracts from wild-type or *ZNF216^{lex/lex}* mice were subjected to immunoblotting using anti-ubiquitin antibody to analyze levels of ubiquitinated proteins. Left and right panels show fasting-induced and denervation-induced muscle atrophy, respectively. Each membrane was re-probed with anti-actin antibody. (D) Association of ZNF216 with the proteasome was increased upon atrophy. The proteasome fractions in muscle extracts from fed (*ad lib*) or fasted (fasting) mice were precipitated with GST-Ubi or GST only as a negative control. Endogenous ZNF216 protein was co-precipitated with the proteasome, which is probed by the anti-S10 antibody.

proteasome inhibitor MG132 (MG132, Figure 8B). The levels of the proteins stabilized by MG132 were comparable among cells transfected with ZNF216 constructs, indicating that protein synthesis of EGFP-CL1 was not significantly affected by ectopic expression of ZNF216 (MG132, Figure 8B). ZNF216WT, and to a lesser extent the mutants M1 and M2 but not M3, attenuated degradation (NT, Figure 8B). Thus, as is the case with other shuttle proteins,

overexpression of ZNF216 inhibits degradation of unstable proteins via the UPS.

Discussion

ZNF216 is an atrogene

In this report, we show that *Znf216^{lex/lex}* mice exhibit resistance to denervation-induced muscle atrophy. It has been

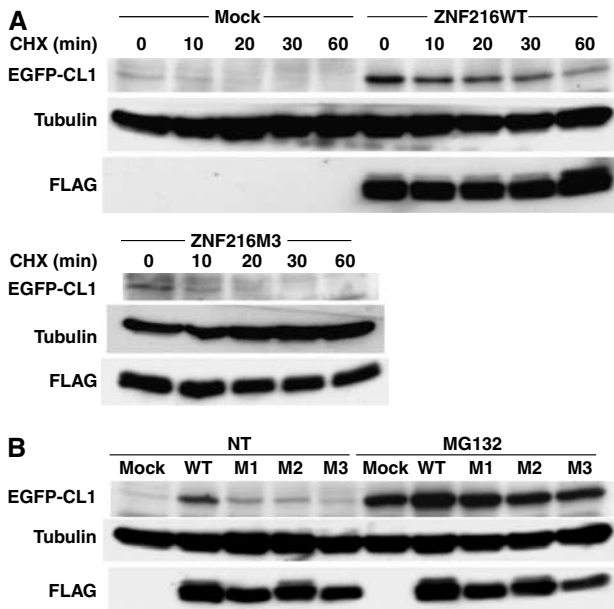


Figure 8 Ectopic expression of ZNF216-affected protein degradation. (A) Degradation of EGFP-CL1 protein was delayed by overexpression of ZNF216. 293 cells stably expressing EGFP-CL1 were transfected with plasmid of ZNF216WT, ZNF216M3 or pcDNA3 (mock). Estimated half-lives of the EGFP-CL1 are 35, 11 and 11 min in ZNF216WT-, ZNF216M3- and mock-transfected cells, respectively. *De novo* protein synthesis was arrested by cycloheximide (CHX). The membrane was re-probed with tubulin antibody to control for protein loading (tubulin) and FLAG antibody to detect ZNF216 expression (FLAG). (B) Degradation of EGFP-CL1 protein in the presence of various ZNF216 constructs. HEK293 cells stably expressing EGFP-CL1 were transfected with plasmids expressing the indicated mutants. Transfected cells were not treated (NT) or MG132-treated (MG132), and EGFP-CL1 protein was detected with an anti-GFP antibody (EGFP-CL1). The membrane was re-probed with tubulin antibody to control for protein loading (tubulin) and FLAG antibody to detect ZNF216 expression (FLAG).

shown that TNF α induces catabolic conditions through UPS during cancer cachexia (Mitch and Price, 2001). Recently, it has been reported that mice deficient in molecules involved in the NF- κ B pathway exhibit resistance to muscular atrophy (Cai *et al*, 2004; Hunter and Kandarian, 2004; McKinnell and Rudnicki, 2004). On the other hand, the IGF-FOXO axis has been suggested to regulate muscle mass through induction of 'atrogenes' such as Murf1 and MAFbx/Atrogin-1 (Sandri *et al*, 2004; Stitt *et al*, 2004). Although we provide evidence that *Znf216* is downstream of FOXO, the NF- κ B pathway could represent an alternative signal inducing ZNF216. Indeed, we have identified *Znf216* as a gene induced by RANKL, a TNF family ligand (Hishiya *et al*, 2005) which activates the NF- κ B pathway through RANK (Anderson *et al*, 1997; Lacey *et al*, 1998). Moreover, TNF α and IL-1 β upregulate expression of ZNF216 in fibroblasts and macrophages (Hishiya *et al*, 2005). These results suggest that *Znf216* may be activated by NF- κ B. Huang *et al* (2004) recently reported that ZNF216 inhibits the NF- κ B pathway. Whereas treatment with TNF α or overexpression of TRAF6 dramatically activated a reporter driven by NF- κ B response elements, ectopic expression of A20/TNFAIP3 but not ZNF216 inhibited NF- κ B activation (not shown). Using mouse embryonic fibroblasts, splenocytes or bone marrow cells from *Znf216*^{lex/lex} or wild-type mice, no significant differences were observed in TNF α -

dependent NF- κ B activation, LPS-induced cytokine expression or proliferation (unpublished data). Therefore, ZNF216 seems to function as a downstream effector (i.e., a component of the UPS) rather than a specific negative regulator of NF- κ B signaling, although ZNF216 function in that pathway is still under investigation. Whereas expression of ZNF216 is not restricted to muscle, such expression was induced upon muscular atrophy and loss of function of *Znf216* promotes resistance to denervation-induced atrophy, thereby suggesting that it fulfills the definition of an 'atrogene'.

As it is in skeletal muscle, ZNF216 is highly expressed in the brain (Scott *et al*, 1998). Aberrations in the UPS have been documented in the pathogenesis of neurodegenerative diseases such as Parkinson's and Huntington's diseases (Ross and Poirier, 2004). Massive accumulation of ubiquitinated proteins, which are often aggregated and impair the UPS leading to neuronal degeneration, has been observed in these pathogenic conditions (Ciechanover and Brundin, 2003; Korhonen and Lindholm, 2004). In cultured cells, blocking the UPS by proteasome inhibitors leads to accumulation of ubiquitinated proteins. These ubiquitinated proteins are then transferred to perinuclear locations and form aggresomes (Johnston *et al*, 1998). As shown here, ZNF216 is localized in aggresomes together with ubiquitinated proteins. Interestingly, proteomic analysis of a protein complex containing HDAC6, a protein often associated with aggresomes (Kawaguchi *et al*, 2003), showed that the complex included AWP1, a structural homologue of ZNF216 (Seigneurin-Berny *et al*, 2001). Although it is unclear whether ZNF216 is involved in aggresome formation, there is great interest in the role of ZNF216 in the pathogenesis of neurodegenerative diseases.

Molecular function of an A20-containing protein, ZNF216

In muscle atrophy, more ubiquitinated proteins accumulate in muscle from *Znf216*^{lex/lex} mice than in muscle from wild-type mice, suggesting an abnormal UPS function. Inhibition of neither polyubiquitinylation nor DUB activity was observed in ZNF216. Although our *in vivo* data showed significant accumulation of polyubiquitinated proteins in muscle from *Znf216*^{lex/lex} mice, there is a possibility that ZNF216 is a ubiquitin-ligase. It has been recently reported that A20/TNFAIP3 protein possesses ubiquitin ligase activity against RIP through its ZnF-A20 repeats (Wertz *et al*, 2004). We asked whether the ZnF-A20 of ZNF216 exhibited activity similar to A20/TNFAIP3, but *in vitro* ubiquitinylation assays were negative (Supplementary Figure S6). In fact, the ZnF-A20 of A20/TNFAIP3 protein does not bind polyubiquitin chains as does the ZnF-A20 of ZNF216 (Supplementary Figure S1). Furthermore, there are seven ZnF-A20 motifs in A20/TNFAIP3 and only the fourth is responsible for E3 activity, suggesting that the ZnF-A20 motif is not inherently active enzymatically (Wertz *et al*, 2004). However, we cannot exclude the possibility that ZNF216 may possess DUB or E3 activity highly specific to an unknown substrate without nonspecific or self-ubiquitinylation activity.

ZNF216 likely acts as a bridging or a shuttle factor of ubiquitinated proteins targeted to the proteasome. Shuttle proteins, such as Rad23p and Dsk2p, share interfaces for ubiquitinated proteins and the proteasome (Hartmann-Petersen and Gordon, 2004; Elsasser and Finley, 2005).

Although shuttle proteins are required for efficient protein degradation, ectopic expression of hHR23 or hPLIC, the human homologues of Rad23p or Dsk2p, respectively, lead to stabilization of p53 protein (Kleijnen *et al*, 2000; Glockzin *et al*, 2003). These outcomes may be caused by titration effects due to overexpression and are commonly observed following misexpression of shuttle proteins in yeast and mammals (Hartmann-Petersen and Gordon, 2004; Verma *et al*, 2004). Here, we show that ZNF216 has a ubiquitin binding domain and can associate with the 26S proteasome even in the absence of ubiquitin binding, and that overexpression of the zinc-finger protein attenuates protein degradation rate. There is no structural counterpart of ZNF216 in the yeast genome. We asked whether ZNF216 could rescue the bridging function of RAD23 or DSK2 mutants by introducing ZNF216 into $\Delta rad23\Delta dsk2$ yeast cells, but the phenotype could not be rescued (data not shown). This suggests that ZNF216 is not the functional orthologue of these proteins. Recently, the presence of an alternative pathway of Rad23p/Dsk2p in protein targeting to the proteasome has been suggested (Bazirgan and Hampton, 2005; Richly *et al*, 2005). It has been reported that tetra-ubiquitin constitutes the minimum proteasomal targeting signal and that the length of polyubiquitin chain may determine the targeting route (Thrower *et al*, 2000; Bazirgan and Hampton, 2005; Richly *et al*, 2005). Notably, ZNF216 preferentially binds polyubiquitin chains longer than di- or tri-ubiquitin (Figure 1D). Therefore, these data suggest that ZNF216 is a novel ubiquitin recognition factor, required for efficient protein degradation via a pathway different from the canonical Rad23p/Dsk2p pathway. Although it is now under investigation, the characterization of ZnF-AN1, an AN1-type zinc-finger domain located at the C-terminus of ZNF216, may reveal the precise molecular function of ZNF216.

Materials and methods

Antibodies

An anti-ZNF216 antibody was raised by immunizing rabbits against synthesized peptide corresponding to the C-terminal sequence of mouse ZNF216. Mouse monoclonal antibodies for FLAG (Sigma, St Louis, MO) and ubiquitin (Santa Cruz Biotechnology, CA), rabbit polyclonal antibodies for ubiquitin (Affiniti Research Products) and actin (Neo Markers, CA), a rat monoclonal antibody for HA (Roche Diagnostics, Mannheim, Germany), and a rabbit polyclonal antibody against S10a/Rpn7p (Affiniti Research Products) were purchased from the indicated manufacturers. For indirect immunofluorescence staining, AlexaFluor 488 goat anti-mouse IgG or AlexaFluor 546 goat anti-rat IgG antibody was obtained from Molecular Probes, OR.

Identification of interacting proteins

RNA was purified from RAW264.7 cells stimulated by RANKL, and used to construct the yeast library (MatchMaker Library Construction & Screening Kit, Clontech). Yeast two-hybrid screening with

pGBKT7-ZNF216 was performed as described previously (Masuda *et al*, 2001). Identification of the co-immunoprecipitated proteins with N- or C-terminally FLAG-tagged ZNF216 (ZA20D2) or AWP1 (ZA20D3) was essentially done by a nano-LC/MS/MS system as previously described (Natsume *et al*, 2002; Komatsu *et al*, 2004).

Experimental models of muscle atrophy

For fasting-induced muscle atrophy, 8-week-old C57BL6 male mice were deprived of food but given free access to water. After 2 days, gastrocnemius muscles were harvested for each experiment. Denervation-induced muscle atrophy was performed by dissecting the sciatic nerve of one hindlimb, and the other hindlimb was sham operated as the control. After 7 days, the contralateral normal and denervated gastrocnemius muscles were harvested for each experiment. All animal experiments were approved in advance by the Ethics Review Committee for Animal Experimentation of the National Institute for Longevity Sciences and the National Center for Geriatrics and Gerontology. Student's *t*-tests were used to evaluate statistical differences between the two groups.

Znf216-deficient mice

Generation of heterozygous *Znf216*^{+/-lex} mice was essentially done by the gene trap method at Lexicon Genetics (Zambrowicz *et al*, 1998). Briefly, ES cells heterozygous for the trapped *Znf216* gene were microinjected into eight-cell-stage ICR mouse embryos and transplanted into uteri. Chimeric mice were crossed to C57BL/6J mice. Northern and immunoblot analyses confirmed disruption of the gene (see text). For genotyping, primers were as follows: KO-A, ACCGACAGGATAGACAATGGCAGAG; KO-B, CGATTTAAGAAAG GAGGCTCTGACC; LTR2, AAATGGCGTTACTTAAGCTAGCTTGC. The wild-type and inserted alleles were detected by PCR using KO-A and KO-B (0.5 kb), and LTR2 and KO-B (0.3 kb), respectively.

EGFP-CL1 degradation assay

The nucleotide sequence encoding the CL1 peptide (ACKNWFSSLSHFVIHL) (Gilon *et al*, 1998) was inserted into the *XhoI/EcoRI* site of pEGFP-C3, and the resulting plasmid was designated pEGFP-CL1. A cell line stably expressing EGFP-CL1 (293EGFP-CL1) was generated by transfection of pEGFP-CL1 into 293 cells. For the degradation assay, ZNF216 expression vectors were transfected into 293EGFP-CL1 cells and cells were harvested 48 h after transfection. MG132 (final 10 μ M) or cycloheximide (final 100 μ g/ml) was added to the culture at 12 or 1 h before harvest, respectively. Protein extraction was as described above.

For more details on supplementary Materials and methods, see Supplementary data

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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