

Supplemental Information

Materials and methods

Plasmid construction

Full-length ZNF216 cDNA was amplified by reverse transcriptase-PCR and subcloned into pEF4/Myc-His A (Invitrogen, Carlsbad, CA, USA) with a FLAG epitope at the N terminus to generate pF-ZNF216. Zinc finger-deleted (ZNF216DN and DC) or mutated ZNF216 (ZNF216M1, M2 and M3) were generated by PCR and subcloned into pEF4/Myc-His A with a FLAG epitope at the N terminus. ZNF216 cDNAs were also subcloned into pGEX-6P-1 (Amersham Biosciences, Uppsala, Sweden) for expression as GST fusion proteins in the *Escherichia coli* strain BL21. As a bait for yeast-two hybrid screening, ZNF216 cDNA was subcloned into pGBKT7 (Clontech, Mountain View, CA, USA). For HA-tagged ubiquitin, ubiquitin cDNA was subcloned into the pcDNA3 plasmid to generate pHA-Ub. All cDNAs and their modified constructs amplified by PCR were fully sequenced (ABI PRISM™ 3100 Genetic Analyzer, Applied Biosystems, Foster City, CA, USA).

Cell culture

HEK293 cells, COS7 cells and C2C12 cells were cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS supplemented with penicillin and streptomycin. For myotube differentiation of C2C12 cells, medium was replaced with DMEM containing 2% horse serum supplemented with 10 ng/ml insulin-like growth factor I (Invitrogen). LipofectAMINE Plus (Invitrogen) reagent or FuGENE6 (Roche Diagnostics) was used for transfection.

In vitro and in vivo binding

To assay binding *in vitro*, a GST pull-down was performed as described previously (Masuda et al., 2001). Briefly, GST and GST fusion proteins were expressed in BL21 cells, induced by 1 mM isopropyl-1-thio- β -galactopyranoside, and purified by affinity chromatography using glutathione-Sepharose beads (Amersham Pharmacia Bioscience, Uppsala, Sweden). Purified K48 linked polyubiquitin chains (Affiniti Research Products) were incubated with GST, GST-ZNF216 and its mutants in RIPA buffer (50 mM Tris-HCl, pH7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate,

0.1% SDS) followed by precipitation with glutathione-Sepharose beads (Amersham Pharmacia Bioscience). Bound proteins were eluted from beads by boiling in SDS sample buffer, separated by SDS-PAGE, and visualized by immunoblotting using anti-ubiquitin antibody.

For *in vivo* association, HEK293 cells were transiently transfected with various plasmids. 24h after transfection, cells were lysed in lysis buffer (50 mM HEPES-NaOH, pH7.5, 150 mM NaCl, 50 mM NaF, 1 mM Na₃VO₄, 1 mM EGTA, 1% NP-40, 10 mM MgCl₂, 0.5 mM DTT) supplemented with a mixture of proteinase inhibitors (Complete™, Roche Diagnostics). Precleared lysates were subjected to immunoprecipitation with anti-FLAG M2-agarose affinity gel (SIGMA). Precipitated proteins were eluted from beads by boiling in SDS sample buffer, separated by SDS-PAGE. Immunoblotting was performed using indicated antibodies.

Aggresome formation

COS7 cells were transfected with plasmids encoding FLAG-ZNF216 and HA-Ub. After 24 hours, cells were treated with the proteasome inhibitor, MG132 (0.5 μM). After an additional 24 hours, cells were fixed using formalin-PBS and permeabilized using 0.2% Triton-PBS. As primary antibodies, anti-FLAG and anti-HA were used, and anti-mouse or anti-rat IgG antibody conjugated with AlexaFluor 488 or 546 was used, respectively, as the secondaries. For nuclear staining, DAPI reagent was used. Images were obtained using epifluorescence microscopy. Photographic slides were converted to digital images using AxioVision (Carl Zeiss, Germany). Processing was by Adobe Photoshop 6.0.

Northern and immuno-blot Analyses

Total RNA isolated using TRIZOL Reagent (Invitrogen) was denatured by glyoxal/DMSO, electrophoresed on a 0.9% agarose gel, and transferred to a nylon membrane (Amersham Pharmacia Biosciences). To detect the indicated mRNA, cDNA probes were labeled with ³²P using DNA labeling beads (Amersham Pharmacia Biosciences).

Extracted protein was subjected to SDS-polyacrylamide gel electrophoresis on a 10% or 12.5% gel, transferred onto polyvinylidene difluoride membranes (Amersham

Pharmacia Biosciences), and probed with indicated antibodies. Signals were visualized using ECL Plus reagents (Amersham Pharmacia Biosciences). Protein concentrations were measured using the BCL protein assay reagent (PIERCE, Rockford, IL. USA).

Measurement of proteasome activity

Proteasome activity of muscle lysates was measured using a fluorogenic substrate, Suc-LLVY-MCA (Peptide Institute, Inc., Osaka, Japan). The reaction consisted of 100 μ M peptide, 2 mM ATP, 5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 30 mM Tris-HCl, pH7.8. The reaction was initiated by adding muscle extract and incubated at 37°C for 1 hour followed by quenching with 60 ml of 10% SDS. Fluorescence was measured on a Perkin-Elmer fluorometer using an excitation wavelength of 380 nm and an emission wavelength of 440 nm.

A novel ubiquitin-binding protein ZNF216 functioning in muscle atrophy

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Supplemental information

Materials and methods for Supplemental data

De-polymerization assay

The deubiquitylation (DUB) assay was performed as described (Hook et al., 2002). Briefly, HEK293 cells were transfected with the ZNF216 constructs and extracted in lysis buffer (50 mM HEPES (pH7.5), 150 mM NaCl, 50 mM sodium fluoride, 1 mM sodium vanadate, 1 mM EGTA, 1% NP-40, 10 mM MgCl₂, 0.5 mM DTT, protease inhibitor cocktail). Lysates were immunoprecipitated with anti-FLAG M2 affinity gel (SIGMA) and the precipitates were washed with wash buffer (50 mM HEPES (pH7.5), 150 mM NaCl, 1 mM EGTA, 0.1% NP-40, 10 mM MgCl₂, 0.5 mM DTT, protease inhibitor cocktail) and Tris-buffered saline. The washed precipitates were eluted with the FLAG peptide (SIGMA) and the eluted protein was confirmed by immunoblotting using anti-FLAG M5 antibody (SIGMA). Immunopurified ZNF216 was incubated with 1.5 µg of K48 polyubiquitin chains in 50 mM Tris (pH8.0), 5 mM MgCl₂, 5 mM DTT at 37°C for 1 hour. After reaction, polyubiquitin was detected by immunoblotting using anti-ubiquitin antibody (MBL, clone 2C5).

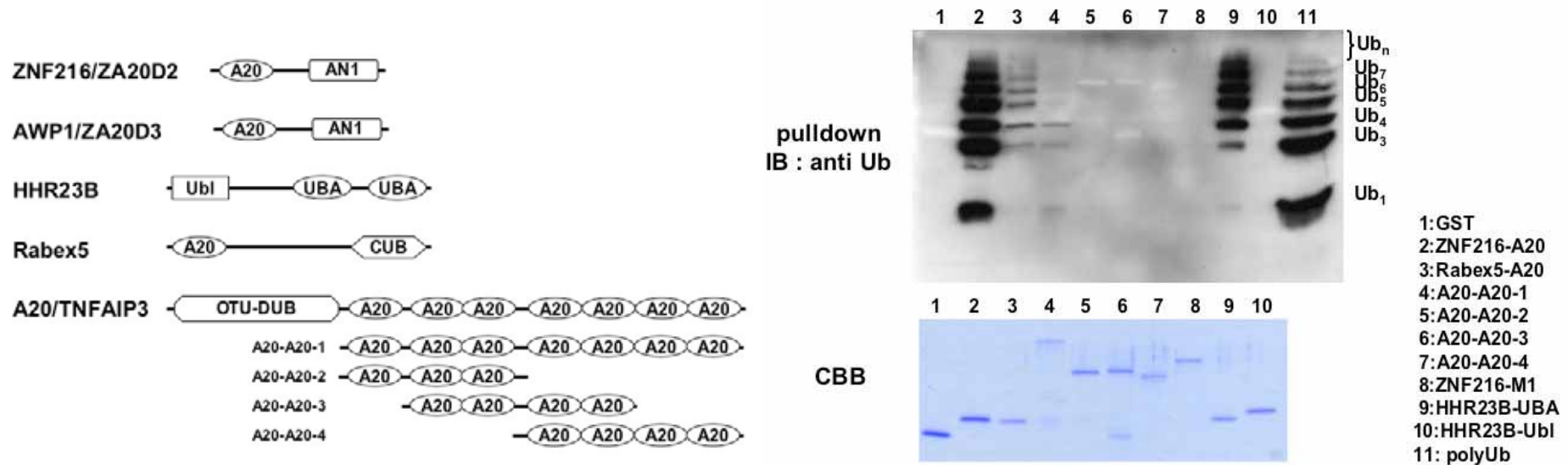
In vitro ubiquitylation

The RING finger domain of Murf1 was synthesized as a GST fusion protein. GST-Murf1 was incubated in E3 reaction buffer containing E1 (40 ng), UbcH5C (200 ng), and N-terminally biotinylated ubiquitin (400 ng) in 30 mM HEPES (pH7.2), 2 mM ATP, 5 mM MgCl₂, 0.2 mM DTT, with or without ZNF216 (200 ng) at 30°C for 1 hour. After reaction, ubiquitin was detected by blotting with avidin-HRP conjugate (BD Pharmingen). E1, UbcH5C, biotinylated ubiquitin were obtained from Boston Biochem. Ubiquitylation by A20 protein was performed as described (Wertz et al., 2004).

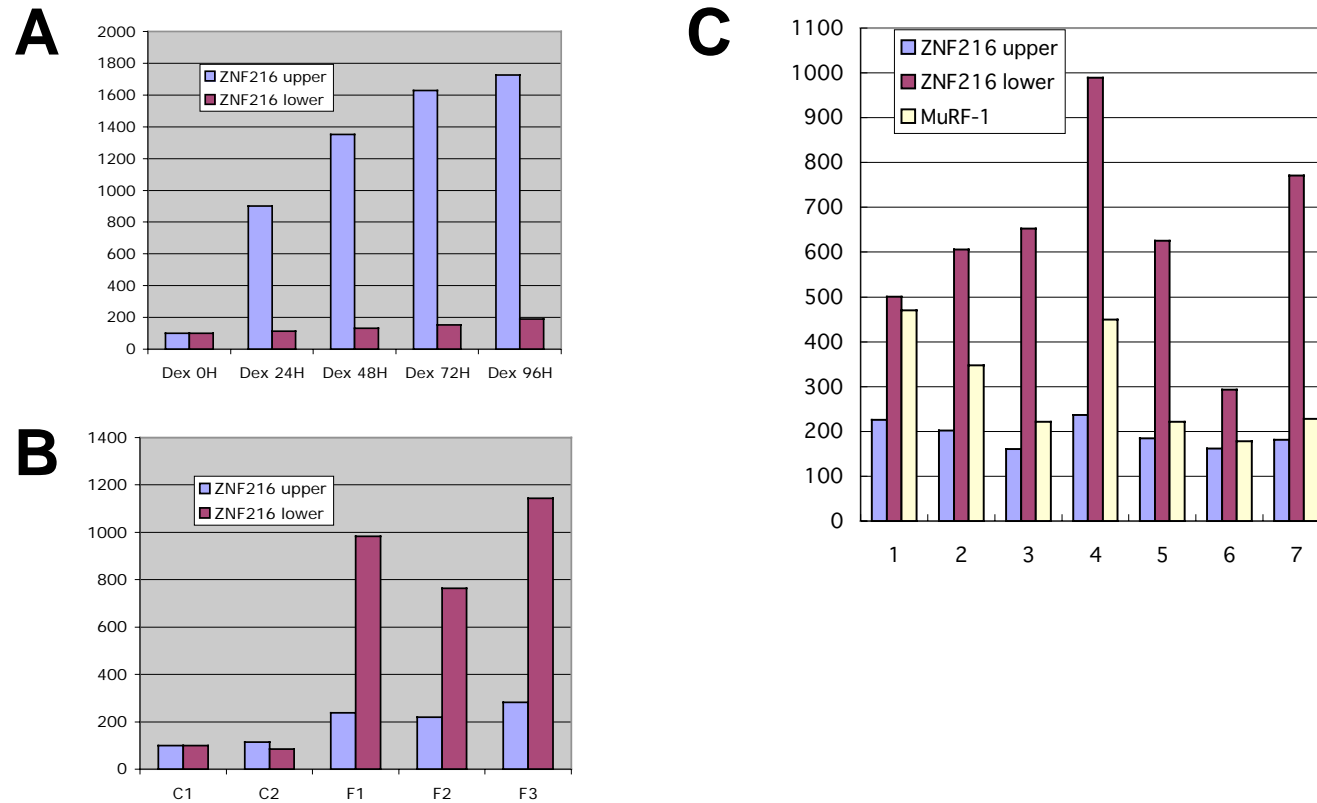
References:

Hook, S. S., Orian, A., Cowley, S. M., and Eisenman, R. N. (2002). Histone deacetylase 6 binds polyubiquitin through its zinc finger (PAZ domain) and copurifies with deubiquitinating enzymes. *Proc Natl Acad Sci U S A* 99, 13425-13430.

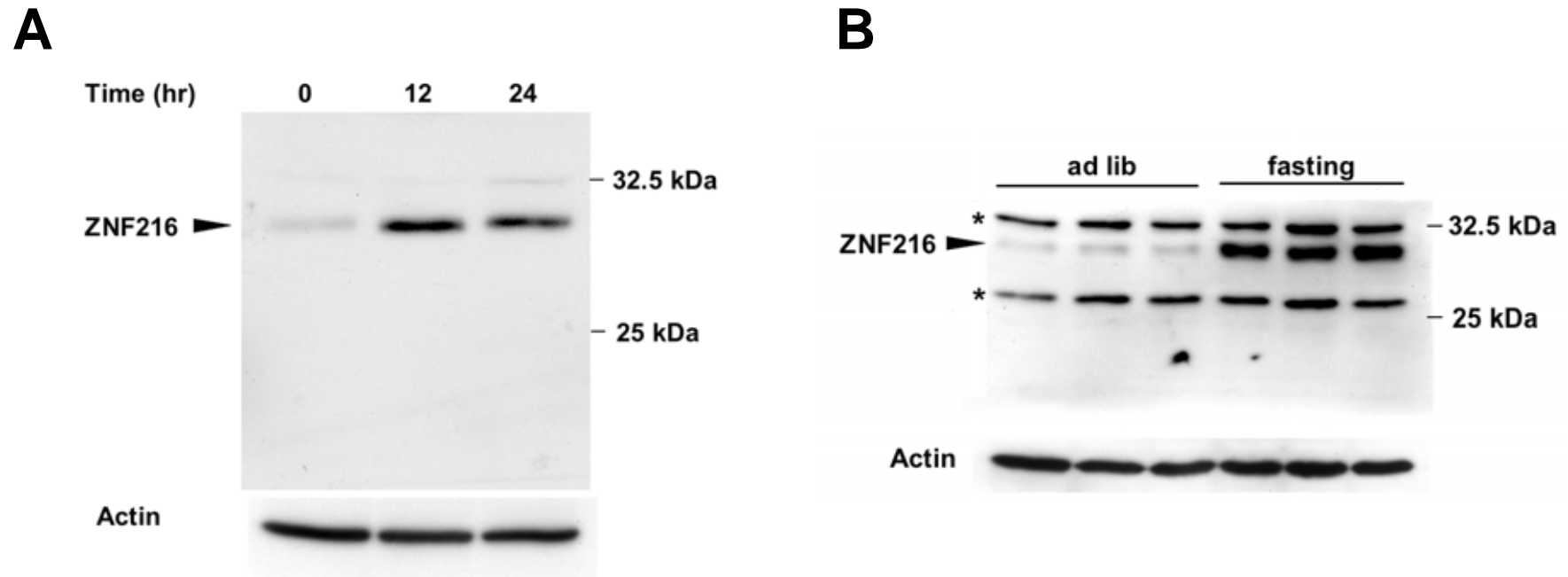
Wertz, I. E., O'Rourke, K. M., Zhou, H., Eby, M., Aravind, L., Seshagiri, S., Wu, P., Wiesmann, C., Baker, R., Boone, D. L., *et al.* (2004). De-ubiquitination and ubiquitin ligase domains of A20 downregulate NF-kappaB signalling. *Nature* 430, 694-699.



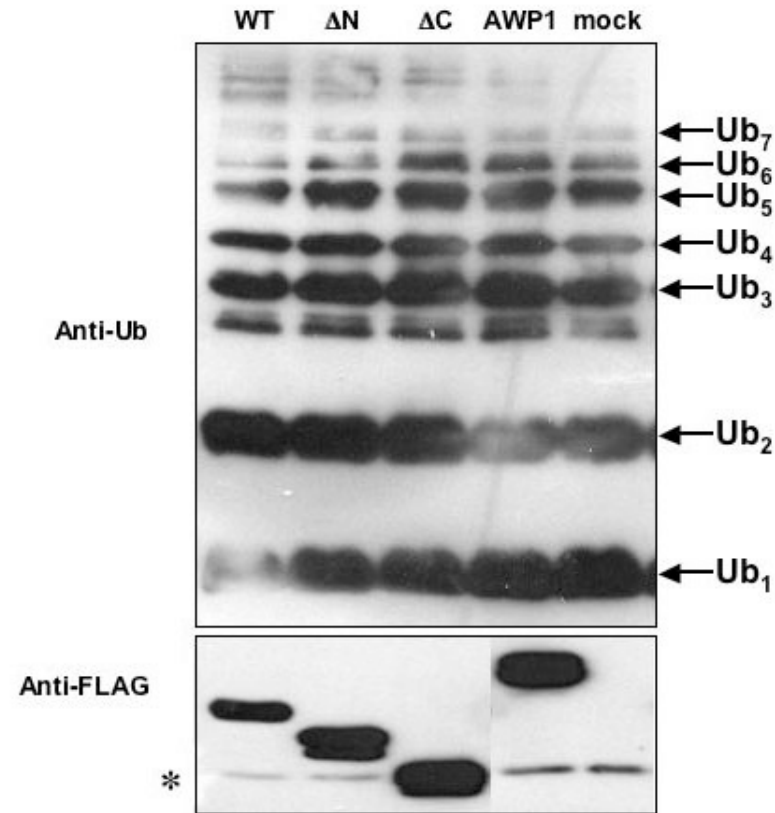
Supplemental Fig. S1. Ubiquitin binding ability of A20-Znf domains in A20-containing proteins. A. Schematic representation of A20 containing proteins and the human Rad23 homologue (HHR23B). B. In vitro ubiquitin binding assay. Purified polyubiquitin was incubated with the indicated A20 domain or HHR23B constructs. The assay was done as described in Experimental Procedures in the text.



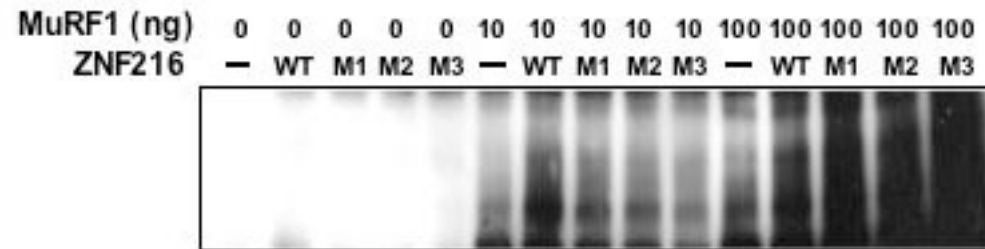
Supplemental Figure S2. Densitometric quantification of two transcripts of Znf216. As shown in Figs. 4 and 6 of the main text, two (2.4- and 1.5kb) transcripts were observed. Percent relative density of the bands are shown. A, dex-induced expression in C2C12 myotube. The signal at t=0 is standardized as 100%. By the treatment, upper (2.4kb) and lower (1.5kb) transcripts were upregulated by 17.3- and 1.9-fold, respectively (at 96 h). B, fasting-induced muscle atrophy. Induction of lower band is more pronounced than that of upper one. C1 and C2, control; F1-3, fasting. C, denervation-induced atrophy. Percent relative density to control (sham-operated) in each mouse was calculated. The numbers (1-7) indicated mice operated (as Fig. 4C). The induction level of the lower transcript was more apparent than that of upper one. Murf1 expression was also evidently upregulated.



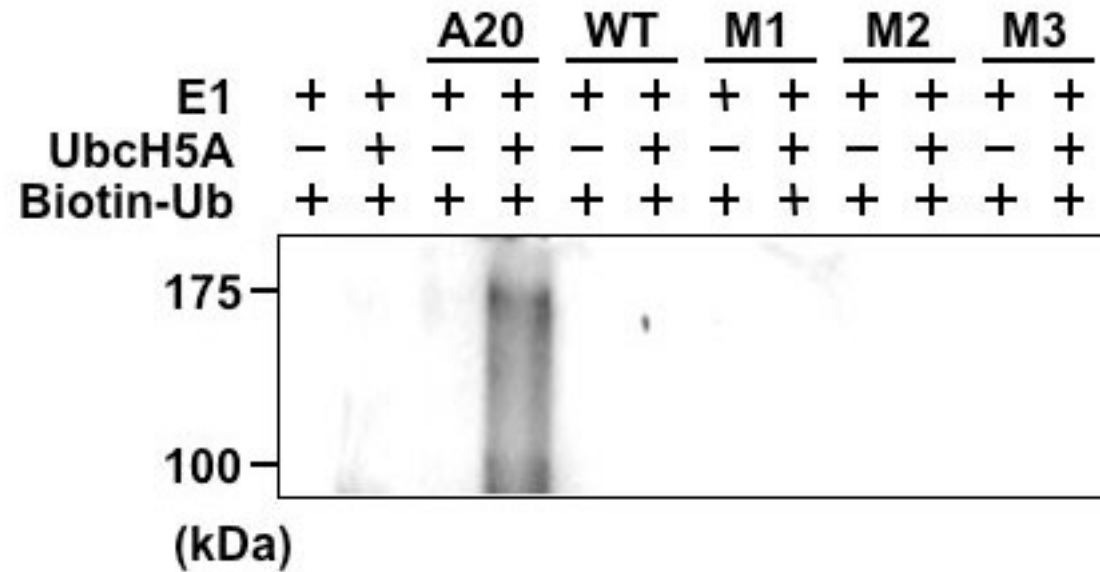
Supplemental Fig. S3. Expression of ZNF216 protein is induced upon muscular atrophy. (A) C2C12 cells were differentiated to myotubes, and then treated with 100 mM Dex for the indicated time. Endogenous ZNF216 protein was detected by immunoblotting with anti-ZNF216 antibody. (B) Fasting-induced muscular atrophy. Muscle extracts were immunoblotted with anti-ZNF216 antibody. As shown, expression of 30-kDa ZNF216 protein was induced by fasting. Nonspecific bands cross-reacted with the antibody are marked by asterisks.



Supplemental Fig. S4. Deubiquitinylation assay. ZNF216, its mutant forms, or AWP1 expressed in 293 cells was immunoprecipitated and subjected to a deubiquitinylation assay. No increase in mono-ubiquitin (Ub₁) or decrease in multi-ubiquitin (Ub₂~) was observed in any lanes compared to mock-transfected controls. A band for an immunoglobulin chain is marked by an asterisk.



Supplemental Fig. S5. Murf1-dependent ubiquitinylation assay. Murf1 was added to the reaction at the indicated levels (0, 10, or 100 ng of protein) with or without ZNF216 protein (200 ng). Levels of ubiquitinylation were increased with increased levels of Murf1 protein. However, no inhibition of poly-ubiquitinylation by Murf1 was observed.



Supplemental Fig. S6. Ubiquitin ligase activity of ZnF-A20. E3 activity of A20-ZnF domain of A20/TNFAIP3 protein and ZNF216 was determined. Polyubiquitinated protein seen as smear was observed only when E1, E2 (UbcH5A), and A20 domain of A20/TNFAIP3 protein were included in the reaction. No polyubiquitinylation was detected in reactions with any ZNF216 constructs.