ZFAND5/ZNF216 is an activator of the 26S proteasome that stimulates overall protein degradation

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ZFAND5/ZNF216, a member of the zinc finger AN1-type domain family, is abundant in heart and brain, but is induced in skeletal muscle during atrophy (although not in proteotoxic stress). Because mice lacking ZFAND5 exhibit decreased atrophy, a role in stimulating protein breakdown seemed likely. Addition of recombinant ZFAND5 to purified 26S proteasomes stimulated hydrolysis of ubiquitinated proteins, short peptides, and ATP. Mutating its C-terminal AN1 domain abolished the stimulation of proteasomal peptidase activity. Mutating its N-terminal zinc finger A20 domain, which binds ubiquitin chains, prevented the enhanced degradation of ubiquitinated proteins without affecting peptidase activity. Mouse embryonic fibroblast (MEF) cells lacking ZFAND5 had lower rates of protein degradation and proteasomal activity than WT MEFs. ZFAND5 addition to cell lysates stimulated proteasomal activity and protein degradation. Unlike other proteasome regulators, ZFAND5 enhances multiple 26S activities and overall cellular protein breakdown.

proteasome | protein degradation | A20 zinc finger | ubiquitin | muscle atrophy

Among the large number of zinc finger proteins in the human genome are the ZFAND family, so named because their zinc finger domains are of the AN1 type. In humans and mice, there are eight ZFAND family members. Two of these, ZFANDs 2a and 5, are of particular physiological interest, because they are induced under conditions that disturb protein homeostasis (i.e., ZFAND2a), or that increase overall protein degradation (i.e., ZFAND5/ZNF216). ZFAND2a, also known as AIRAP, is induced under conditions where misfolded proteins accumulate, including arsenite exposure, heat shock, or proteasome inhibition (1, 2). Following arsenite exposure, AIRAP binds to proteasomes and stimulates their peptidase activity (3). The closely related AIRAP-like protein, AIRAPL (ZFAND2b), is constitutively expressed, but it also associates with proteasomes upon arsenite exposure (4) and was assumed to also enhance their activity. A yeast homolog of ZFANDs, Cuz1 was shown to interact with the proteasome subunit Rpn2 and with cdc48 (the yeast homolog of p97) and to be essential for cell growth upon endoplasmic reticulum (ER) stress or after arsenite treatment (5, 6). In plants, there are more than 14 ZFAND proteins, which are mainly stress-associated proteins. Their expression is induced by dehydration, high salinity, cold, or high temperature, and they increase plant tolerance to such insults (7).

In mice, the ZFAND5/ZNF216 gene is normally expressed strongly in the eye, brain, and heart (8), but only weakly in skeletal muscle. However, upon fasting or denervation, which cause muscle atrophy, its expression in muscle is induced (9). Similarly, in myotubes the glucocorticoid dexamethasone, which stimulates overall protein breakdown and causes muscle fiber atrophy (10), elevates ZFAND5 content. Moreover, knockout mice lacking ZFAND5 show reduced muscle atrophy upon denervation. In cell lysates, ZFAND5 associates with both proteasomes and ubiquitin (Ub) (9). These findings strongly suggest ZFAND5's involvement in protein breakdown (9), especially in the accelerated proteolysis responsible for skeletal muscle wasting. Accordingly, down-regulation of ZFAND5 (and several other components of the ubiquitin proteasome system) in mouse muscle was recently found to raise the levels of a mutant form of dystrophin found in Duchenne muscular dystrophy (11). In addition, in the macrophage line, RAW264.7, ZFAND5 expression is induced by RANKL, TNFα, and IL-1β, and its expression inhibits their differentiation into octeoclasts (12). ZFAND5 also inhibits the activation of transcription by NF-κB, TNFα, or IL-1β in HEK293 cells (13), although the mechanisms underlying its various intriguing actions are unclear.

A role of ZFAND5 in degradation of ubiquitinated proteins is strongly suggested by the presence of the A20-type zinc finger domain at its N terminus (9, 14). This domain allows ZFAND5 to associate with polyubiquitinated proteins (9), although it is unclear whether ZFAND5 binds the Ub conjugates directly, and if this association is specific to certain types of Ub chains. Unlike the A20 deubiquitinase in the NF-κB pathway (15), ZFAND5 does not have DUB activity, but there are conflicting reports on whether it has Ub ligase activity (9, 16). We have explored here the possible effects of ZFAND5 on proteasome function, the specific roles of its two Zn finger domains, and its possible influence on intracellular protein degradation.

Major advances have been made in recent years in our understanding of proteasome mechanisms and regulation (17). While ubiquitinated substrates can bind with high affinity directly to 26S proteasomes through the ubiquitin receptor subunits, Rpn1, Rpn10, or Rpn13 (18), in cells Ub conjugates may also associate with the 26S proteasome through "shuttling factors," hHR23a, hHR23b, or ubiquilins (PLICs), which contain both a Ub-binding [Ub-association (UBA)] domain and a proteasome binding [Ub-like (UBL)] domain (17, 19). Because ZFAND5 interacts with both Ub and proteasomes, it was proposed that ZFAND5 may also

Significance

ZFAND5/ZNF216, a member of the ubiquitous ZFAND protein family, contains two zinc finger domains. It is induced in skeletal muscle during atrophy and was shown to be essential for the resulting large loss of muscle mass. We show here that purified ZFAND5 stimulates the 26S proteasomes' capacity to degrade peptides and ubiquitinated proteins. In cells, it promotes the degradation of endogenous cell proteins through its ubiquitin-binding zinc finger domain (A20). Unlike several ZFAND proteins, it is not induced in proteotoxic stresses. Unlike other proteasome activators, ZFAND5 actually stimulates overall protein breakdown by the ubiquitin proteasome pathway.

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serve as a shuttling factor (9). The present studies have attempted to clarify the precise roles of ZFAND5 in regulating proteasome function and intracellular protein degradation. We show here that ZFAND5, unlike the related proteins ZFAND2a and ZFAND2b, can enhance multiple proteasomes activities and strongly promotes overall proteolysis by the Ub proteasome pathway.

Results

ZFAND5 Expression Under Different Conditions. To monitor the levels of ZFAND5/ZNF216, we generated a rabbit polyclonal antibody against the purified recombinant ZFAND5, which was expressed in *Escherichia coli*. This antibody reacted strongly with recombinant ZFAND5 and the specificity of the antibody was verified using endogenous ZFAND5 induced in C2C12 myotubes by dexamethasone (Fig. 1*A*), as had been reported previously (9). In mice, the levels of ZFAND5 mRNA vary widely between tissues (8). With this antibody, we confirmed that, in normal mice, the content of this protein was much higher in heart and brain than in skeletal muscles, and even lower in liver (Fig. 1*B*). Although a number of other cell protein bands reacted weakly with it, ZFAND5 was the single dominant band in cell extracts (Fig. 1 *C* and *D*). The induction of ZFAND5 upon exposure to dexamethasone is in accord with its increased expression during atrophy of adult muscle, which is induced either by fasting, denervation, or glucocorticoid administration (9).

Because ZFAND5 content in myoblasts isolated from β4-FLAG (PSMB4-FLAG) knockin mice was relatively high, these cells were used to determine its half-life. Following cycloheximide addition, the amount of ZFAND5 decreased rapidly with an apparent half-life of ∼2.5 h at 37 °C (Fig. 1*C*). Additional experiments tested whether ZFAND5, like ZFAND2a/AIRAP, is induced upon heat shock. Surprisingly, its levels decreased quickly after a shift to 43 °C in contrast to a typical heat shock protein, Hsp70, which increased (Fig. 1*D*). In fact, at 43° C in the presence of cycloheximide, ZFAND5 was degraded significantly faster (half-life, ∼1.2 h) than at 37 °C (Fig. 1*C*). Thus, unlike ZFAND2a whose content rises at 43 °C as part of the heat shock response (1), ZFAND5 is not a heat shock protein and is even rapidly eliminated under this stressful condition. Also, ZFAND5 was not induced upon treatment of myotubes with tunicamycin (Fig. 1*E*), which blocks protein glycosylation in the ER and induces the unfolded protein response. Thus, ZFAND5 expression correlates with conditions where total cellular proteolysis increases, but not in proteotoxic conditions, where misfolded proteins accumulate.

ZFAND5 Binds Directly to Proteasomes and Decreases Their Electrophoretic Mobility. When recombinant ZFAND5 was incubated with lysates of mammalian cells and immunoprecipitated, or when an epitope-tagged ZFAND5 transfected into mammalian cells was immunoprecipitated, it was found to associate with both proteasomes and Ub conjugates (9). To determine whether these associations are direct or mediated by other proteasome-interacting proteins, we expressed GST-ZFAND5 in *E. coli*, and after purification, it was used directly, or after the GST was cleaved off with PreScission protease (*SI Appendix*, Fig. S1*A*). Upon chromatography on a size exclusion column, ZFAND5 was eluted as a monomer of about 30 kDa, which corresponds roughly to its molecular weight of 23,058 Da (213 aa) (*SI Appendix*, Fig. S1*B*). To test direct binding to proteasomes, GST-ZFAND5 bound to a sepharose resin was incubated with 26S proteasomes affinity-purified from HEK293 or HeLa cells by the UBL method (20) at 4 °C for 30 min. After washing with 150 mM NaCl, the presence of proteasomes bound to GST-ZFAND5 was clearly evident by Western blot (Fig. 2*A*). Conversely, when 26S proteasomes were immobilized using a resin-bound antibody against the 20S subunit, α1, and ZFAND5 was added and incubated for 30 min at 4 °C, ZFAND5 became bound to the 26S particles (Fig. 2*B*).

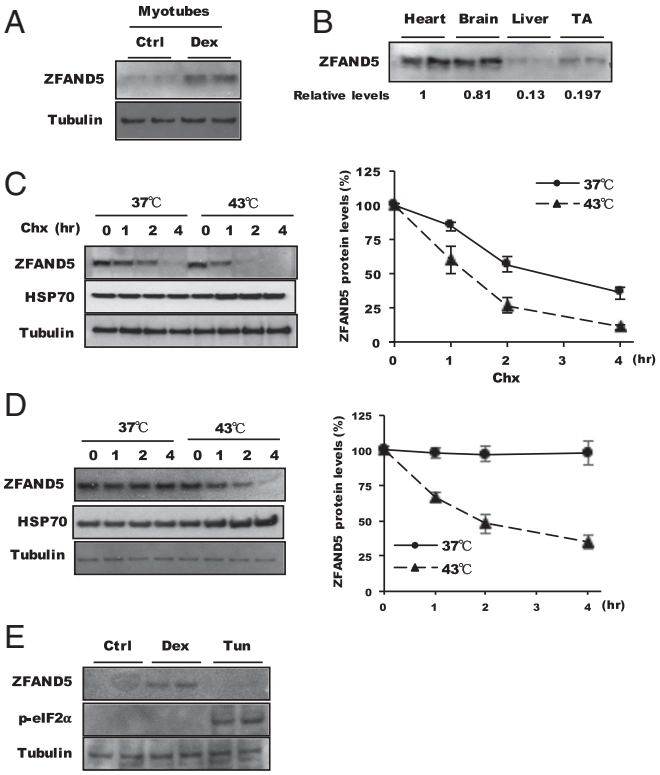

Fig. 1. ZFAND5 expression is induced by dexamethasone, but not by heat shock or tunicamycin treatment. (*A*) The expression of ZFAND5 was induced in C2C12 myotubes by dexamethasone (Dex) as shown previously (9). C2C12 myotubes were treated with dexamethasone (100 μM) for 48 h, and the increase in ZFAND5 was determined by Western blots using our polyclonal rabbit antibody raised against the recombinant protein. (*B*) The levels of ZFAND5 protein were higher in mouse brain and heart than in skeletal muscle (tibialis anterior) and much higher than in liver. Equal amounts (10 μg) of mouse tissue lysates were subjected to SDS/PAGE, and the content of ZFAND5 was determined by Western blots, as in *A*. The numbers represent the relative concentrations of ZFAND5 in different tissues normalized to the same amount of soluble tissue proteins loaded in each lane. (*C*) After addition of cycloheximide, ZFAND5 was degraded with a half-life of about 2.5 h at 37 °C, but more rapidly (half-life, ∼1.2 h) upon heat shock (43 °C). (*D*) The amounts of ZFAND5 in myoblasts fall rapidly after a shift to high temperature (43 °C), which induces the heat shock proteins, such as Hsp70. Myoblasts established from skeletal muscles of mice expressing β4-FLAG (*Materials and Methods*) were cultured in 37 °C or 43 °C in the presence (*C*) or absence (*D*) of cycloheximide (20 μg/mL) and harvested. Equal amounts (20 μg) of the lysates were loaded onto SDS/PAGE, and ZFAND5 contents were determined by Western blot. These data (*C* and *D*) are based upon measurements of ZFAND5 levels in three independent experiments. (*E*) Unlike dexamethasone, ER stress induced with tunicamycin does not induce ZFAND5 in C2C12 myotubes. These cells were treated with dexamethasone (100 μM) for 48 h or tunicamycin (0.5 μg/mL) for 24 h, and the levels of ZFAND5 were measured by Western blot.

Upon binding of ubiquitinated proteins, 26S proteasomes undergo major conformational changes (17), as shown by cryo-electron microscopy (cryo-EM) (21, 22) and by changes in their migration in native PAGE (23). The association of ZFAND5 with the proteasomes also caused a clear decrease in their migration in native PAGE. Addition of ZFAND5 slowed migration of both singly capped (SC) and doubly capped (DC) 26S species in the gels (Fig. 2*C*). Moreover, we also observed slower migration of proteasomes when the cell lysates were incubated with recombinant ZFAND5 (Fig. 2*C*). No further shift in mobility occurred with increasing amounts of ZFAND5 (Fig. 2*C* and *SI Appendix*, Fig. S1*C*). Thus, it appears to be a single saturable transition. Migration of free 19S in these native gels was also slowed by ZFAND5 (*SI Appendix*, Fig. S1*C*), although the migration of

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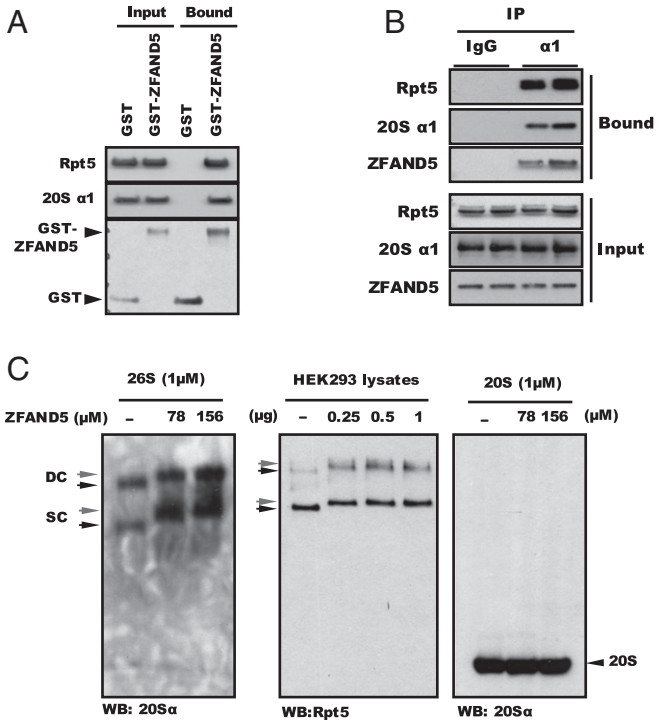

Fig. 2. ZFAND5 associates with 26S proteasomes and slows their migration in native PAGE. (*A*) Purified 26S proteasomes bind directly to ZFAND5. 26S proteasomes (200 nM) purified from HEK293 cells were incubated for 30 min at 4 °C with GST-ZFAND5 (100 nM) bound to a glutathione resin. After washing, the GST-ZFAND5 with bound proteasomes was eluted with glutathione, and the eluates were analyzed by SDS/PAGE amid the amounts of proteasomes and GST-ZFAND5 determined as in Fig. 1. (*B*) Coimmunoprecipitation assay also showed that 26S proteasomes associate directly with ZFAND5. Purified 26S proteasomes (100 nM) were immobilized using an antibody to the 20S subunit α1 or control (IgG) and incubated with ZFAND5 (200 nM) for 30 min at 4 °C. The resin-bound 26S proteasomes and ZFAND5 in the eluates were measured. (*C*) The association with ZFAND5 reduced the migration of 26S proteasomes in native PAGE. Purified 26S (1 μM) or 20S proteasomes (1 μM) were incubated with or without ZFAND5 (78 or 156 μM) for 37 °C for 30 min. As shown with purified 26S proteasomes, ZFAND5 also slowed the migration of 26S in HeLa lysates in native PAGE. Increasing amounts of ZFAND5 (0.25–1 μg) were incubated with HeLa lysates (15 μg) for 30 min. The mixture was then resolved in the native gel, and singly capped (SC) or doubly capped (DC) 26S were detected by the antibodies to 20S α-subunits for purified proteasomes or to Rpt5 subunit for proteasomes in the lysates.

purified 20S was not affected (Fig. 2*C*). These changes in mobility are much larger than would be anticipated from addition of a single 23-kDa protein to 26S proteasomes (Fig. 2*C* and *SI Appendix*, Fig. S1*C*). Although the slower migration possibly resulted from binding of many ZFAND5 molecules, it is much more likely they involve a major conformational change in the particle, because increasing amounts of ZFAND5 caused a discrete shift in mobility without any intermediate bands (Fig. 2*C* and *SI Appendix*, Fig. S1*C*). Together, these observations show that ZFAND5 binds to the 19S particle, and the resulting change in the electrophoretic mobility strongly suggests a large alteration in proteasome structure.

ZFAND5 Markedly Stimulates the 26S Proteasomes' Three Peptidase Activities. When purified 26S proteasomes were incubated with recombinant ZFAND5, hydrolysis of LLVY-amc increased about fivefold, and of the larger, related substrate RPFHLLVY-amc, about twofold (Fig. 3*A*). With increasing concentrations of ZFAND5, the 26S particles' chymotrypsin-like activity increased up to sixfold, the caspase-like activity about 3.6-fold, and the trypsin-like activity 3-fold. Stimulation of these three activities increased and appeared to reach maximal activity at similar concentrations of ZFAND5 (Fig. 3*B*). This coordinated increase in all three peptidase activities makes it very likely that ZFAND5 acts by enhancing substrate entry and gate opening into the 20S particles (24).

The nonhydrolyzable ATP analog, ATPγS, stimulates peptide hydrolysis more than ATP does because it maintains an open state of the gated entry channel through the ATPase into the 20S particle (21, 22, 25, 26). In addition, the binding of ubiquitinated proteins to proteasomes enhances peptide hydrolysis (26–28), and this effect can be mimicked by adding Ub chains or Ub aldehyde, which bind to Usp14 or Uch37 (27, 29). In the presence of ZFAND5, the proteasomes' peptidase activity was increased similarly as with ATPγS, hexa-Ub or Ub aldehyde, but ZFAND5 did not stimulate further when added together with hexa-Ub or Ub aldehyde (*SI Appendix*, Fig. S2 *A–C*).

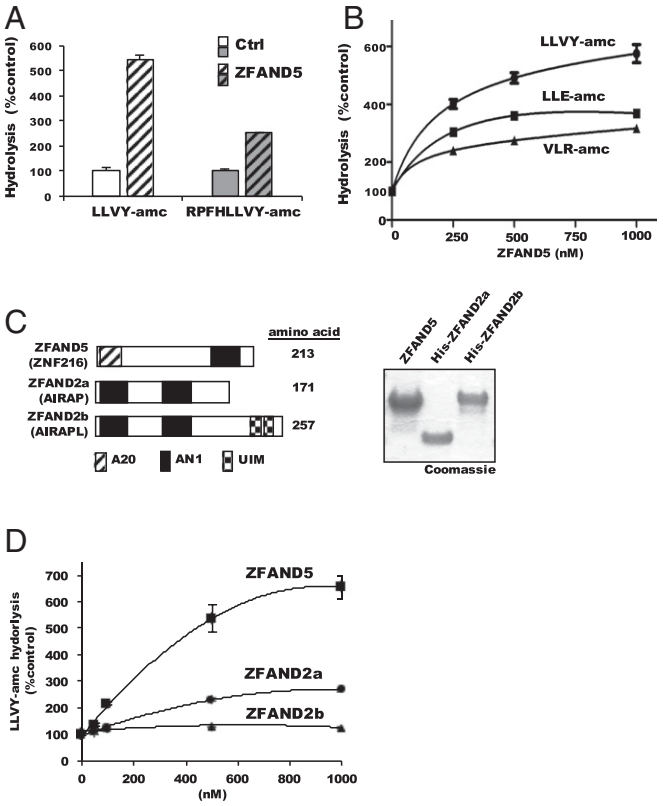

Fig. 3. ZFAND5 markedly stimulates peptidase activities of 26S proteasomes, much more than ZFAND2a and 2b. (*A*) ZFAND5 enhanced hydrolysis of short peptides by 26S proteasomes. To assay their chymotrypsin-like activity, the hydrolysis by 26S proteasomes (2 nM) of the fluorogenic peptides of different lengths, Suc-LLVY-amc (10 μM) or Suc-RPFHLLVY-amc (10 μM), was measured in the absence (Ctrl) or presence (ZFAND5) of recombinant ZFAND5 (0.5 μM). The activity without ZFAND5 was taken as a 100%, and the activation by ZFAND5 is shown. (*B*) ZFAND5 stimulated all three proteasomal peptidase activities. The caspase-like activity was measured with Z-LLE-amc and the trypsin-like activity with Z-VLR-amc (10 μM) in the presence of increasing amounts of ZFAND5. (*C*) Comparison of the zinc finger domains of three ZFAND proteins. Recombinant, His-ZFAND2a and His-ZFAND2b were expressed in *E. coli*, and the purified proteins were stained with Coomassie Brilliant Blue (CBB). (*D*) ZFAND5-stimulated peptide hydrolysis was much more than ZFAND2a or ZFAND2b. When the effects of increasing amounts of His-ZFAND2a, His-ZFAND2b, or ZFAND5 on chymotrypsin-like activities of 26S (2 nM) were compared, ZFAND5 stimulated about approximately sixfold and ZFAND2a (about twofold), although their affinities for proteasomes appeared quite similar ($K_a \sim 275$ nM for both ZFAND5 and ZFAND2a). Surprisingly, ZFAND2b did not enhance proteasome activity. All values are the means of three experiments ± SD.

The activation of proteasomes by Ub conjugates, HexUb, or Ub-al requires interaction with Usp14 or Uch37, which are 26S-associated DUBs with key regulatory functions (27, 29). We tested whether the stimulation of peptidase activity by ZFAND5 also requires USP14 by using proteasomes from USP14KO mouse embryonic fibroblast (MEF) cells, which show less response to Ub conjugates or Ub-al than 26S from wild-type (WT) MEF cells (29). Proteasomes lacking USP14 exhibited slightly higher peptidase activity than WT (*SI Appendix*, Fig. S2*D*, lane 1 vs. 3) (30) and also differ significantly from the WT in their structure, as shown by cryo-EM (31). Addition of ZFAND5 stimulated the USP14-deficient particles' peptidase activity even more than WT 26S (*SI Appendix*, Fig. S2*D*, lane 2 vs. 4), perhaps because the lack of USP14 enhanced ZFAND5 binding, although under this condition we were unable to demonstrate differences in binding by coimmunoprecipitation. Thus, ZFAND5 markedly stimulates the proteasome's peptidase and ATPase activities by a mechanism that is unclear, although this activation appears to be associated with a large conformational change in the 19S complex, as discussed above.

ZFAND5 Is a Much More Potent Proteasome Activator than ZFAND2a or ZFAND2b. Upon arsenite treatment of cells, two other ZFAND proteins, ZFAND2a and ZFAND2b (Fig. 3*C*), were reported to associate with proteasomes (3, 4). ZFAND2a is induced in various conditions that cause an accumulation of misfolded or damaged proteins, including exposure to arsenite, heat shock, or proteasome inhibitors (1, 4). By contrast, ZFAND2b is constitutively expressed in cells and is not arsenite inducible (4). To determine whether these ZFAND proteins affect proteasome activity in a similar fashion as ZFAND5, recombinant ZFAND2a and ZFAND2b were expressed in *E. coli*, purified (Fig. 3*C*), and incubated with 26S proteasomes. As reported previously (3), ZFAND2a was found to enhance peptidase activity by up to twofold, but this stimulation was much less than that by similar concentrations of ZFAND5 (Fig. 3*D*). Even though ZFAND2b also contains AN1 and UIM domains (Fig. 3*C*) (4), in these assays, it caused no stimulation of peptidase activity.

ZFAND5's AN1 Domain, but Not the A20, Is Required for the Stimulation of Peptidase Activity. ZFAND5 contains two zinc finger domains, A20 at its N terminus and AN1 at its C terminus (9) (Fig. 4*A*). A20 is essential for ZFAND5 binding to Ub conjugates (9), and

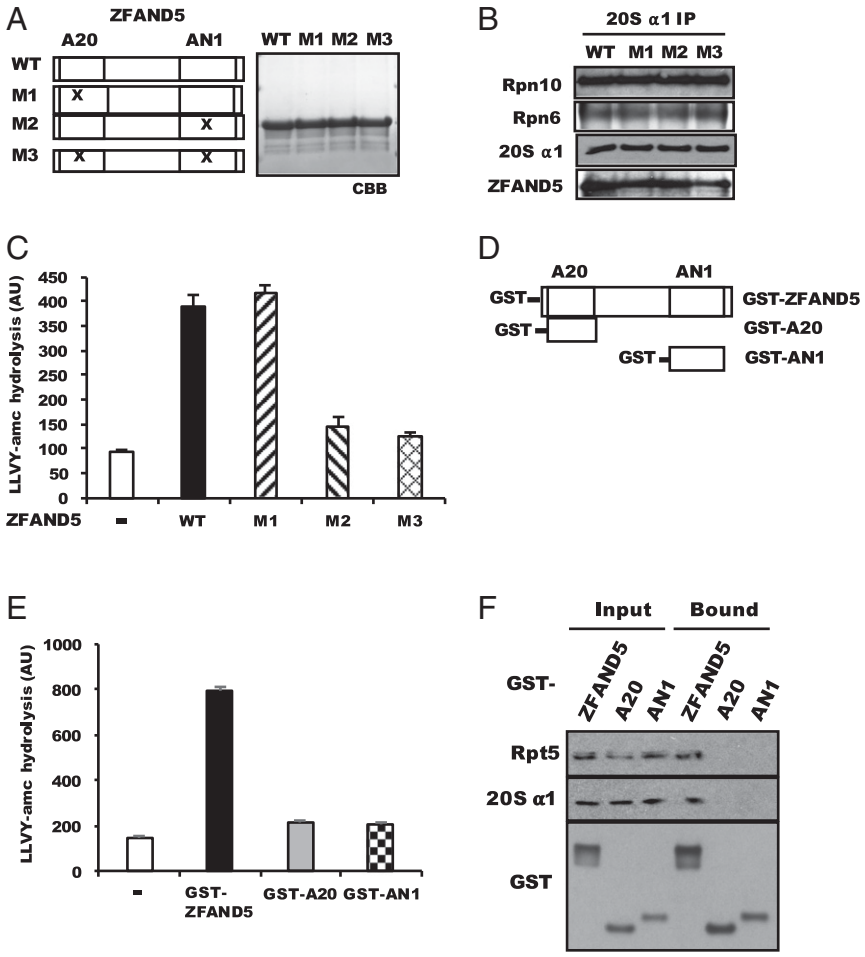

Fig. 4. The AN1 domain is essential for stimulation of the proteasomes' peptidase activity. (*A*) Point mutations in the two zinc finger domains of ZFAND5. ZFAND5 was mutated at C30A and C33A in the A20 domain (M1), or at C170A and C175A in the AN1 (M2), or at these residues in both domains (M3). The WT and mutant GST-ZFAND5 were expressed in *E. coli*, the GST was removed by PreScission, and the ZFAND5 was purified. Equal amounts (0.3 μg) of each protein were subjected to SDS/PAGE and stained with Coomassie Brilliant Blue (CBB). (*B*) Mutations in the zinc finger domains did not disrupt the interactions of ZFAND5 with 26S proteasomes. Coimmunoprecipitation assays were performed with WT and ZFAND5 mutants as in Fig. 2*B*. Interaction of ZFAND5 with 26S proteasomes was monitored by Western blots. (*C*) Mutation in AN1 (M2) failed to activate peptide hydrolysis. Peptidase activity of 26S proteasomes (2 nM) was measured with or without ZFAND5 or mutants (0.5 μM). (*D*) The isolated zinc finger domains fused to GST. (*E*) Neither the AN1 nor A20 domain alone was able to activate proteasomes unlike GST-ZFAND5. Peptidase activity of proteasomes (2 nM) was measured with GST-ZFAND5, GST-A20, or GST-AN1 (0.5 μM). (*F*) The GST-A20 or GST-AN1 domain alone did not bind to 26S proteasomes.

the complex of the A20 of ZFAND5 with Ub has been analyzed by NMR (14). By contrast, the role of the AN1 domain remains unknown. To investigate whether ZFAND5's zinc finger domains are essential for the activation of proteasomes, we expressed recombinant proteins bearing the C30A and C33A mutations in the A20 domain (termed M1), and C170A and C175A in the AN1 domain (termed M2), which should prevent the coordination of zinc (Fig. 4*A*) (9, 14). To determine whether these domains are necessary for the association with 26S proteasomes (Fig. 2 *A* and *B*), these particles were immobilized with a resin-bound antibody against the 20S subunit, α1, and then incubated at 4 °C with the WT ZFAND5 or these point mutants. The WT and ZFAND5s bearing mutations in the A20 (M1) or AN1 (M2) domain all bound to the 26S similarly (Fig. 4*B*). Even when both zinc finger domains (termed M3) were disrupted, it associated with the 26S, although slightly less than the single mutants. Thus, these two zinc fingers are not required for proteasomal binding.

When the A20 mutant was incubated with the proteasomes, peptide hydrolysis was enhanced as much as with the WT protein (Fig. 4*C*). However, the AN1 mutation alone or together with the A20 mutation completely failed to activate peptide hydrolysis (Fig. 4*C*). Thus, the C-terminal AN1 zinc finger is required to stimulate peptide entry into 26S proteasomes. To learn whether the AN1 domain by itself can enhance peptidase activity, we generated, expressed in *E. coli*, and purified a GST-tagged AN1 (GST-AN1) and GST-tagged A20 (GST-A20) (Fig. 4*D*). Unlike the large stimulation by ZFAND5 or GST-ZFAND5 (*SI Appendix*, Fig. S3*A*), neither GST-AN1 nor GST-A20 had a significant stimulatory effect (Fig. 4*E*). This result led us to determine whether AN1 or A20 domain alone can bind to proteasomes. GST pull-down assays showed that AN1 and A20 domains could not by themselves associate with the 26S particles (Fig. 4*F*). These findings suggest that the central region between A20 and AN1 is required for ZFAND5's stimulatory action of AN1, which is in accord with our observation (Fig. 4*B*) that the double mutant still binds proteasomes.

To test this possibility, the A20 (ΔA20) or the AN1 (ΔAN1) domains was deleted from ZFAND5. The intact protein and fragments containing the central region plus only one zinc finger domain were expressed and purified, and their effects on peptidase activity were examined. Surprisingly, even the ΔA20 mutant, which contains the central region and the AN1, failed to stimulate peptide hydrolysis (*SI Appendix*, Fig. S3*B*), probably because none of these deletion mutants associated with the proteasomes (*SI Appendix*, Fig. S3*C*). Thus, full-length ZFAND5 seems to be required for its binding to the 26S complex, and a functional AN1 domain is essential for the stimulation of peptide hydrolysis.

ZFAND5 Enhanced Hydrolysis of ATP and Ubiquitinated Proteins. The 26S proteasome is an ATP-dependent protease complex, and its rate of degradation of ubiquitinated proteins is proportional to its rate of ATP hydrolysis (32–34). We therefore tested whether ZFAND5 influences the proteasomes' basal ATPase activity (33). ATP hydrolysis by 26S proteasomes was elevated by about 80% after addition of ZFAND5 at a concentration that stimulates peptidase activity (Fig. 5*A*). Next, we examined whether the zinc finger domains are required for the activation of ATP hydrolysis. Mutations in either zinc finger domain reduced the stimulation of ATP hydrolysis seen with WT ZFAND5 (Fig. 5*B*), although there was still a small increase in ATP activity by these mutant forms (Fig. 5*B*).

An enhancement of ATPase activity by itself should lead to more rapid degradation of ubiquitinated proteins. To determine whether proteasomal degradation of ubiquitinated proteins can be stimulated directly by ZFAND5, ubiquitinated dihydrofolate reductase (DHFR) (Ub_5-DHFR) was radiolabeled using [^{32}P]ATP as described previously (27), and the hydrolysis of ^{32}P-Ub_5-DHFR to TCA-soluble peptides was measured. After addition of the ZFAND5, 26S proteasomes consistently hydrolyzed this ubiquitinated protein more rapidly (Fig. 5*C*). In contrast to this stimulation by ZFAND5, ZFAND2a did not increase the degradation of this model ubiquitinated substrate (*SI Appendix*, Fig. S4), as was also reported previously (3). Since Ub conjugates are the proteasomes' physiological substrates, these findings strongly suggest a role of ZFAND5 in promoting proteolysis in cells (see below).

The A20 Domain Is Essential for the Increased Degradation of Ubiquitinated Protein and for Binding K48- and K63-Linked Ub Conjugates. We then tested whether ZFAND5's two Zn finger domains are required for the stimulation of Ub conjugate degradation. No activation of the hydrolysis of ^{32}P-Ub_5-DHFR was observed with the A20 mutant protein (Fig. 5*C*), even though it can increase peptidase activity (Fig. 3*C*). Therefore, surprisingly, the enhancement of peptide hydrolysis is not required to accelerate Ub conjugate degradation. By contrast, the AN1 mutant (M2) stimulated the degradation of ubiquitinated DHFR in a similar fashion as the WT (Fig. 5*C*). Accordingly, when both A20 and AN1 (M3) were mutated, no stimulation was observed. Thus, the A20 domain (M1), although not required to increase peptidase activity, is essential for the increased Ub conjugate hydrolysis, presumably because this domain allows ZFAND5 to bind Ub conjugates (9).

To learn whether ZFAND5 associates preferentially with K48- or K63-linked Ub chains, we used the GST-linked Ub ligases, E6AP and NEDD4, and allowed them to autoubiquitinate (29, 34) by incubation with E1, the E2 UbcH5b, Ub, and ATP. E6AP forms on itself homogeneous K48-, and NEDD4 forms K63-polyubiquitin chains, and these ubiquitinated proteins were purified (35). ZFAND5 was then incubated with immobilized polyubiquitinated or unmodified GST-E6AP, and the nonbound materials were washed away. Both the WT ZFAND5 and the AN1 mutant bound to the ubiquitinated E6AP, but not to the unmodified E6AP (Fig. 6*A*). By contrast, the A20 mutant and ZFAND5 with mutations in both A20 and AN1 did not associate with Ub_n-E6AP. Thus, binding of ZFAND5 to K48-linked Ub chain requires the A20 zinc finger domain (Fig. 6*A*). To test whether ZFAND5 binds also to K63 chains, similar assays were performed with the polyubiquitinated NEDD4. The WT ZFAND5 and AN1 mutant also associated with ubquitinated NEDD4, but ZFAND5 mutated in the A20 domain did not (Fig. 6*A*). These findings with ubiquitinated proteins are consistent with a prior report that the A20 domain can associate with di-Ub containing either a K48 or K63 linkage (14).

Because ZFAND5 associates with Ub, we investigated whether ZFAND5 also binds the UBL domain, which are found in several shuttling factors. GST fused to the UBL domain of RAD23b was purified (20) and incubated with ZFAND5 alone. In contrast to a prior report that failed to find such an interaction (9), we found that ZFAND5 binds directly to the UBL domain, even without proteasomes present (Fig. 6*B*).

Protein Degradation Is Decreased in MEF Cells Lacking ZFAND5. The attenuation of muscle wasting observed in mice lacking ZFAND5 (9) and the stimulation of the degradation of ubiquitinated proteins shown here (Fig. 5*C*) strongly suggest that ZFAND5 enhances protein degradation in vivo by the Ub proteasome pathway. To test this hypothesis, we measured the overall rate of breakdown of long-lived proteins (the bulk of cell proteins) in MEF cells from WT mice or mice lacking ZFAND5 (ZFAND5KO). To radiolabel MEF cell proteins, these cells were grown in the presence of radioactive ^{3}H-phenylalanine for 1 d, then washed and cultured for 2 h in chase medium containing a large excess of nonradioactive phenylalanine to prevent the reincorporation of released ^{3}H-phenylalanine back into proteins (36). The total rates of proteolysis at different times

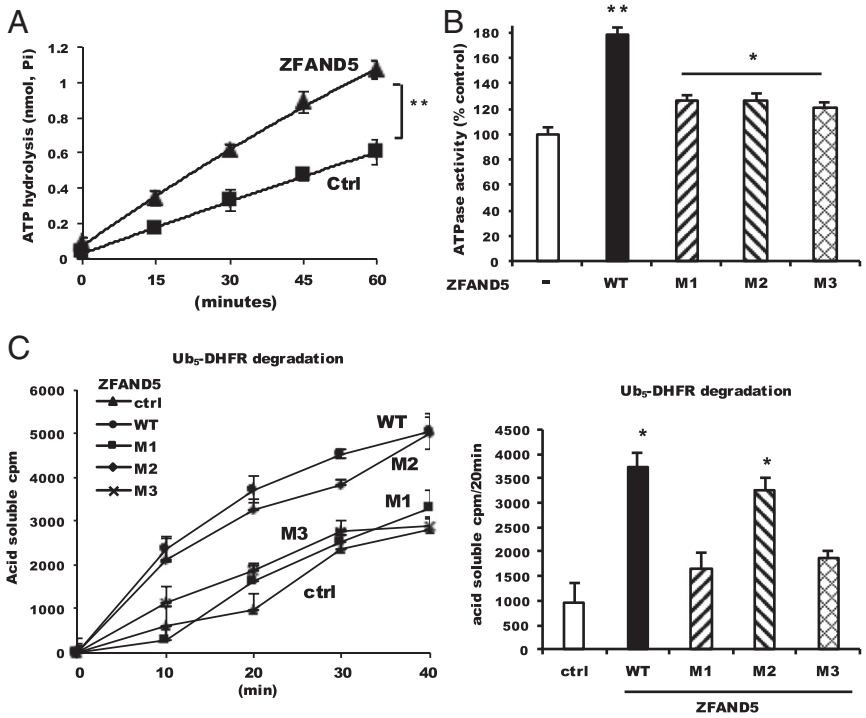

Fig. 5. ZFAND5 enhanced basal ATPase activity and degradation of Ub conjugates by pure 26S proteasomes. (*A*) ZFAND5 increased ATP hydrolysis by 26S proteasomes, which (10 nM) were incubated with or without ZFAND5 (2.5 μM) at 37 °C. At each time point, production of inorganic phosphate (Pi) was measured using the malachite green assay. All values are the means of at least three independent experiments ± SD. $^{**}P < 0.01$. (*B*) Point mutations in either or both zinc fingers of ZFAND5 activated ATP hydrolysis much less than WT ZFAND5. The WT or ZFAND5 mutants were incubated with proteasomes, and the production of Pi was measured. $^{**}P < 0.01$, WT ZFAND5 vs. control. $^{*}P < 0.05$, ZFAND5 mutants vs. control. (*C*) ZFAND5 accelerated the breakdown of ubiquitinated DHFR, but only if the A20 domain was intact. Degradation of ^{32}P-labeled ubiquitinated DHFR (Ub_5-DHFR) by 26S proteasomes (1 nM) was measured with or without the WT or the ZFAND5 mutants (0.25 μM) at 37 °C. The reactions were stopped at the indicated time points by addition of TCA, and the TCA-soluble radioactivity was measured (*Left*). The mean values of Ub_5-DHFR degradation at 20 min were shown for comparison (*Right*). All values are the means of at least three independent experiments ± SD.

were determined by measuring the conversion of ^{3}H-labeled proteins to TCA-soluble radioactivity (i.e., amino acids) in the medium. The MEF cells lacking ZFAND5 showed about a 35% lower rate ($P < 0.01$) of overall protein degradation than the WT MEF cells (Fig. 7*A*).

In addition, in the extracts of the MEF cells lacking ZFAND5, there was a slower hydrolysis of peptides by the proteasome's chymotrypsin-like site (Fig. 7*B*). This lower proteasome activity in the cells lacking ZFAND5 was not due to a lower content of proteasomes as the levels of 19S subunits, Rpn6, Rpn10, and the 20S α-subunits did not differ by Western blot in WT and ZFAND5KO cells (Fig. 7*C*), nor did the levels of doubly and SC 26S particles differ on native PAGE (Fig. 7*D*). In addition, the total levels of Ub conjugates were similar in the two types of MEF cells (Fig. 7*D*). Thus, the absence of ZFAND5 seems to account for the lower rate of proteolysis.

ZFAND5 Stimulates Degradation of Endogenous Proteins in Cell Extracts. Our repeated attempts to overexpress active ZFAND5 in HEK293 cells were not successful for reasons that remain unclear. Although the Flag-tagged protein was expressed strongly, it had modest stimulation of proteasomes, in contrast to the large activation seen upon addition of recombinant ZFAND5 (Fig. 3). Therefore, to test whether ZFAND5 can promote degradation of endogenous cell proteins by the Ub proteasome pathway, we determined whether adding increasing amounts of ZFAND5 to crude cell extracts could also stimulate proteasome activity and the breakdown of endogenous cell proteins. Because the lack of ZFAND5 reduced the overall rate of proteolysis in MEF cells (Fig. 7*A*), lysates of these cells were studied. As expected, when recombinant ZFAND5 was added to lysates of either WT and ZFAND5KO cells, ZFAND5 stimulated the activity of endogenous proteasomes. In fact, the added ZFAND5 increased this activity in the ZFAND5-deficient extracts above the level in the WT extracts, which were further stimulated by the exogenous ZFAND5 (Fig. 7*E*).

To test whether the addition of recombinant ZFAND5 to MEF cell lysates could also elevate overall protein degradation, we labeled proteins in the MEF cells with ^{3}H-phenylalanine, as in Fig. 7*A*, but instead of allowing the cells to degrade the labeled proteins, we prepared cell lysates. Adding increasing amounts of ZFAND5 to the labeled extracts of both the WT and KO cells stimulated the degradation of the ^{3}H-labeled endogenous proteins (Fig. 7*F*). (The absolute increase in proteolysis in the KO MEFs appeared slightly lower than in WT, but the total incorporation of ^{3}H-phenylalanine was also lower in the KO cells.) This increase in proteolysis seemed proportional to the amount of ZFAND5 added. Curiously, at very high concentrations, proteolysis fell off precipitously. This decrease must be due to a lower affinity interaction with some cellular component (perhaps ubiquitin conjugates or Rad23) that inhibits proteolysis, since no such decline was seen in the hydrolysis of Ub conjugate by purified proteasomes (Fig. 5*C*). A similar anomalous inhibition of proteolysis by very high concentrations of ZFAND5 was also observed in lysates of HeLa and HEK293 cells.

We then tested whether ZFAND5 could also stimulate the hydrolysis of endogenous proteolysis in extracts of other cell lines (i.e., HeLa and HEK293 cells) where ZFAND5 is not detectable by Western blot. When ZFAND5 was added to the HeLa lysates, peptide hydrolysis increased up to sevenfold (Fig. 7*G*), as we had

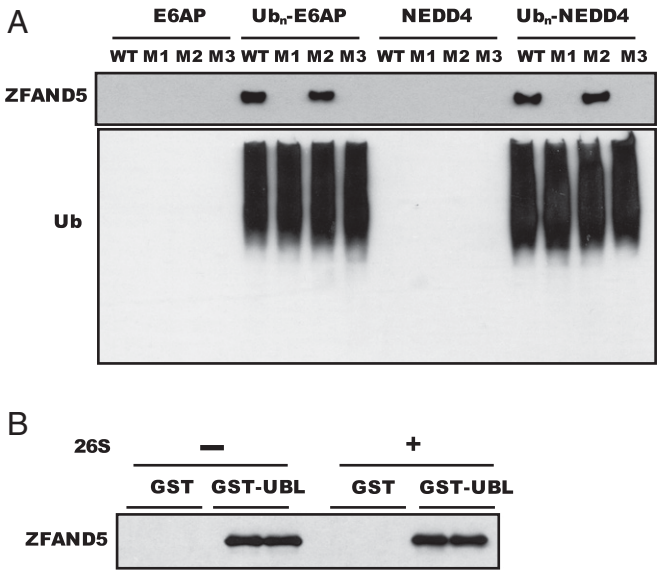

Fig. 6. ZFAND5 binds both K48- and K63-polyubiquitin conjugates and the UBL-domain. (*A*) Binding of ZFAND5 to K48- and K63-linked Ub conjugates required the A20 domain. K48-polyubiquitinated GST-E6AP and K63-polyubiquitinated GST-NEDD4 were prepared as described previously (29). The ubiquitinated proteins (150 nM) bound to a glutathione resin were incubated with WT or the zinc finger mutants (450 nM) for 30 min at 4 °C. After washing to remove the unbound proteins, the association of ZFAND5 with Ub conjugates was analyzed by Western blots. (*B*) In addition to Ub conjugates, ZFAND5 also binds to an isolated UBL domain. The resin-bound GST-UBL was incubated with ZFAND5. After washing, ZFAND5 bound to GST-UBL was determined by Western blot.

observed with purified 26S (Fig. 3), and this stimulation also required the AN1 domain. Moreover, increasing amounts of ZFAND5 stimulated degradation of radiolabeled cell proteins in the HeLa lysates (Fig. 7*H*) as well as in radiolabeled extracts of HEK293 cells (*SI Appendix*, Fig. S5). The AN1 mutant, which fails to activate peptide hydrolysis, could also stimulate the degradation of endogenous proteins, but surprisingly this effect was evident at much lower concentrations. The stimulation of proteolysis by this mutant reached a similar maximal level as was observed with the WT proteins. (Interestingly, the supramaximal concentrations of this mutant protein caused a similar sharp fall in degradation, as was seen with very high concentrations of WT ZFAND5.) By contrast, the ZFAND5 mutant that lacked the Ub-binding domain A20 (M1) was unable to enhance endogenous protein breakdown (Fig. 7*H*) in accord with its inability to stimulate hydrolysis of ubiquitinated DHFR by pure 26S proteasomes (Fig. 5*C*). Thus, ZFAND5 enhances overall breakdown of endogenous proteins and can do so in many cell types, even in ones where this protein is not normally present. Thus, the presence or absence of ZFAND5 can regulate overall protein breakdown in cells and extracts.

Discussion

ZFAND5 Differs from Known Proteasome Activators. The biochemical properties and cellular functions of ZFAND5 differ in multiple respects from those of other factors that can activate the 26S or 20S proteasome. The term "proteasome activator" (PA) has been used to refer to a variety of specific factors or treatments (e.g., phosphorylation by PKA) that enhance different activities of the 20S or 26S particles (37). The important early studies of DeMartino and Rechsteiner and their coworkers identified several PA complexes that stimulate peptidase activity of the 20S proteasome, including PA700 (the 19S regulatory complex), PA28αβ and PA28γ [the 11S regulators (REG)], and PA200 (Blm10) (38). The PA700/19S complex stimulates hydrolysis of peptides and is essential for ATP and Ub conjugate degradation, while PA28αβ and PA28γ and PA200 stimulate peptide hydrolysis, but not protein or conjugate degradation by 20S and by SC 26S particles. By contrast, ZFAND5 activates 26S complexes both singly and doubly capped and enhances their multiple enzymatic activities, but not the activity of the 20S proteasomes.

ZFAND5 differs completely in structure, function, and expression pattern from these other activating factors. Binding of Ub conjugates to the 26S proteasome enhances these same enzymatic activities as ZFAND5 through an interaction with the associated DUBs, Usp14 and Uch37, and this activation can be mimicked by Ub-al or a Ub chain (27, 29). However, in the presence of ZFAND5, neither Ub-al, hexa-Ub, nor ATPγS, could stimulate proteasome activity further (*SI Appendix*, Fig. S2 *A*–*C*). These findings suggest that these different agents activate peptide hydrolysis by inducing similar structural changes; that is, they enhance gate opening in the 20S and enlarge the substrate channel through the ATPases. However, unlike Ub chains or Ub-a1, which act via binding to Usp14 (29), ZFAND5 stimulates even more effectively proteasomes lacking Usp14 (*SI Appendix*, Fig. S2*D*). Thus, its initial mechanism must be quite different from that of ubiquitinated substrates or Ub-al.

ZFAND5's stimulatory actions are very different from those of the two other ZFAND5 proteins tested (see below), but do resemble the changes seen upon 26S phosphorylation by cAMP and PKA (39) or by DYRK2, a protein kinase active during the cell cycle (40), although ZFAND5 in no way resembles a protein kinase. Unlike cAMP, which selectively enhances the breakdown of short-lived cell proteins (39), the presence of ZFAND5 stimulates the breakdown of the bulk of cell proteins (i.e., long-lived components). Also unlike these kinases, which act catalytically in modifying 26S particles, ZFAND5 acts in a saturable stoichiometric manner to stimulate the proteasome (Figs. 2*C* and 3*B*). Under conditions that activate proteasomes, ZFAND5 is monomeric (*SI Appendix*, Fig. S1*B*) but appears to induce large conformational changes in the 26S, as indicated by the changes in electrophoretic mobility of SC and DC particles as well as free 19S particles (Fig. 2*C* and *SI Appendix*, Fig. S1*C*). During proteasome activation and proteolysis, the 19S complex assumes at least six different structural states (41), but exactly where ZFAND5 binds and how it alters 19S structure and this sequence of conformational changes must await cryo-EM analysis.

The UBL-UBA proteins, for example, RAD23a and b, or ubiquilin/PLIC, are believed to serve shuttling factors that help deliver ubiquitinated substrates to proteasomes (42, 43). Despite appreciable evidence for such a role in vivo, various cell-free studies have thus far failed to demonstrate a clear stimulation of Ub-dependent proteolysis by RAD23a or b, in contrast to the large stimulation shown here with ZFAND5, because ZFAND5 also binds to both Ub conjugates and the 26S particle (Figs. 2 and 6*A*), and both of these interactions are essential for its stimulation of degradation of Ub conjugates and cellular proteins (Figs. 5*C* and 7 *F* and *H* and *SI Appendix*, Fig. S5). ZFAND5 resembles a shuttling factor that can promote association of ubiquitinated substrates with the proteasome, while also serving as a proteasome activator.

The Functions of the Different Domains in ZFAND5. Point mutations revealed the functions of ZFAND5's two zinc finger domains in the degradation of Ub conjugates and small peptides. The AN1 domain is clearly essential for the large enhancement of peptidase activity (Fig. 4*C*), but the isolated AN1 domain by itself cannot activate or even bind to proteasomes (Fig. 4 *E* and *F*). In contrast, the A20 domain is essential for ZFAND5's interaction with the Ub chain and for the stimulation of Ub conjugate degradation, as shown with ubiquitinated DHFR (Fig. 5*C*)

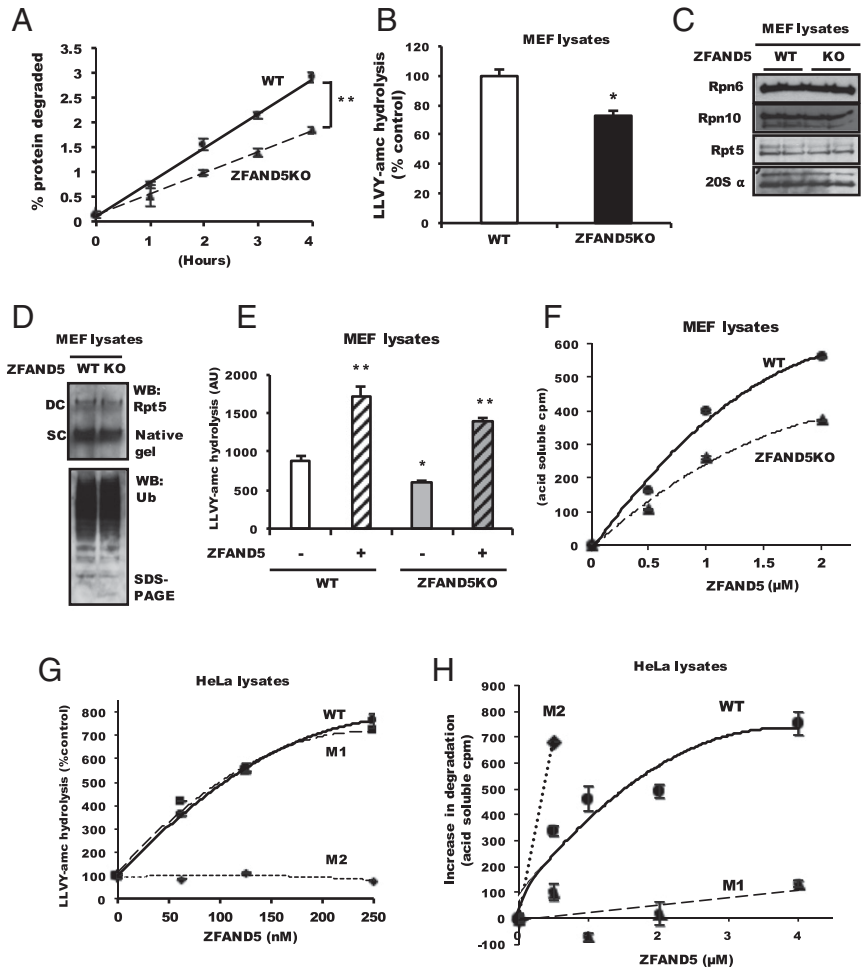

Fig. 7. ZFAND5 stimulates proteasomes and promotes overall protein degradation in MEF, HeLa, and HEK293 cells. (*A*) Overall proteolysis is reduced in MEF cells lacking ZFAND5. WT and ZFAND5 knockout cells (ZFAND5KO) were incubated with ^{3}H-phenylalanine for 24 h to label most cell proteins. After washing with chase medium containing a large excess of nonradioactive phenylalanine (2 mM), cells were incubated for 2 h to allow degradation of short-lived proteins. After fresh chase medium was added, the amounts of acid-soluble radioactivity appearing in the medium were measured at each time point and expressed as a percentage of total radioactivity initially incorporated into cell proteins. $^{**}P < 0.01$. (*B*) Peptidase activity of proteasomes in ZFAND5KO cells was also lower than in WT MEF cells than in the ZFAND5 knockout. Peptide activity was assayed in crude lysates, and the activity of WT MEF cells was taken as 100%. $^{*}P < 0.05$. (*C*) ZFAND5 knockout does not alter the amounts of proteasome subunits in these cells, as measured by Western blots. (*D*) The levels of doubly capped (DC) or singly capped (SC) 26S proteasomes also did not differ in the lysates of WT and KO MEF cells as shown by native PAGE. The total content of Ub conjugates were also similar as evaluated by SDS/PAGE and Western blots. (*E*) Addition of recombinant ZFAND5 (250 nM) to the lysates (2 μg) of WT or ZFAND5KO MEF cells elevated peptidase activity of endogenous 26S proteasomes. *WT versus ZFAND5KO (in the absence of added ZFAND5). **The stimulation by ZFAND5. $^{*,**}P < 0.05$. (*F*) Addition of ZFAND5 stimulated hydrolysis of endogenous proteins in the lysates of WT or ZFAND5KO MEF cells. Proteins in MEF cells were labeled with ^{3}H-phenylalanine as in *A*, and cell lysates (200 μg) were incubated with increasing amounts of ZFAND5WT for 30 min at 37 °C to allow degradation of endogenous proteins. The increases in proteolysis, as measured by the increases in TCA-soluble radioactivity above that in the absence of added ZFAND5 is shown. (*G*) Recombinant ZFAND5 promoted also peptide hydrolysis by proteasomes (2 μg) in HeLa cell lysates, which do not express ZFAND5. As shown with purified proteins in Fig. 3*C*, AN1 is essential for the activation of proteasomal peptidase in cells. Increasing amounts of recombinant ZFAND5WT or mutants (M1 or M2) were added, and the activity of 26S without ZFAND5 was taken as 100%. (*H*) Addition of ZFAND5 to HeLa lysates greatly stimulated the breakdown of endogenous proteins, and this effect required the A20 domain. The lysates (200 μg) of HeLa cells, labeled by with ^{3}H-phenylalanine as in *F*, were incubated with increasing amount of ZFAND5WT or the mutants for 30 min at 37 °C to allow degradation of endogenous proteins. The increased production of TCA-soluble radioactivity from labeled cell proteins after addition of ZFAND5WT or the mutants was plotted as in *F*. All values are the means of at least three independent experiments ± SD.

and endogenous proteins in cell homogenates (Fig. 7 *F* and *H*). Thus, ZFAND5 seems to have to bind the ubiquitinated substrate to enhance its degradation. Moreover, neither the central region alone nor the central region plus AN1 was able to bind and stimulate proteasomal activity (*SI Appendix*, Fig. S3 *B* and *C*). In other words, full-length ZFAND5 is required for binding both ubiquitinated substrates and proteasomes and for stimulating proteolysis.

It was surprising to find that the structural requirements for the ZFAND5's enhancement of peptide hydrolysis are different from those for the stimulation of Ub conjugate degradation (Figs. 4 and 5*C*). Thus, the increased peptide hydrolysis is not necessary for the increase in hydrolysis of ubiquitinated proteins. This finding was unexpected, since other treatments known to enhance proteasomal degradation of Ub conjugates, including phosphorylation by DYRK2 or cAMP-PKA and ATP hydrolysis, also promote peptide entry and hydrolysis. However, other well-known proteasome activators (PA28αβ, or PA28γ, and PA200) can also markedly enhance peptidase activities but do not increase Ub conjugate hydrolysis and thus by themselves do not appear capable of promoting protein degradation in vivo, unlike ZFAND5.

The maximal stimulation of peptide hydrolysis by ZFAND5 was many-fold larger than that by ZFAND2a, and ZFAND2b had no

stimulatory effect (Fig. 3*D*), even though these latter proteins do contain two AN1 domains. The AN1 domains of ZFAND2a and ZFAND2b are very similar (61% identity) as shown using "blastp suite" (https://blast.ncbi.nlm.nih.gov/Blast.cgi), but they showed only 30% identity with the AN1 domain of ZFAND5, which presumably explains their having little or no effect on peptidase activity. Most importantly, ZFAND2a, unlike ZFAND5, did not stimulate degradation of a ubiquitinated protein (*SI Appendix*, Fig. S4), and thus its role in vivo is unclear.

ZFAND5's Roles in Vivo. A number of ZFAND proteins, including ZFAND2a, ZFAND2b, AIP-1, and Cuz-1, were reported to associate with proteasomes or with CDC48/p97/VCP and were proposed to facilitate the degradation of misfolded proteins upon exposure to arsenite or heat shock and to reduce their toxic consequences (1–3, 6, 44). In fact, a recent study showed that upon arsenite treatment ZFAND1 recruits p97 and proteasomes to the stress granules and appears to promote clearance of the granules (45). Although originally proposed to function as proteasome activators, ZFAND2a had little and ZFAND2b had no effect on proteasomal peptidase activity, and neither stimulated proteasomal degradation of Ub conjugates (*SI Appendix*, Fig. S4). These studies suggest that ZFAND proteins may require other key regulator of protein degradation such as p97 in addition to proteasomes for efficient hydrolysis of misfolded or aggregated proteins. Unlike ZFAND2a and the ZFAND proteins in plants, ZFAND5 does not appear to function in proteotoxic stresses. Although inducible by catabolic hormones, it is not induced during the unfolded protein response nor during heat shock (Fig. 1 *C*–*E*). In fact, during heat shock, ZFAND5, normally a short-lived protein, is degraded even faster, and its level falls rapidly (Fig. 1 *C* and *D*). It is also noteworthy that the absence of ZFAND5 in MEF KO cells decreases and its addition to cell lysates increases the degradation of normal long-lived cell proteins and not the misfolded proteins that trigger proteotoxic stress.

The tissue distribution of ZFAND5 is intriguing, but difficult to explain. Its presence in high amounts in heart, eye, and brain presumably correlates with the high rates of protein turnover in these tissues, although liver, which lacks ZFAND5, also is very active in proteolysis by the UPS. Protein breakdown rates normally are low in skeletal muscle, except during atrophy when the UPS is activated and accounts for most of increased protein degradation (10). Using ZFAND5 knockout mice, Hishiya et al. (9) demonstrated that ZFAND5 is essential for the rapid muscle atrophy induced by fasting, denervation, or high doses of glucocorticoids. They also showed that these conditions all cause ZFAND5 induction, as was confirmed here with dexamethasone treatment of myotubes (Fig. 1*A*). Our finding that ZFAND5 promotes overall protein degradation in cells accounts nicely for its induction during atrophy and its critical role in the loss of muscle mass. Most of the protein loss during atrophy is due to degradation of myofibrillar proteins, and possibly the rapid hydrolysis of these large fibrous proteins requires a more potent form of the proteasome than typical cell proteins. The disassembly and degradation of the myofibrillar apparatus by the UPS requires the p97/VCP complex as well as proteasomes (46). Possibly ZFAND5, like ZFAND2b (44) and Cuz1 (6), can enhance p97 function in addition to 26S proteasomes.

The marked loss of muscle mass in these various conditions requires the transcription by FoxO 1 and 3 of several atrophy-specific Ub ligases (e.g., Atrogin-1/MAFBx, and MuRF1), which increase Ub conjugation generally (10). The simultaneous induction of ZFAND5 by enhancing the proteasomes' degradative activity should promote the clearance of these ubiquitinated proteins, presumably to promote the efficient clearance of ubiquitinated proteins. Moreover, some forms of Duchenne and Becker muscular dystrophy are due to missense mutations in dystrophin that lead to its rapid degradation by the UPS, and recently down-regulation of ZFAND5 was shown to raise the levels of this critical protein (11). Because down-regulation of ZFAND5 reduced muscle wasting and proteolysis by the UPS, pharmacological antagonists of ZFAND5 might represent a therapy to ameliorate the debilitating loss of muscle protein in various cachectic conditions, as well as in these muscular dystrophies.

Materials and Methods

Cell Lines and Antibodies. HeLa, HEK293, and MEF cells lacking ZFAND5 or WT cells (kindly provided by Ken Watanabe, National Center for Geriatrics and Gerontology, Obu, Japan) were cultured in DMEM with 10% FBS at 37 °C. The primary antibodies used are commercially available: 20S α (BML-PW8195; Enzo Life Science), PSMC3 (A303-538A; Bethyl Laboratories), PSMA1 (A303-809; Bethyl Laboratories), PSMD11 (14303; Cell Signaling Technology), PSMD4 (A303-855A; Bethyl Laboratories), Ub (sc-8017; Santa Cruz Biotechnology), GST (sc-138; Santa Cruz Biotechnology), HSP70 (ADI-SPA-810-D; Enzo Life Science), and tubulin (T8328; Sigma). The polyclonal antibody to ZFAND5 was raised against recombinant mouse ZFAND5. The antibody to PSMA6 was a generous gift from Alexei Kisselev, Auburn University, Auburn, AL.

Plasmids. The mouse gene for ZFAND5 was isolated by RT-PCR from tibialis anterior muscle of mice deprived of food for 2 d. Full-length ZFAND5 or deletion mutants lacking zinc finger domains or sequences encoding only A20, AN1, or central region (GST-ΔA20, -ΔAN1, and -CR; GST-A20, -AN1) were cloned as GST fusions into pGEX-6P-1. The bacterial expression plasmids encoding the mouse ZFAND5 gene (both WT and zinc finger point mutants) fused to GST were kindly provided by Ken Watanabe. The bacterial plasmid expressing His-ZFAND2a was a kind gift from David Ron, University of Cambridge, Cambridge, UK. ZFAND2b gene was amplified by RT-PCR and cloned into a bacterial expression plasmid (pET28a; Qiagen).

Myoblasts Expressing PSMB4-FLAG from Transgenic Mouse. Transgenic mice expressing PSMB4-FLAG were produced in collaboration with Regeneron Pharmaceuticals. After harvesting skeletal muscles, myoblasts were established, and expression of PSMB4-FLAG was confirmed using FLAG (M2) antibody (F1804; Sigma). All mice were housed with the approval of Institutional Animal Care and Use Committee-Harvard Medical School and are in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (47).

Purification of ZFAND2a, ZFAND2b, ZFAND5, and 26S Proteasomes. GST-tagged ZFAND5 and the various mutants were expressed in *E. coli*, and the lysate was incubated with glutathione Sepharose 4B (17075601; GE). After washing with PBS, resin-bound GST-ZFAND5 proteins were eluted with 10 mM glutathione or GST was removed with PreScission (27084301; GE). His-ZFAND2a and His-ZFAND2b were purified with Ni-NTA resin (Qiagen). Proteasomes were purified from lysates of HEK293 or HeLa cells with GST-UBL, as described previously (20).

Purification of Ubiquitin Conjugates and GST Pull-Down Assay with ZFAND5WT or Mutants. To make K48 or K63 linked Ub chains, GST-tagged E6AP and NEDD4 were allowed to autoubiquitinate, as described previously (35). Briefly, E3 (600 nM) was incubated with E1 (50 nM), E2/UbcH5b (600 nM), ATP (2 mM), Ub (30 μM), and DTT (1 mM) at 37 °C for 4 h. Glutathione resin-bound unmodified or polyubiquitinated Ub ligases (150 nM) were washed with buffer (25 mM Hepes, pH 8, 125 mM potassium acetate, 2.5 mM $MgCl_2$, and 0.025% Triton X-100) and then incubated with ZFAND5 WT or mutants (450 nM) for 30 min at 37 °C. After washing the resin with 150 mM NaCl, GST-E6AP or NEDD4 was eluted with 10 mM reduced glutathione, and binding of ZFAND5 was detected by Western blots.

Measurement of Peptidase Activity. Chymotrypsin-like site activity of 26S proteasomes (2 nM) was examined with fluorogenic substrates [Suc-LLVY-7-amino-4-methylcoumarin (AMC) (10 μM) or Suc-RPFHLLVY-AMC (10 μM), the caspase-like site with Z-LLE-AMC (10 μM), the trypsin-like with Z-VLR-AMC (10 μM)] in a buffer containing 50 mM Tris, pH 7.5, 40 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 1 mM ATP, and 50 μg/mL BSA. Hydrolysis of the peptides was monitored continuously by fluorescence from released AMC (λ_{ex}, 380 nm; λ_{em}, 460 nm) for 60 min at 37 °C. The rate of degradation was determined by the slope of the linear phase of the reaction curve. Each figure shows data from at least three independent experiments, each with three replicates.

Measurement of ATPase Activity. The effect of ZFAND5 on the ATPase activity of 26S proteasomes was measured using the malachite green assay, which

detects production of inorganic phosphate (Pi). Proteasomes (10 nM) in the absence or presence of ZFAND5 (2.5 μM) were incubated in a buffer containing 25 mM Hepes (pH 8), 2.5 mM $MgCl_2$, 125 mM potassium acetate, 0.025% Triton X-100, 1 mM ATP, 1 mM DTT, and 0.1 mg/mL BSA as previously described (39). Each figure shows data from at least three independent experiments with three replicates.

Measurement of Protein Degradation in Cells and Cell Lysates. The method for measurement of protein degradation rate has been described in details previously (36). WT MEF or MEF cells lacking ZFAND5, HeLa cells, or HEK293 cells were incubated with ^{3}H-phenylalanine (4 μCi/mL; PerkinElmer) for 24 h to label long-lived proteins. After washing with a chase medium containing nonradioactive 2 mM phenylalanine, the cells were incubated for 2 h to limit reincorporation of released radioactive phenylalanine. Then, fresh chase medium was added and 10% of the medium was collected at the indicated times. Proteins were precipitated with 10% trichloroacetic acid (TCA), and the radioactivity in the TCA-soluble supernatant (amino acids) was measured. Protein degradation was expressed as the radioactivity released (i.e., amino acids generated) as a percentage of the total ^{3}H-phenylalanine incorporated into proteins (i.e., the sum of radioactivity in TCA-soluble fraction at each time point and radioactivity remaining in the cell proteins).

To assess the effect of recombinant ZFAND5 on protein degradation in the lysates of HeLa cells or HEK293 cells, increasing amounts of ZFAND5WT or a zinc finger mutant were incubated with 200 μg of lysates for 30 min at 37 °C. Proteins were precipitated with TCA, and radioactivity in the TCA-soluble fractions was measured as described above for intact cells. The increase in protein degradation (TCA-soluble counts per minute) by different concentrations of ZFAND5 was expressed by subtracting the radioactivity generated without added ZFAND5. Each figure shows data from at least three independent experiments with three replicates.

Measurement of the hydrolysis of radiolabeled Ub_5-DHFR was described in detail previously (39). The generation of TCA-soluble radioactivity by 26S proteasomes with or without WT ZFAND5 or mutants was then determined.

Statistical Analysis. Statistical analysis was performed using unpaired Student's *t* tests, and significant differences are marked by asterisks.

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