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The Slow Wallerian Degeneration Protein, WldS, Binds Directly to VCP/p97 and Partially Redistributes It within the Nucleus


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Slow Wallerian degeneration (WldS) mutant mice express a chimeric nuclear protein that protects sick or injured axons from degeneration. The C-terminal region, derived from NAD+ synthesizing enzyme Nmnat1, is reported to confer neuroprotection in vitro. However, an additional role for the N-terminal 70 amino acids (N70), derived from multiubiquitination factor Ube4b, has not been excluded. In wild-type Ube4b, N70 is part of a sequence essential for ubiquitination activity but its role is not understood. We report direct binding of N70 to valosin-containing protein (VCP; p97/Cdc48), a protein with diverse cellular roles including a pivotal role in the ubiquitin proteasome system. Interaction with WldS targets VCP to discrete intranuclear foci where ubiquitin epitopes can also accumulate. WldS lacking its N-terminal 16 amino acids (N16) neither binds nor redistributes VCP, but continues to accumulate in intranuclear foci, targeting its protein with diverse cellular roles including a pivotal role in the ubiquitin proteasome system. Interaction with WldS activity but its role is not understood. We report direct binding of N70 to valosin-containing protein (VCP; p97/Cdc48), a protein with diverse cellular roles including a pivotal role in the ubiquitin proteasome system. Interaction with WldS lacking its N-terminal 16 amino acids (N16) neither binds nor redistributes VCP, but continues to accumulate in intranuclear foci, targeting its intrinsic NAD+ synthesis activity to these same foci. Wild-type Ube4b also requires N16 to bind VCP, despite a more C-terminal binding site in invertebrate orthologues. We conclude that N-terminal sequences of WldS protein influence the intranuclear location of both ubiquitin proteasome and NAD+ synthesis machinery and that an evolutionary recent sequence mediates binding of mammalian Ube4b to VCP.

INTRODUCTION

The E4 ubiquitination factor Ube4b (or Ufd2a) has a 123-amino acid N-terminal region that is essential for ubiquitination activity (Mahoney et al., 2002). It is unclear why this region is essential because it does not contain the U box, and it appears to be absent in invertebrate orthologues that ubiquitinate effectively (Koegl et al., 1999; Hatakeyama et al., 2001; Mahoney et al., 2002; Hoppe et al., 2004; Richly et al., 2005). It is important to understand the molecular mechanism of Ube4b because it has a key role in the ubiquitin proteasome system (UPS; Hoppe, 2005), it is neuroprotective in polyglutamine disorders (Matsumoto et al., 2004) and an important candidate gene for neuroblastoma (Krona et al., 2003). Information on the substrates of Ube4b is beginning to emerge (Hoppe et al., 2004; Okumura et al., 2004; Spinette et al., 2004; Richly et al., 2005) but there is much still to learn about its regulation.

In the slow Wallerian degeneration mutant mouse (WldS), 70 amino acids of this essential domain of Ube4b form the N-terminus of a chimeric protein that delays Wallerian degeneration of injured axons in mice and rats by 10-fold (see Figure 1A; Lunn et al., 1989; Mack et al., 2001; Adalbert et al., 2005). The chimeric protein is absent in wild-type mice. This sequence (N70) is fused in WldS protein to the full coding sequence of nicotinamide mononucleotide adenylyltransferase (Nmnat1; Conforti et al., 2000; Emanuelli et al., 2001; Mack et al., 2001), implicating the UPS or NAD+ metabolism.
in regulating axon degeneration. Wld<sup>S</sup> also delays axon degeneration in a wide range of neurodegenerative disorders and acute retrograde axonal degeneration after spinal injury, indicating that axon degeneration mechanisms are more closely related than previously thought (Wang et al., 2002; Ferri et al., 2003; Samsam et al., 2003; Coleman, 2005; Kerschensteiner et al., 2005; Mi et al., 2005). Surprisingly, Wld<sup>S</sup> in vivo has only been found in nuclei, suggesting that downstream axonal effector(s) mediate its remarkable effect on axon degeneration (Mack et al., 2001; Samsam et al., 2003; Sajadi et al., 2004).

Nmnat1 activity was reported to preserve injured axons for 3 d in vitro (Araki et al., 2004; Wang et al., 2005). However, axons in vivo are far longer, have a profoundly different environment, and are protected by full-length Wld<sup>S</sup> for up to 3 wk (Crawford et al., 1995; Adalbert et al., 2005). A contribution of N70 to strengthening the neuroprotective phenotype in vivo is suggested by the fact that transgenic mice overexpressing N70 could contribute to neuroprotection by perturbing synthesis activity, as Wld<sup>S</sup> protein clusters to clear sites or immediately is passed to downstream components when Wld<sup>S</sup> protein is present. VCP, one of the AAA family of proteins (Meyer et al., 2005), accumulates in neuronal nuclei in a range of neurodegenerative diseases and can influence neurodegeneration both positively and negatively (Hirabayashi et al., 2001; Higashiyama et al., 2002; Mizuno et al., 2003; Watts et al., 2004; Schroder et al., 2005). Its intranuclear roles include interaction with Werner syndrome proteins, including lipids, was resuspended in 40 ml NIM supplemented with protease inhibitor cocktail (Sigma). Unbroken cells and connective tissue were filtered through a 100-μm nylon mesh and centrifuged at 100 g for 10 min. The supernatant containing solubilized nuclear proteins was preincubated with protein G agarose (Roche) for 2 h at 4°C under constant stirring. The supernatant was washed four times with PBS (0.1 M sodium phosphate, 0.5 M NaCl, pH 7.4) and then resuspended in PBS (0.1 M sodium phosphate, 0.5 M NaCl, pH 7.4). Proteins fished by a pulldown assay were eluted with double-concentrated PEG buffer and separated on SDS-PAGE gels. From the gel individual Coomassie blue-stained protein bands were excised with a scalpel and subjected to in-gel trypsin digestion. Peptides were extracted with 50% acetonitrile/0.1% formic acid and analyzed by MALDI-TOF mass spectrometry using an ABI 4700 Proteomics Analyzer (Applied Biosystems, Foster City, CA). For the subsequent immunoprecipitation, protein lysates were preincubated with 30 μl of protein G agarose (Roche) for 2 h at 4°C under constant

### MATERIALS AND METHODS

#### Constructs

VCP full-length cDNA was PCR-amplified with the Expand High Fidelity PCR System (Roche, Mannheim, Germany) from a mouse fibroblast cDNA library kindly provided by R. Lange using the 5’ BamHI- and 3’ SalI-tagged primers (restriction sites in bold): 5’-ATATGAAGATCCCTATGGAGACGCGGCTGT-3’ and 5’-GGCTGATCTTGTCTCAGTGCTGCTTC-3’. The products were cloned with BamHI/SalI into pGEX5X-1 (Amersham Biosciences, Freiburg, Germany), and with NcoI/SalI into the pGIPZ vector (BD Biosciences Clontech, Heidelberg, Germany). The Wld<sup>S</sup> protein, N70 and further truncation products were similarly PCR-amplified and cloned using the Wld<sup>S</sup> transgene construct (Mack et al., 2001) as template and appropriate primers based on the Wld<sup>S</sup> cDNA (GenBank AF260924). Human Ube4b was amplified from a construct kindly provided by Professor James Mahoney using primers 5’-ATCCCCGAAATTCATGAGGACGCGGCTGT-3’ and 5’-GCGTGGGTCTTGTCTCAGTGCTGCTTC-3’. The PCR-amplified enzyme (Pfu) (Stratagene, Heidelberg, Germany) and appropriate combinations of the following primers (1 + 2, 4 + 2, and 1 + 3, respectively) were used in a reaction buffer containing 10% restriction enzyme tags, added for cloning purposes, are shown in bold and the first or last three bases of sequence derived from the Wld<sup>S</sup> gene are underlined. A single base change to repress the stop codon of Wld<sup>S</sup> Rev allows read-through of the C-terminal GFP is double-underlined: 1) 5’- TAGATCCCAAGCTTAAACCTTCACATTAAGGGAAAGGACGATG-3’; 2) 5’-GGCGGATCCGCGGCCAGGAGGAAATGTCGTCAGTCG-3’. The activities of the pEGFP-N1 (BD Biosciences), all sequences were verified using the Taq FS BigDye-terminator cycle sequencing method on a ABI 3730077 primers and the corresponding ABI software. The sequences were then cloned into the pCGG vector (program Wisconsin Package Version 10.2, Accelrys, San Diego, CA). The full-length Wld<sup>S</sup> cDNA was subcloned into the HindIII/ BamHI insert from pEGFP-N1 (above) into pDsRed2-N1 (BD Biosciences). Wld<sup>S</sup> cDNA was PCR-amplified from the human DNA sequence encoding full-length Wld<sup>S</sup> and 50 μM Tris, pH 7.4, and 0.1% deoxycholate containing protease inhibitor mix (Sigma, Taufkirchen, Germany). After incubating for 30 min at 37°C, insoluble material was removed by centrifugation (15,000 × g, 30 min) and the supernatant was dialyzed against binding buffer (50 mM Tris, pH 7.4, and 0.1% Triton X-100) overnight at 4°C. Homogenates were incubated overnight at 4°C with GST fusion proteins bound to glutathione Sepharose 4B. The beads were washed four times with phosphate-buffered saline (PBS) (0.1% Triton X-100) and resuspended in 1X binding buffer. The beads were then recrystallized by addition of 1 μl of protein G agarose (Roche) for 2 h at 4°C under constant stirring. The beads were filtered through a 100-μm nylon mesh and centrifuged at 100 g for 10 min. The supernatant containing solubilized nuclear proteins was preincubated with protein G agarose (Roche) for 2 h at 4°C under constant

##### Pulldown Assays

CS/BL/6 mouse brain homogenates were used in this experiment to avoid contamination from endogenous Wld<sup>S</sup> protein. Brains were collected and homogenized in 50 volumes of 50 mM Tris, pH 9.0, and 1% deoxycholate containing protease inhibitor mix (Sigma, Taufkirchen, Germany). After incubating for 30 min at 37°C, soluble material was removed by centrifugation (15,000 × g, 30 min) and the supernatant was dialyzed against binding buffer (50 mM Tris, pH 7.4, and 0.1% Triton X-100) overnight at 4°C. Homogenates were incubated overnight at 4°C with GST fusion proteins bound to glutathione Sepharose 4B. The beads were washed four times with phosphate-buffered saline (PBS) (0.1% Triton X-100) and resuspended in 1X binding buffer. The beads were then recrystallized by addition of 1 μl of protein G agarose (Roche) for 2 h at 4°C under constant stirring. The beads were filtered through a 100-μm nylon mesh and centrifuged at 100 g for 10 min. The supernatant containing solubilized nuclear proteins was preincubated with protein G agarose (Roche) for 2 h at 4°C under constant

##### MALDI-TOF Mass Spectrometry

Proteins fished by a pulldown assay were eluted with double-concentrated SDS-PAGE sample buffer and separated on SDS-PAGE gels. From the gel individual Coomassie blue-stained protein bands were excised with a scalpel and destained by washing with 25 mM NH<sub>4</sub>HCO<sub>3</sub>/30% acetonitrile for MALDI-TOF mass spectrometry analysis, the samples were dissolved in 5 μl 0.1% aqueous trifluoroacetic acid. MALDI-MS was carried out in linear mode on a Bruker Reflex IV equipped with a video system (Rheinstetten, Germany), a nitrogen UV laser (Omax ~ 337 nm), and a HiMass detector. One microliter of the sample solution was placed on the target and 1 μl of a freshly prepared saturated solution of sinapinic acid in acetonitrile/H<sub>2</sub>O (2:1) with 0.1% trifluoroacetic acid was added. The spot was then recrystallized by addition of another 1 μl acetonitrile/H<sub>2</sub>O (2:1), which resulted in a fine crystalline matrix. For recording of the spectra an acceleration voltage of 20 kV was used, and the detector delay was adjusted to 1.9 kV. Approx. 600 single laser shots were summed into an accumulated spectrum. Calibration was carried out using the single and doubly protonated ion signal of bovine serum albumin for external calibration. Identification of the mass fingerprint spectra was performed using the Mascot program available from Matrix Science on the World Wide Web (http://www.matrixscience.com/home.html).

##### Isolation and Immunoprecipitation of Nuclear Proteins

Six mouse brains (ca. 2.5 g) were each homogenized using a Dounce homogenizer in 40 ml precooled nuclear isolation medium (NIM; 0.25 M sucrose, 25 mM HEPES, 5 mM MgCl<sub>2</sub>, 10 mM Tris (pH, 7.4)) supplemented with protease inhibitor cocktail (Sigma). Unbroken cells and connective tissue were removed by filtration. The filtered homogenate was then diluted with an equal volume of ice-cold NIM, centrifuged (10 min, 8000 × g) and the pellet, including lipids, was resuspended in 40 ml NIM supplemented with protease inhibitor cocktail. After repeating the centrifugation, the new pellet was resuspended in 8 ml NIM, 2 ml sucrose density barrier (SDB; 2.3 M sucrose, 25 mM KCl, 5 mM MgCl<sub>2</sub>, 10 mM Tris (pH, 7.4)) solution was added and mixed vigorously. 10 min later 1/2 of the suspension was added to 1.3 M sucrose and centrifuged for 1 h at 100,000 × g in a Beckman SW41 Ti rotor (Krefeld, Germany). The pellet, together with remaining lipids, was resuspended in PBS containing 1% Triton X-100 and incubated for 2 h at 4°C with constant agitation at 400 rpm. 500 single laser shots were summed into an accumulated spectrum. Calibration was carried out using the single and doubly protonated ion signal of bovine serum albumin for external calibration. Identification of the mass fingerprint spectra was performed using the Mascot program available from Matrix Science on the World Wide Web (http://www.matrixscience.com/home.html).
agitation. Samples were centrifuged (5 min, 500g) to remove proteins unspecifically bound to the protein G agarose and 3 µL of anti-WldS rabbit serum and another 30 µL of protein G agarose were then added and incubated overnight at 4°C with constant agitation. The precipitated proteins were then washed four times with the same buffer. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5% low fat milk/Tween-20 and incubated with primary antibodies and horseradish peroxidase-conjugated secondary antibody (1:2000) overnight at 4°C. After washing with PBS, the membranes were incubated at room temperature in serum blocker consisting of 4% milk, 0.5% Tween-20 and 0.1% Triton X-100. Samples were then analyzed by SDS-PAGE and immunoblotting.

In Vitro Binding Assays

GST fusion proteins were purified and coupled to glutathione-Sepharose 4B according to the protocol of the manufacturer (Amersham Biosciences). The pGKBK7 constructs containing the WldS promoter were in vitro transcribed and translated incorporating [35S]-methionine using the TNT T7 Reticulocyte Lysate Coupled Transcription/Translation kit from Promega (Promega GmbH, Mannheim, Germany). The obtained proteins were mixed with equal amounts of GST fusion proteins and the binding assay was performed as previously described (Dai et al., 1998). Reactions were analyzed by SDS-PAGE, and the gel was fixed with 10% methanol/10% acetic acid before autoradiography. Purified His-tagged VCP was a kind gift from Dr. Sarah Spinette (Johns Hopkins).

Cell Culture and Transfection

Plasmid DNA was isolated using the endonuclease free plasmid kit (QIAGEN, Hilden, Germany). DNA was transfected using LipofectAMINE 2000 (Invitrogen) into COS-7, PC12, or HeLa cells immediately before differentiation by culturing in 100 ng/µl NGF on a type IV collagen substrate (Sigma). The “TV” PC12 subline, stably transfected with a tet-off inducible C-terminal EGFP-tagged VCP construct (Kobayashi et al., 2002) was grown in 1.0 µg/ml doxycycline (Sigma), which was removed to induce VCP/EGFP expression. Protein location was observed 1-5 d after transfection. Spinal motor neurons from embryonic day 14 (E14) embryonic rats were cultured and electroporated in suspension as previously described (Henderson et al., 1995; Raum et al., 2002).

Immunocytochemistry

Slices of 100 µm were cut from the cerebellum of WldS mice and fixed in 4% paraformaldehyde (Fisher, Schwerte, Germany). Lumbar spinal cord dorsal root ganglia (DRG) were removed from WldS mice or rats and fixed in 4% paraformaldehyde, and then 20-µm sections cut on a cryostat. Slices were incubated for 2 h at room temperature in serum blocker consisting of 4% bovine serum albumin (Sigma) and 0.5% Triton X-100 (Sigma) in WldS-18 antibody (Samsam et al., 2003; 1:500 dilution) plus VCP antibody (against amino acids 9–130; BD Biosciences; 1:200 dilution; or mouse monoclonal to VCP (ab11433); AbCam, Cambridge, United Kingdom, 1:500 dilution) were applied simultaneously in serum blocking solution overnight at 4°C. After washing with PBS, slices were incubated overnight at 4°C in a solution containing TRITC-conjugated anti-rabbit (DAKO, Hamburg, Germany; 1:20 dilution in PBS) plus Alexa488-conjugated anti-mouse (Molecular Probes, Leiden, Netherlands; 1:200) secondary antibodies. Slices were then washed in PBS and incubated in To-pro3 (Molecular Probes) for 10 min before mounting in Mowiol/DABCO preparation. Staining was visualized on a laser scanning confocal microscope (Bio-Rad Radiance 2000, Hemel Hempsted, United Kingdom). DNA was stained using TRITC-conjugated anti-rabbit (DAKO) or tetramethylrhodamine isothiocyanate-conjugated anti-mouse (Molecular Probes, Eugene, Oregon) antibodies. Images were taken on a Zeiss LSM 510 META confocal system (Oberkochen, Germany; LSM Software Release 3.2) coupled to a Zeiss Axiovert 200 microscope.

Western Blotting

WldS protein expression was analyzed in mouse cerebellum homogenized in five volumes of RIPA buffer, respectively, plus 1X Complete protease inhibitor cocktail (Roche) or in PC12 cells scraped from the dish in a minimal volume of the same buffer. Proteins were separated by SDS-PAGE and semidry blotted onto nitrocellulose (Bio-Rad). Blocking and incubation with primary antibodies and horseradish peroxidase-conjugated secondary antibodies (1:5000; Serotec, Heidelberg, Germany) were performed in PBS plus 0.02% Tween-20 and 5% low fat milk. Proteins were visualized using the ECL detection kit (Amersham Biosciences) according to the manufacturer’s instructions.

RESULTS

WldS Protein Binding Directly to VCP through its N-terminal Domain

A screen for WldS binding partners revealed that a protein of ca. 97 kDa can be pulled down in large quantities from mouse brain homogenate by GST-WldS (Figure 1B). N70 is sufficient for this activity. Isolation of this protein from an SDS-PAGE gel followed by MALDI-TOF mass spectrometry protein identification revealed several tryptic peptides exactly matching sequences from murine VCP (Figure 1C). To confirm that binding of WldS to VCP is biologically relevant, we communoprecipitated VCP specifically from nuclear extracts of WldS mouse brain using antibody Wld-18 and confirmed the identity of VCP by Western blotting (Figure 1D). VCP could not be communoprecipitated from wild-type mouse brain, which lacks WldS protein. The reciprocal co-immunoprecipitation was inconclusive because VCP itself was not immunoprecipitated by the BD Biosciences anti-VCP antibody, but these data strongly suggest that the WldS/VCP complex is biologically relevant. To determine whether the binding was direct or mediated by other factors, we precipitated purified His-tagged VCP (a kind gift from Dr. Sarah Spinette and Prof. Antony Rosen, Johns Hopkins) using GST-tagged N-terminal fragments of WldS (Figure 1E; see also Figure 3). The ability of WldS-derived peptides to precipitate VCP was retained in the absence of other relevant proteins, showing that VCP binds directly to the shared N-terminus of WldS and Ube4b. Because WldS protein has been suggested to alter the UPS (Zhai et al., 2003; Coleman and Ribchester, 2004), we then tested by Western blotting of WldS and wild-type brain homogenates whether WldS alters turnover of VCP, and thus its steady-state level, and found that it does not (Figure 1F). Thus, VCP directly binds to WldS protein through its N70 domain, and this binding does not significantly alter steady-state level of VCP in brain.

 summary
iological conditions, including in at least some neurons that display the WldS phenotype.

VCP Binds within the First 16 Residues of WldS
We show above that WldS binds to and partially redistributes VCP. To establish that binding causes redistribution, we mapped and then deleted key residues of WldS required for VCP binding. Truncated N70 constructs were expressed as GST-fusion proteins and used to pull down IVTT-expressed VCP. First the N-terminal 23 amino acids were found to be necessary and sufficient for VCP binding, and this was subsequently narrowed to the N-terminal 16 amino acids (N16; Figure 3A). Ube4b lacking this sequence also did not bind VCP. Two WldS/DsRed2 constructs were then made: one full-length and one lacking N16, and transiently transfected into the TV PC12 stable subline that expresses VCP/EGFP fusion protein in a tet-off-inducible manner (a kind gift from Prof. Akira Kakizuka). Full-length WldS/DsRed2 was able to bring about partial redistribution of VCP/EGFP into intranuclear foci in transfected cells (arrowhead; Figure 3, B–E) but removal of N16 containing the VCP binding site prevented this (Figure 3, F–I). Interestingly, the ability of WldS protein to accumulate in intranuclear foci does not require binding to VCP (Figure 3F). Thus, VCP has to bind WldS N16 to be partially redistributed in the manner we describe.

The Sequence Targeting WldS Protein to Intranuclear Foci Is Not Nmnat1
Because VCP binding is not required to target WldS to intranuclear foci, we asked whether this is a property of the Nmnat1 sequence by fusing Nmnat1 to EGFP and transiently transfecting it (Figure 4). Nmnat1, but not N70, was restricted to the nucleus, confirming previous reports that
Nmnat1 is a nuclear protein and suggesting that nuclear targeting of WldS protein is due to the putative nuclear localization signal of Nmnat1 (Raffaelli et al., 2002; Araki et al., 2004; Magni et al., 2004). However, unlike WldS/EGFP, intranuclear distribution of Nmnat1/EGFP was homogeneous apart from its exclusion from nucleoli. The fraction of N70/EGFP that entered the nucleus was also homogeneously distributed, suggesting that WldS protein is targeted to intranuclear foci either by a conformational change involving both N70 and Nmnat1 sequence or by the short unique sequence that separates these two parts of the protein. Cell and nuclear shape indicated that the cells were healthy in all cases. We conclude that Nmnat1 and WldS protein differ in their intranuclear distribution, but as shown above, their differing abilities to bind VCP do not underlie this difference.

WldS Also Can Partially Relocalize Ubiquitin inside the Nucleus

VCP binds long ubiquitin chains (Dai and Li, 2001) so one consequence of its partial redistribution by WldS protein could be redistribution of bound polyubiquitinated proteins. This was supported by immunocytochemistry of PC12 cells transiently transfected with WldS/EGFP fusion construct (Figure 5, A–H). As with VCP, the normal distribution of ubiquitin throughout the nucleus and cytoplasm of untransfected cells is joined by a punctate intranuclear pattern in transfected cells, where ubiquitin puncta colocalize with WldS/EGFP. Cell and nuclear shape indicated that the cells were healthy (see also Supplementary Figure 3). The association of ubiquitin epitopes with WldS also depended on the N16 sequence, suggesting it is secondary to VCP binding to WldS (Figure 5, I–P).

An alternative explanation for the accumulation of ubiquitin epitopes at these sites could be ubiquitination of the WldS/EGFP fusion protein, so we looked for these putative ubiquitinated species by Western blotting (Figure 6). Wld-18 antibody detected only a single band of the expected size, even on long-exposure ECLs after a generalized accumulation of ubiquitination products due to proteasome inhibition. The intensity of the WldS band did increase after proteasome inhibition, suggesting that WldS protein is degraded by the UPS but despite this, ubiquitinated WldS did not reach detectable levels on the Western blots. Although we cannot rule out the possibility that ubiquitinated WldS contributes to the ubiquitin speckles, the inability to detect this putative species on a Western blot and the probable dependence on VCP binding suggest that putative ubiquitination of WldS is unlikely to be the sole explanation for the ubiquitin speckles. Thus, we propose that polyubiquitinated proteins may accumulate in the WldS/VCP complex.

VCP Binding to N16 Is an Evolutionary “Recent” Development

An alignment of human Ube4b with known orthologues shows that an N-terminal extension containing the N16 sequence is present in mammals, birds, fish, and insects, but this entire region is absent in nematodes, slime molds, and yeasts (Figure 7). Nevertheless, the VCP ortholog Cdc48
does bind Ufd2 in Saccharomyces cerevisiae (Koegl et al., 1999; Richly et al., 2005) and Caenorhabditis elegans (C. Kähler and T. Hoppe, personal communication) so the interaction in these species must have a different molecular basis. The Cdc48 binding site in S. cerevisiae has been mapped (Richly et al., 2005) and mammals do have a sequence homologous to it. However, this sequence appears to be insufficient for VCP binding in human, because N-terminally truncated Ube4b did not bind VCP (Figure 3A). Mammalian Ube4b was previously reported to bind VCP (Meyer et al., 2000; Kaneko et al., 2003) but the interacting sequence was not fully mapped. Our data show that VCP binds to N16, and this appears to be a recent event in evolutionary terms with distantly related higher eukaryotes employing a different mode of interaction.

DISCUSSION

We report direct binding between VCP and the N-terminal 16 amino acids of WldS protein (N16) that drives partial redistribution of VCP in nuclei in vivo and in vitro when full-length WldS is present. N16 is necessary and sufficient for VCP binding and is necessary for relocation of VCP, but is not required to target WldS to discrete intranuclear foci. Thus, sequences outside N16 cause WldS to accumulate at these sites and VCP is redistributed through binding to N16. The N-terminal domain of WldS also influences the intranuclear distribution of the covalently attached Nmnat1 sequence and hence the distribution of nuclear NAD+ synthesis machinery.

A recent report indicates that Nmnat1, but not N70, is sufficient to confer a WldS-like phenotype on neurons in vitro (Araki et al., 2004). However, the relative strengths of the neuroprotective phenotypes induced by Nmnat1 and WldS were not tested and Nmnat1 was not shown to protect in vivo, where WldS robustly protects longer axons for far greater time periods (Mack et al., 2001; Adalbert et al., 2005). Our data show that N70 is not an inert addition to Nmnat1, but instead concentrates VCP, NAD+ synthesis activity and probably its associated ubiquitinated proteins into sub-
nuclear sites, functions that each have potential to influence the strength of the WldS phenotype. Thus, WldS joins a growing list of chimeric proteins whose biological activity amounts to more than the sum of their parts (Fujimoto et al., 1996; Campbell et al., 1997; Blume-Jensen and Hunter, 2001).

Further studies are needed to test whether VCP binding is required for the neuroprotective WldS phenotype, but this possibility was upheld in two important tests. First, WldS and VCP show colocalization in at least some DRG neurons known to express the neuroprotective phenotype, as well as in cerebellum where the phenotype in vivo remains to be tested. Second, colocalization was conserved across species in DRG of WldS rats (Adalbert et al., 2005). The more heterogeneous intranuclear distribution of WldS in motor neurons in vivo makes colocalization in this cell type more difficult to test (Mack et al., 2001; Samsam et al., 2003). Cultured motor neurons electroporated with WldS/EGFP show WldS puncta without obvious colocalization of VCP but this does not exclude a role for VCP in the neuroprotective phenotype in vivo (Supplementary Figure 5).

Colocalization studies cannot ultimately answer the question of whether VCP is required for the WldS phenotype. Unfortunately, VCP knockout mice are not available to test the hypothesis and a more complex strategy may be needed. Given the essential role of VCP in endoplasmic reticulum-associated protein degradation (ERAD) and several reports of damaging effects of VCP RNAi, mutation, and deletion in other species or cell lines, it is unlikely that such knockout mice would be viable (Hirabayashi et al., 2001; Kobayashi et al., 2002; Wojcik et al., 2004; Yamanaka et al., 2004). In contrast, WldS alters VCP in a way that leaves all cells and organisms healthy, with no overt harmful effect in the orig-

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**Figure 4.** Nmnat1 is not sufficient to target WldS protein to intranuclear foci. C-terminal EGFP fusion constructs transiently transfected into COS cells. (A) WldS/EGFP localizes primarily to discrete intranuclear foci (arrowhead), although some remains homogeneously distributed inside the nucleus (arrow). (B) Nmnat1/EGFP is homogeneously distributed, apart from exclusion from nucleoli. N70/EGFP (C) and EGFP (D) alone are homogeneously distributed throughout the cell but EGFP has a preference for the nucleus as previously described (Alonso et al., 2004). Neither is targeted to intranuclear foci. Scale bar, 10 μm. Figures are representative of three experiments.

**Figure 5.** Full-length WldS can also partially relocalize ubiquitin inside the nucleus. (A) PC12 cells transiently transfected with WldS/EGFP fusion construct show a punctate intranuclear distribution (arrowheads), whereas untransfected cells show no signal (arrow). (B) Ubiquitin immunofluorescence shows colocalizing punctate signal in transfected cells (arrowheads), together with a widespread nuclear and cytoplasmic signal. Untransfected cells show only the more homogeneous distribution (arrow). (C) DAPI. (D) Overlay. (E and F) Control experiment in which the first antibody against ubiquitin was omitted. Transfected cells (arrow) show no signal in the red channel. Panel order corresponds to A–D as indicated. Scale bar, 10 μm. (I–P) A repeat of the experiment in Figure 3 but immunostaining for ubiquitin instead of VCP. Ubiquitin shows the same dependence on N16 for colocalization with WldS/DsRed2 in intranuclear spots. (I–L) Transfection with full-length WldS/DsRed2 results in partial colocalization, which is retained when the confocal image stack is rotated (arrowheads in L). (M–P) Transfection with N-terminally truncated WldS/DsRed2 does not result in colocalization. The small region of overlapping signal (arrow in P) is not retained when the confocal image stack is rotated. (I and M) DAPI staining shows position of nuclei; (J and N) ubiquitin immunostain. (K and O) DsRed2 signal fused, respectively, to full-length WldS and WldS lacking N16. (L and P) Overlays.
VCP plays pivotal roles in ERAD, nuclear envelope reconstruction, cell cycle, postmitotic Golgi reassembly, and suppression of apoptosis (Kondo et al., 1997; Dai and Li, 2001; Hetzer et al., 2001; Rabinovich et al., 2002; Wang et al., 2004). Its nuclear functions are equally diverse. First, a nuclear transport role is suggested by its association with adapter proteins Ufd1 and Npl4, and by VCP-dependent transport of T-cell-specific adaptor protein into eukaryotic nuclei (Meyer et al., 2000; Martin and King, 2005). Second, VCP controls nucleolar retention of Werner syndrome helicase and its release after DNA damage (Indig et al., 2004). Interestingly, our data fit with the proposal of these authors that other nuclear binding partners regulate VCP distribution and control other pathways. Third, the nucleus has a quality control pathway (Gardner et al., 2005) likely to involve VCP. Interaction and colocalization with expanded polyglutamine and other intranuclear inclusions suggests VCP may be trying to clear misfolded nuclear proteins (Hirabayashi et al., 2001; Doss-Pepe et al., 2003; Mizuno et al., 2005). Finally, VCP regulates the stability of the transcription factor SPT23 (Richly et al., 2005), consistent with the tight regulation of transcription by the UPS (Muratani and Tansey, 2003).

VCP is altered in several neurodegenerative disorders so its interaction with the neuroprotective WldS protein is particularly interesting. VCP missense mutations cause inclusion body myopathy with Paget disease of bone and frontotemporal dementia, a disease characterized by ubiquitin-containing nuclear inclusions and white matter pathology (Watts et al., 2004; Schroder et al., 2005). VCP is present in Lewy-like inclusions in amyotrophic lateral sclerosis, in nigral Lewy neurites in Parkinson’s disease (Ishigaki et al., 2004), and in ubiquitin-positive intraneuronal inclusions in motor neuron disease with dementia, ballooned neurons in Creutzfeldt-Jakob disease, and dystrophic neurites of senile plaque in Alzheimer’s disease (Mizuno et al., 2003). In polyglutamine disorders such as Huntington’s disease and Machado-Joseph disease, specific binding to expanded polyglutamine targets VCP to intranuclear inclusions (Hiraba-

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**Figure 6.** Ubiquitinated WldS protein cannot be detected. (A) Western blotting with antibody to WldS protein did not detect any ubiquitinated WldS/EGFP fusion protein or ubiquitinated WldS in transfected PC12 cells (left) or in WldS mouse cerebellum (right), respectively. The 70- and 43-kDa bands correspond to the predicted molecular weights of the unmodified proteins. (B) The proteasome inhibitor MG-132 (10 μM for times shown) caused ubiquitination products to build up in PC12 cells (left), but ubiquitinated WldS/EGFP (>70 kDa) still could not be detected even on an overexposed ECL (right). Figures are representative two experiments.

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**Figure 7.** VCP binding to N16 is an evolutionary “recent” event. (A) Full-length alignment of Ube4b and Ufd2 sequences. (B) Amino acid alignment of N16-like or other N-terminal sequences in the same proteins. The database accession numbers are also shown. Mammals, birds, fish, and insects have sequence showing significant homology to the VCP binding site in N16 at positions denoted by the asterisk (*). Nematodes, slime molds, and yeasts lack the entire N-terminal extension containing this sequence. The Cdc48 binding site of S. cerevisiae Ufd2p (Richly et al., 2005), the U box (Hatakeyama et al., 2001), and the sequence essential for in vitro ubiquitination activity (Mahoney et al., 2002) are also marked. Black boxes, regions of significant homology to human Ube4b; open boxes, regions not homologous to human Ube4b; bold, amino acids identical to human sequence; underlined, conservative substitutions.
yashi et al., 2001). This interaction appears to influence disease severity, as loss-of-function mutations in the Drosophila VCP ortholog, ter4, dominantly suppress neurodegeneration caused by expanded polyglutamine (Higashiyama et al., 2002).

It is now important to determine whether Wld\textsuperscript{S} protein, or N70 can interfere with the effect of VCP in any of these neurodegenerative disorders. Ectopic expression in Drosophila of mammalian Ube4b suppresses polyglutamine disease, and loss of function mutations in VCP have a similar effect (Higashiyama et al., 2002; Matsumoto et al., 2004). It is also now clear that Wld\textsuperscript{S} and expanded polyglutamine both bind VCP directly and partially relocalize it to intranuclear foci (Hirabayashi et al., 2001). Taken together, these observations raise the intriguing possibility that N70 and VCP antagonize each other in some circumstances as a result of the binding interaction we describe.

In summary, we report direct interaction between VCP and the N-terminal 16 amino acids of both Wld\textsuperscript{S} and Ube4b proteins. This interaction drives focal intranuclear clustering of VCP in Wld\textsuperscript{S} neurons, probably together with associated multiubiquitinated proteins, and helps understand the function of an important, evolutionarily recent regulatory sequence in the Ube4b protein. It is now important to determine whether the redistribution of covalently attached Nmnat1 or of bound VCP influences the strength of the Wld\textsuperscript{S} phenotype in vivo and to understand how the N-terminal binding of VCP to Ube4b influences this ubiquitin ligase.

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