MOLECULAR AND CELLULAR BIOLOGY, Feb. 2011, p. 897–909 Vol. 31, No. 4
0270-7306/11/$12.00 doi:10.1128/MCB.00799-10

Basic Leucine Zipper Protein Cnc-C Is a Substrate and Transcriptional Regulator of the *Drosophila* 26S Proteasome▽†

Kristian Björk Grimberg,‡ Anne Beskow,‡ Daniel Lundin, Monica M. Davis, and Patrick Young*

Department of Molecular Biology and Functional Genomics, Stockholm University, Stockholm S-10691, Sweden

Received 11 July 2010/Returned for modification 31 July 2010/Accepted 1 December 2010

While the 26S proteasome is a key proteolytic complex, little is known about how proteasome levels are maintained in higher eukaryotic cells. Here we describe an RNA interference (RNAi) screen of *Drosophila melanogaster* that was used to identify transcription factors that may play a role in maintaining levels of the 26S proteasome. We used an RNAi library against 993 *Drosophila* transcription factor genes to identify genes whose suppression in Schneider 2 cells stabilized a ubiquitin-green fluorescent protein reporter protein. This screen identified Cnc (cap 'n' collar [CNC]; basic region leucine zipper) as a candidate transcriptional regulator of proteasome component expression. In fact, 20S proteasome activity was reduced in cells depleted of *cnc*. Immunoblot assays against proteasome components revealed a general decline in both 19S regulatory complex and 20S proteasome subunits after RNAi depletion of this transcription factor. Transcript-specific silencing revealed that the longest of the seven transcripts for the *cnc* gene, *cnc-C*, was needed for proteasome and p97 ATPase production. Quantitative reverse transcription-PCR confirmed the role of Cnc-C in activation of transcription of genes encoding proteasome components. Expression of a V5-His-tagged form of Cnc-C revealed that the transcription factor is itself a proteasome substrate that is stabilized when the proteasome is inhibited. We propose that this single *cnc* gene in *Drosophila* resembles the ancestral gene family of mammalian nuclear factor erythroid-derived 2-related transcription factors, which are essential in regulating oxidative stress and proteolysis.

The ubiquitin/proteasome system (UPS) degrades intracellular proteins and is essential for regulating a wide range of cellular pathways. The UPS plays a critical role in the regulated degradation of proteins involved in tumor development and cell cycle control. Proteins destined for proteasomal degradation are modified by conjugation of ubiquitin moieties through the concerted action of E1, E2, and E3 enzymes. Repeated rounds of conjugation lead to the formation of a polyubiquitin chain attached to the target protein, making it a preferred substrate for the 26S proteasome. The 26S proteasome, which hydrolyzes the targeted proteins, is composed of a 20S proteolytic core flanked by one or two 19S regulatory particles (17). The 19S regulatory particle functions to acquire ubiquitylated substrates and direct them into the proteolytic chamber (18, 40).

Proteasome inhibitors have been shown to possess strong antitumor activity and are used in the treatment of multiple myeloma. One such inhibitor, bortezomib (Velcade), was the first approved compound in this new category of cancer treatments (43). Proteasome inhibitor treatment can result in increased proteasome levels. Recently, an adaptive feedback mechanism was identified where long-term treatment of human lymphoma cells with bortezomib induced increased *de novo* biogenesis of proteasomes (14). This allowed the cells to survive proteasome inhibition and to become hyperproliferative and apotosis resistant. A number of cancer cell types have been shown to have abnormally high proteasome levels, including certain human hematopoietic tumor cells (26). Identifying the factors that participate in transcription of proteasome subunit mRNAs would be valuable in understanding the regulation of ubiquitin proteasome activity and help explain the antitumor activity of proteasome inhibitors.

In *Saccharomyces cerevisiae*, a well-defined transcription negative feedback loop controls proteasome levels (11, 31, 53). The transcription factor RPN4 binds to promoter PACE elements and promotes transcription of the proteasome and related genes. RPN4 is a rapidly degraded proteasome substrate and is present at low levels when proteasome activity is sufficient for degradation of UPS substrates. Surprisingly, the RPN4 negative feedback network has only been identified in *Saccharomyces cerevisiae* and the closely related yeast *Hemiascomycetes* sp. (30).

Treatment of mammalian or *Drosophila melanogaster* cells with proteasome inhibitors results in the upregulation of proteasome subunits (29, 34). In both *Drosophila* adults and cell cultures, the depletion of one of the proteasome ubiquitin receptors, S5a/Pros54 (PSMD4), strongly increases the specific transcription and overproduction of proteasome subunits (29, 48). Importantly, *Drosophila* cells depleted of S5a do not upregulate stress or heat shock genes, suggesting that a proteasomal gene-specific transcriptional regulatory pathway exists in *Drosophila*.

Genome-wide RNA interference (RNAi) libraries have proven to be a powerful tool to identify new essential genes in many pathways (9). We have used one such genome-wide RNAi library of transcription-related genes to identify the

* Corresponding author. Mailing address: Department of Molecular Biology and Functional Genomics, Stockholm University, Stockholm S-10691, Sweden. Phone: 46-(0)8-52487387. Fax: 46-(0)8-6420460. E-mail: gly@me.com.

† Supplemental material for this article may be found at http://mcb.asm.org/.

‡ K. B. Grimberg and A. Beskow are co-first authors.

▽ Published ahead of print on 13 December 2010.

transcription factors that may control metazoan proteasome levels. Initial validations of our screen identified a transcription factor, Cnc-C, that contributes to the expression of proteasomal components in *Drosophila*. It has been shown that *Drosophila* Cnc-C plays a role in oxidative stress tolerance, similar to the mammalian Nrf2 transcription factor (nuclear factor erythroid 2 p45 subunit-related factor 2), and it has been proposed to be a direct homolog of mammalian Nrf2 (46). In this work we show by phylogenetic analysis that duplication of the Nrf genes occurred during vertebrate evolution, and we instead propose that Cnc-C resembles the ancestral complex common to the mammalian Nrf transcription factor gene family.

Heat stress results in strong upregulation of proteasomal activity in human fibroblasts (2). However, it has been shown that proteasome genes are not coregulated by the same transcription factor, Hsf1, as heat shock proteins in response to cellular stress in mammalian cells (49). Recently, the Nrf1 transcription factor (TCF11) was shown to be important for induction of proteasome gene transcription in mammalian cells (41, 45). Our current results demonstrate a functional similarity between the mammalian Nrf1 transcription factor and *Drosophila* Cnc-C, suggesting that there is an evolutionarily conserved function in proteasomal gene transcription for this specific transcription factor. By using a V5 antibody-His-tagged Cnc-C construct, we show that the Cnc-C protein is degraded by the proteasome and is also stabilized by depletion of the proteasome ubiquitin receptor S5a. We present a model where both oxidative stress tolerance and proteasome induction are controlled by a single transcription factor in *Drosophila*, Cnc-C.

MATERIALS AND METHODS

RNAi interference library screen: screening of 384-well plates. For screening, 384-well plates with prealiquoted double-stranded RNA (dsRNA) were thawed for a few minutes and then spun at 200 × *g* for 1 min. Ub^{G76V}-GFP is a widely used proteasomal reporter in which the single G76V mutation inhibits cleavage of the ubiquitin from green fluorescent protein (GFP) and converts the GFP into a ubiquitin fusion domain (UFD) proteasome substrate. Ub^{G76V}-GFP stable cells were counted, spun at 100 × *g* for 5 min, and washed twice, after which they were resuspended in serum-free medium at a concentration of 2.5×10^6 cell/ml. The adhesive seal on the plates was removed, and 10 μl of cells was plated in each well. The plates were incubated for 2 h, after which 30 μl complete medium was added to each well. The plates were sealed and incubated for 4 days, with screening on days 2, 3, and 4 for each plate. Each plate was screened each day by two persons, and each well was given a score of 0 to 4, with 0 being no stabilization of Ub^{G76V}-GFP and 4 being high stabilization of Ub^{G76V}-GFP.

Follow-up screen. Using the MEGAscript kit from Ambion, dsRNA for positive knockdowns from the first and second screenings was synthesized. Oligonucleotides with T7 RNA polymerase promoter regions were constructed from gene sequences found in FlyBase. Fragments of approximately 500 to 700 bp were amplified using *Drosophila* genomic DNA extracted from S2 cells, after which dsRNA was synthesized from fragments. All dsRNA was examined by agarose gel electrophoresis after annealing. Oligonucleotide sequences used to generate fragments were as follows: T7 5′ cncC#1, 5′-GAATTAATACGACTCACTATAGGGAGAGGCTGCAAGCTTCCGCCAAGATTCAACG; T7 3′ cncC#1, 5′-GAATTAATACGACTCACTATAGGGAGAGTGCTGAGGGGTGCTCCACTGCCGC; T7 5′ cncC#2, 5′-GAATTAATACGACTCACTATAGGGAGAATTGGAAAATCGGGGAGATAGGCCGTGG; T7 3′ cncC#2, 5′-GAATTAATACGACTCACTATAGGGAGAACAAAAGTGCTGCCGTTGAATCTTGGCG; T7 5′ cnc-All, 5′-GAATTAATACGACTCACTATAGGGAGAGGCTACAGCTGCCTCCAATGCTGTTTCG; T7 3′ cnc-All, 5′-GAATTAATACGACTCACTATAGGGAGATCCAGAAGGGACAACACCATGCAAGACG; T7 5′ ssrp, 5′-GAATTAATACGACTCACTATAGGGAGAGCCCGCAGCGGCGGATCC-3′; T7 3′ ssrp, 5′-GAATTAATACGACTCACTATAGGGAGAGCTCCTTCCACATCTCGCCGCCC-3′; T7 5′ spen, 5′-GAATTAATACGACTCACTATAGGGAGAGGGCTTTGGAAAATCCATGCCCACC-3′; T7 3′ spen, 5′-GAATTAATACGACTCACTATAGGGAGAGGGCATGGATGTGCTCGCTGGTACC-3′; T7 5′ cenp-c, 5′-GAATTAATACGACTCACTATAGGGAGAGCCGCCACCGAGAAAGTCAATGAGC-3′; T7 3′ cenp-c, 5′-GAATTAATACGACTCACTATAGGGAGAGCCAGGCTGGGTCGTGACTTTGTCG-3′; T7 5′ kr-h1, 5′-GAATTAATACGACTCACTATAGGGAGAGGTGTCGCAGGTGCGCAAGCCC-3′; T7 3′ kr-h1, 5′-GAATTAATACGACTCACTATAGGGAGACGGGCACTTGTACGGCTTCTCGCC-3′.

For knockdowns, *Drosophila* S2 cells were diluted to a concentration of 1×10^6 cells/ml in Express Five serum-free medium (Invitrogen). After cell attachment, medium was replaced twice with 1 ml serum-free medium, and the cells were grown in the serum-free medium for 1 to 2 h. dsRNA was introduced into cells by adding 20 to 60 μg of dsRNA directly to the medium under constant agitation. After 12 h, 2 ml complete S2 medium (Invitrogen) was added, and the cells were cultured for 3 to 4 days prior to isolation.

Synthesis of dsRNA. A second round of double-stranded RNAs (approximately 600 bp in length) was generated for candidate transcription factors and proteasome genes. For each treatment, a total of 5 μg of dsRNA was added as a supplement to the medium of a stable *Drosophila* cell line expressing the UFD reporter Ub^{G76V}-GFP by using a 96-well format. Triplicates were carried out for controls and all RNAi treatments. After 3 days of growth the cells were measured by fluorescence-activated cell sorting.

Fluorometric assays of proteasome activities. Spectrofluorometric assays were performed in the presence of Suc-LLVY-AMC, succinyl-Leu-Leu-Val-Tyr-AMC succinyl-Leu-Leu-Val-Tyr-7-amino-4-methyl-coumarin, and 10 μg of each crude extract from RNAi-treated *Drosophila* S2 extracts in 100 μl TS buffer (10 mM Tris-HCl [pH 8.5], 25 mM KCl, 10 mM NaCl, 1.1 mM $MgCl_2$, and 0.1 mM EDTA). Before initiating the proteasome assays, equivalent amounts of extracts were diluted into TS buffer with 0.03% SDS and preincubated for 15 min at room temperature in the absence of ATP to allow activation of the 20S proteasomes. The concentration of fluorescent peptide was 200 μM. Assay volumes were 100 μl, and mixtures were incubated at 37°C for 1 h. Assays were stopped by the addition of 1 ml ice-cold water. Fluorescence was measured using an excitation wavelength of 380 nm and an emission wavelength of 440 nm. Suc-LLVY-AMC was purchased from AFFINITI Research Products.

RNAi knockdowns and RNA purification. On day 1, Schneider's *Drosophila* medium-cultured S2 cells were plated on Corning Costar six-well plates (2.25×10^6 cells in each well). The cells were washed four times with Express Five serum-free medium (Invitrogen) with a 1-hour incubation in between washes. A total of 20 μg dsRNA was applied to the cells in 1 ml serum-free medium. When two different dsRNAs were added, a 20-μg aliquot of each of the dsRNAs was used. On day 2, 2 ml Schneider's medium was added to each well. On day 3, total RNA was isolated with the TRIzol Plus RNA purification kit (Invitrogen).

QPCR and RT-PCR. For quantitative PCR (QPCR) and reverse transcription-PCR (RT-PCR), first the isolated RNA was treated with DNase (Turbo DNA-free kit; Ambion). To make cDNA, the high-capacity cDNA reverse transcription kit was used (Applied Biosystems), and 490 ng RNA was used in a 20-μl reaction mixture. The resulting cDNA was then used in QPCRs, which were performed with the TaqMan gene expression assay system. The following TaqMan-predesigned probes were used: Drosophila prosalpha7 (Dm01812483_g1), Rpt3 (Dm01837846_g1), cnc (Dm02150448_g1), p97 (TER94) (Dm01812530_g1), and Rpn11 (Dm01804595_g1). Ribosomal protein L32 (Dm01812483_g1) was used as an internal control for all measurements. Each of the three biological replicate experiments was examined in triplicate. Optimized amounts of cDNA were originally tested for the samples to measure the different gene levels. One individual sample of the triplicate for maf-s and cnc contained 85 ng cDNA, for prosalpha7, Rpt3, p97, and Rpn11 27 ng was used, and for the ribosomal protein 54 ng was used. The QPCRs were performed on the ABI Prism 7000 apparatus (Applied Biosystems), and the data were analyzed according to the ΔC_T method.

Phylogenic tree of cap 'n' collar proteins. Sequences were collected from GenBank by using PSI-BLAST with the *Drosophila melanogaster* Cnc isoform A protein sequence (NCBI accession number NP_732835) as seed and an E-value cutoff of 1e−40. The data set was reduced by deletion of sequences from closely related species and isoforms that were very similar to other sequences in the data set. Subsequently, the data set was complemented with sequences from *Caenorhabditis* spp., *Nematostella cectensis*, and *Trichoplax adhaerens* that did not pass the E-value cutoff of the initial run. A multiple alignment was constructed using Probcons 1.10 (10), and phylogenetic trees were estimated using PhyML 3.0 (19, 20).

Expression of a V5-His-tagged form of the unique Cnc-C region. The cDNA clone IP15234 was obtained from the *Drosophila* Genomics Resource Center and fully sequenced to confirm the open reading frame. The partial cDNA contained the complete region for the Cnc-C-specific region of Cnc but lacked the bZIP

DNA binding domain. Through PCR an open reading frame sequence was obtained with flanking EcoRI and NotI restriction sites and cloned into the EcoRI/NotI sites of the pAcV5His-B plasmid (Invitrogen), which translates into a C-terminally V5-tagged protein. The oligonucleotides used for the PCR were the following: 5′-ATTAAGAATTCGACTACAAGAGTCACCCACGCACCC ATTCGC-3′ and 5′-TAATTAGCGGCCGCCCTCGTCTAGTTCCTTCTTGG CGTCCTCATCTAAG-3′. The resulting clone was sequenced, and site-directed mutagenesis was carried out to fix two unwanted mutations present in the PCR clone.

Oligonucleotides used for mutagensis were V309I, 5′-GATTACGAGGGCG AGCTGATCGGTGGAGTGGCCAACG-3′, and K517E, 5′-CGGATTGTTTC GGAAACCGGCGAGGATTTACTCAGTGGC-3′. The resulting clone expressed a 611-amino-acid residue C-terminal V5-His tagged C region. The Cnc-C region itself was 576 amino acid residues in length and only contained the Cnc-C specific sequence and not the bZIP binding domain found in all Cnc transcripts.

RESULTS

Inhibition of Ub^{G76V}-GFP proteasomal degradation with screening of a genome-wide RNAi library of transcription factors. To screen for factors that are required for the specific transcription of proteasome genes, a *Drosophila* S2 stable cell line (29) expressing the ubiquitin fusion protein Ub^{G76V}-GFP (8) was used in an RNAi screen (Fig. 1A). Ub^{G76V}-GFP is constitutively expressed at high levels via an actin promoter in these cells and results in low fluorescence levels when UPS activity is at wild-type levels. The Ub^{G76V}-GFP reporter requires both the 26S proteasome and preprocessing by the p97 ATPase for degradation (3). We predicted that RNAi depletion of a factor that is specifically responsible for transcription of proteasome pathway genes, rather than a part of the general transcription machinery, would deplete the cell of proteasomes but would not interfere with the ability to express the Ub^{G76V}-GFP reporter.

The dsRNAs used in this screen were from a *Drosophila* transcription factor RNAi sublibrary (DRSC TRXN) (42). This sublibrary targets *Drosophila* genes that have either proven or predicted roles as transcription factors. The library contains on average 2 amplicons per gene and a total of 993 targeted genes. After 2 days of RNAi treatment the library was screened using inverted fluorescence microscopy and scored by two independent screeners for three consecutive days. Variable autofluorescence from the well surfaces compromised the results obtained by automatic fluorescent plate reading, and therefore manual screening was used. Knockdown of the p97 ATPase or proteasome subunits was used to set a maximum positive score value. The assay used a blind screening method, in which the identities of the genes targeted by the dsRNA in the wells were unknown. Each day two screeners scored a 384-well plate. On the following day the screeners were switched, so that all examiners scored every RNAi well in the RNAi library at least once. The scores were averaged and compared in a heat map format (Fig. 1B; see also Fig. S1 in the supplemental material). For the heat map, only scores that were marked positive at some level by both independent screeners are shown. Initially, a total of 52 RNAi treatments resulted in increased levels of Ub^{G76V}-GFP. The majority of the 52 targeted genes showed only minor stabilization of the UPS reporter, while a small number showed greater increases. The identified genes were grouped into three classes: in group I, stabilization was observed consistently over the 3-day screening period; in group II, stabilization of the Ub^{G76V}-GFP reporter occurred on 2 of the 3 days; in group III, stabilization occurred only on a single day. The class I group of RNAi-targeted transcription factors had the highest levels of Ub^{G76V}-GFP reporter stabilization.

For the majority of transcription factors screened, two distinct dsRNAs were present in the library. However, for the majority of candidates found to be positive, only a single dsRNA led to visual stabilization of Ub^{G76V}-GFP. The exceptions that showed multiple positive hits in the library screen were the *Drosophila* genes *cnc*, *spen*, *cenp-C*, *foxo* and the little-studied genes CG34406, similar to the human zinc finger protein 84 (ZNF84), and CG5366, which has high similarity to the cullin-associated NEDD8-dissociated protein 1 TIP120/CAND. The proposed small cosubunit to Cnc, the Maf protein Maf-S, was also initially observed to stabilize Ub^{G76V}-GFP. The small size of the *maf-S* gene meant that the RNAi library only contained a single amplicon for *maf-S*. The fact that the other candidates did not show consistent stabilization in different amplicon targets suggests that the majority of observed changes were likely the result of indirect effects, such as inhibition of general cellular homeostasis and blockage of turnover of the highly expressed reporter gene. A second possibility is that the difference seen between the amplicon pair for a single gene represents a real variation in the role of the alternative transcripts of that gene. Further characterization of the remaining candidate transcription factors is required to address false positives versus the real contributors to maintenance of proteasome levels.

To confirm that the candidate genes were required for the expression of genes of the ubiquitin proteasome system, larger-scale RNAi targeting was carried out on the top positive candidate genes by using dsRNA sequences that did not overlap the target sites from the DRSC TRXN sublibrary. Depletion of *cnc*, *spen*, and *ssrp* was confirmed to stabilize the Ub^{G76V}-GFP reporter. RNAi depletion of the Cnc transcription factor resulted in significant stabilization of the Ub^{G76V}-GFP reporter (Fig. 1B; see also Fig. S2 in the supplemental material). Comparison of the specific regions targeted for the *cnc* gene in the original library screen indicated that only dsRNA directed to the *cnc-C* transcript was sufficient to inhibit proteasome degradation of Ub^{G76V}-GFP (top hit in Fig. 1B; see also Fig. S2). Somewhat surprisingly, a second round of treatment with specific dsRNAs directed to *maf-S* or *kr-h1* was unable to show significant stabilization of the proteasome reporter. Previous studies suggested that Maf-S should be essential for Cnc-C to function as an obligate heterodimer. Additional studies are required to address the irreproducibility of the original hit for *maf-S* to understand if this was due to insufficient RNAi depletion or was biological in nature.

The *Drosophila cnc* gene resembles the ancestral gene for the mammalian Nrf transcription factor with diversified functions in a single locus. The *Drosophila cnc* gene encodes seven different mRNAs (Fig. 2A). The majority of these transcriptional forms are due to different transcriptional start sites and not alternative splicing. The related mammalian homologs of *Drosophila cnc* include the p45 NFE2 (nuclear factor erythroid-derived 2) and the Nrf family of bZIP genes (NFE2-related factors Nrf1, Nrf2, and Nrf3) (Fig. 2B) (47). These four related transcription factors typically function as transcriptional activators. Two additional related vertebrate bZIP fac-

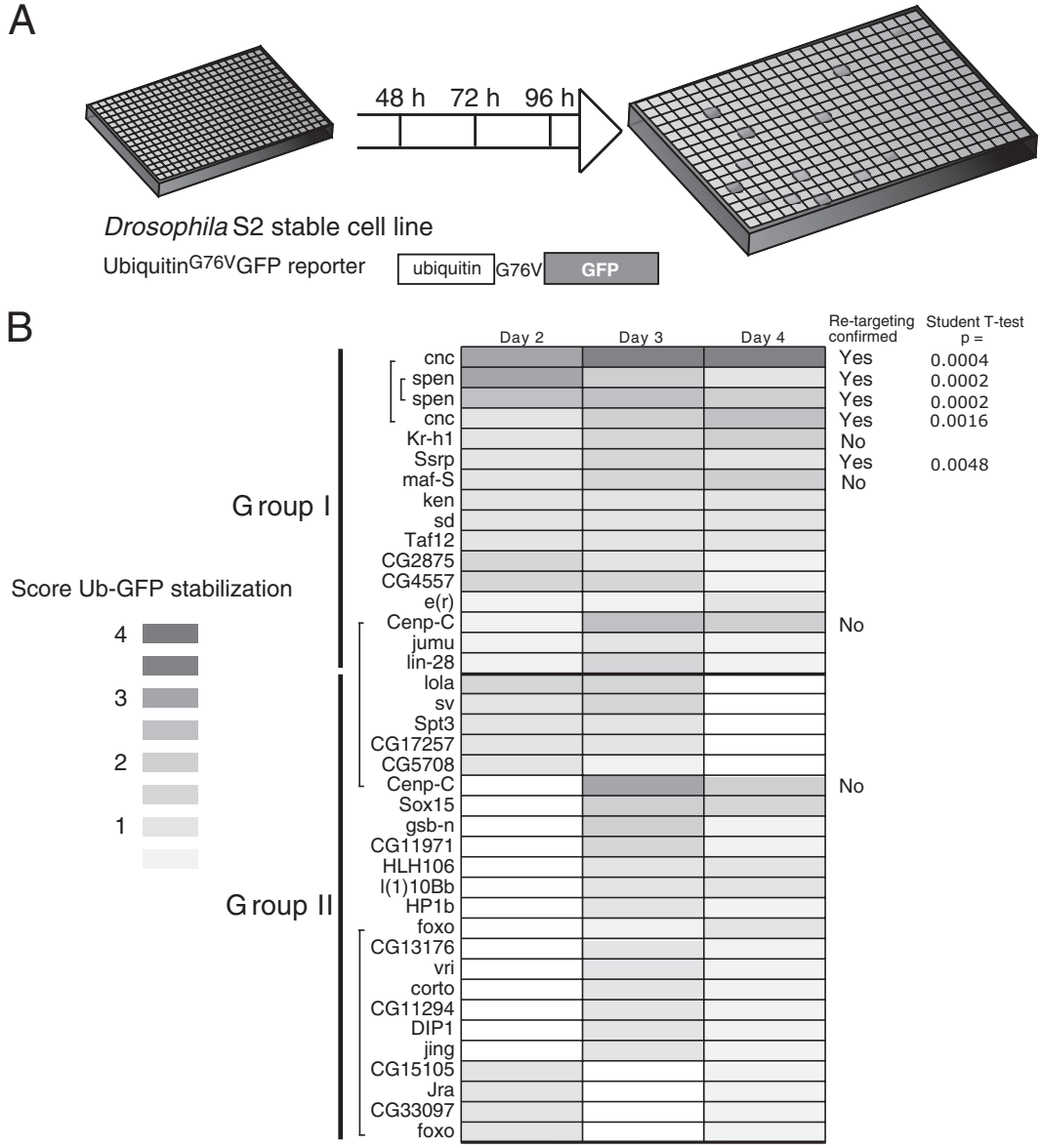

FIG. 1. (A) Overview of the genome-wide RNAi screen to identify transcription factors involved in regulating the ubiquitin proteasome system. The *Drosophila* Transcription Factor RNAi Library (DRSC TRXN) was manually screened using a stable S2 cell line. A total of 993 gene target wells were examined for the library screen. A *Drosophila* S2 stable cell line that constitutively expressed the Ub^{G76V}-GFP reporter was used in the library screening; it is not detectable in *Drosophila* cells that have adequate proteasome levels but is observed in cells with depleted proteasomes levels. (B) Rankings for stabilization of the proteasome reporter Ub^{G76V}-GFP from an RNAi genome-wide screening of *Drosophila* transcription factors. The positive hits were ranked on a heat map and grouped into categories based on the number of days that Ub^{G76V}-GFP was observed to be stabilized. Group I represents targets that showed consistent stabilization for all three screening periods. Group II genes stabilized Ub^{G76V}-GFP for two consecutive days. Brackets show target genes that were found twice in the library screen with different target dsRNAs. The strongest stabilization of Ub^{G76V}-GFP was found with a *cnc* dsRNA target that mapped to a specific transcript of the gene, *cnc-C*, shown as the top row. The top candidate genes were retargeted with new dsRNA treatments to new exonic regions. Triplicate treatments were analyzed by flow cytometry and measured for Ub^{G76V}-GFP stabilization relative to controls. For the *cnc* gene, retargeting specifically to unique sequences specific for the *cnc-C* transcript was functional in stabilizing Ub^{G76V}-GFP. *P* values (based on Student's *t* test) are shown.

tors, Bach1 and Bach2, function as transcriptional repressors (39). Similar to its mammalian homologs, the proteins encoded by the alternative transcripts of *cnc* have been proposed to serve various functions in *Drosophila*, with Cnc-A functioning as a putative repressor similar to the Bach factors, Cnc-B functioning as a developmental transcriptional regulator similar to p45 NFE2, and Cnc-C acting as a response mediator similar to the Nrfs (47).

Our RNAi screen implicated the protein encoded by the longest transcript of *cnc*, *cnc-C*, in *Drosophila* proteasome regulation. RNAi depletion using dsRNA targeted specifically to *cnc-C* was equivalent or better at inhibiting proteasome deg-

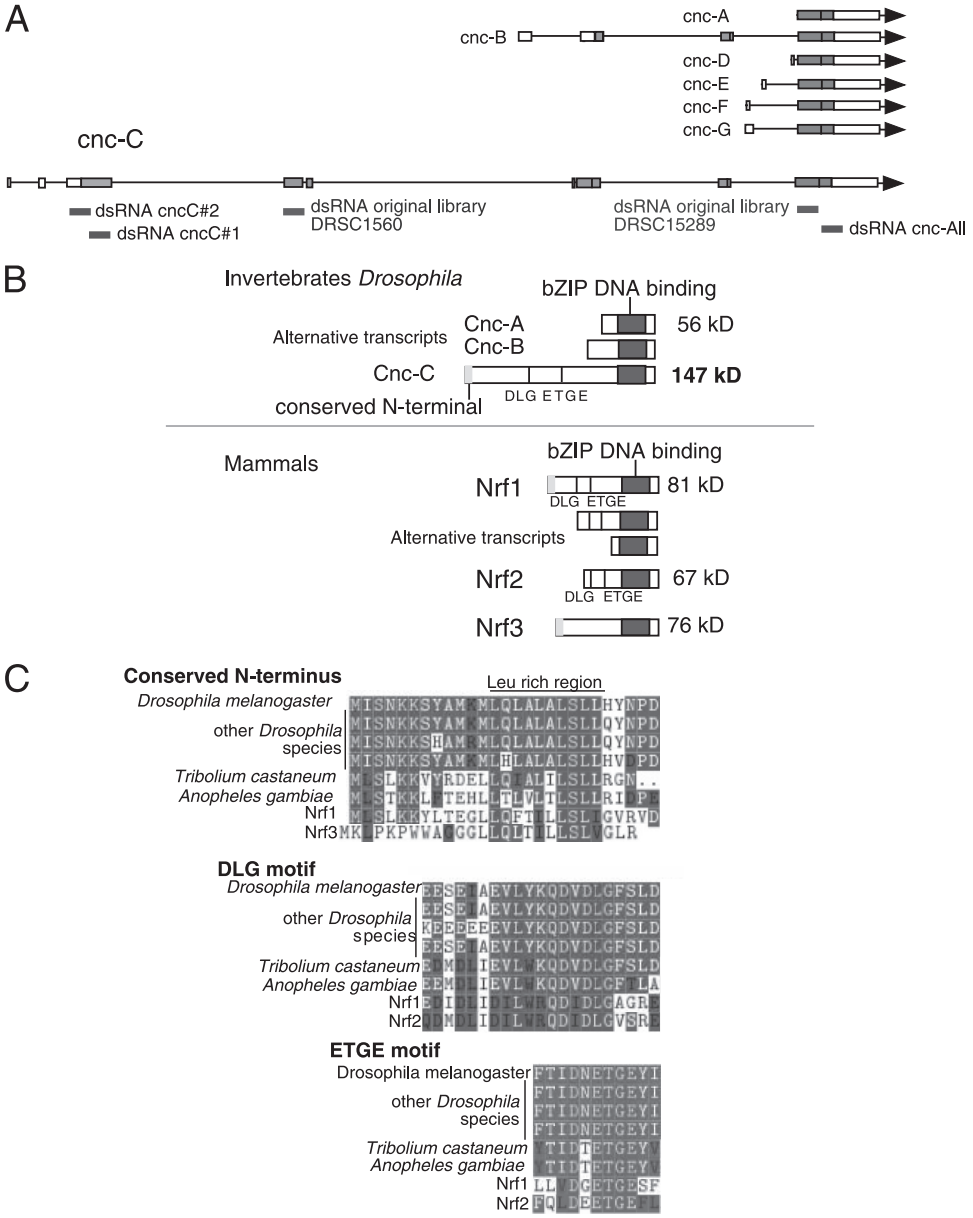

FIG. 2. Comparison of the *cnc* family of transcription factors in mammals versus *Drosophila*. (A) Schematic diagram of alternative splice forms of the basic leucine zipper gene *cnc*. Translated regions are shown as gray boxes, while untranslated regions are shown as white filled boxes. The positions of the dsRNA sequences from the initial library and confirmative dsRNAs are shown as short lines. Double-stranded RNA was designed to target all alternative splice forms, Cnc-All, or was specific for the Cnc-C transcripts. (B) At the protein level, the ETGE domain is part of a conserved region that binds to Keap1 homologs and prevents import to the nucleus for Nrf2 in mammalian cells and is also proposed to function in binding *Drosophila* Cnc-C during downregulation of antioxidant gene transcription. The mammalian system contains three distinct genes, Nrf1, Nrf2, and Nrf3. (C) The N termini of Nrf1 and Nrf3 function as signal peptide sequences (38, 57) and anchor the proteins to membranes. The *Drosophila* Cnc-C N terminus shows similarities to both mammalian Nrf1 and Nrf2 critical domains. The *Drosophila* Cnc-C and other insect Cnc sequences show similarities to the mammalian Nrf1 N-terminal region, which has been shown to target Nrf1 to the ER and cell membranes but is absent in Nrf2 transcription factors. The *Drosophila* Cnc-C also contains two positionally conserved Keap1 binding domains that target mammalian Nrf2 to the 26S proteasome.

radation than dsRNA constructs that targeted all *cnc* transcripts. The *cnc-C* transcript contains a long unique 5′ sequence that is absent in the other transcripts (Fig. 2A). Translation from this mRNA results in a Cnc-C protein containing 1,383 amino acid residues, of which the N-terminal 578 amino acids are unique among the Cnc isoforms. The *Drosophila* Cnc-C protein has been proposed to have Nrf2-like functions and act as the central mediator of the antioxidant response system (46). This classification is based on the ability of Cnc-C to promote transcription of antioxidant genes and the observation that Cnc-C can also be regulated by a homologous cytoplasmic binding repressor, Keap1, as found with the vertebrate Nrf2 transcription factor. The vertebrate Nrf1 transcription factor also has Keap1 binding domains; however,

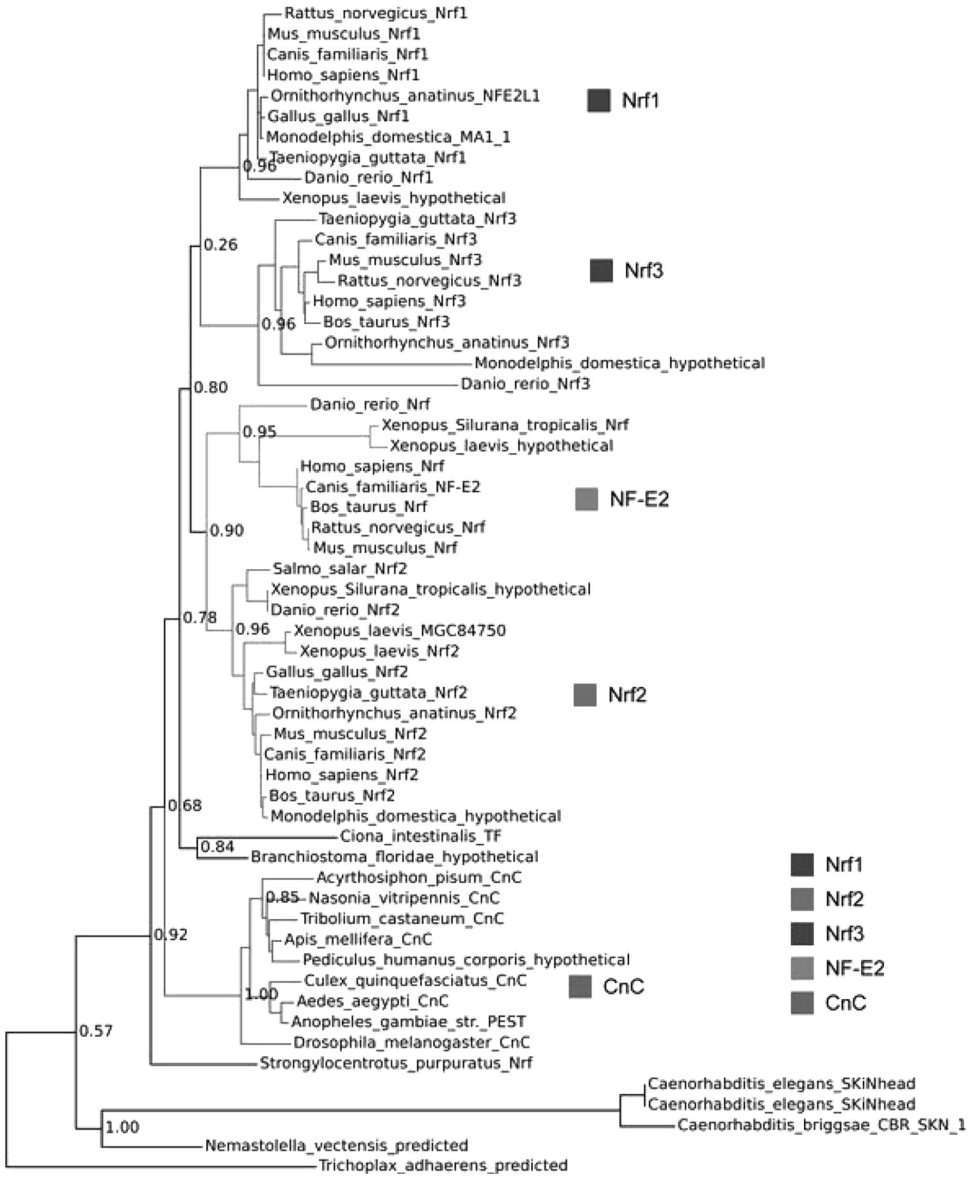

FIG. 3. Phylogeny of Cnc and Nrf proteins from metazoa rooted with the most basal lineage included, *Trichoplax adhaerens*. The tree was estimated using PhyML (19, 20) and the LG substitution model (28), with four-category gamma correction of different mutation rates along sequences. Support values shown are the SH-like values described in reference 1. Except for the grouping of *Caendorhabditis* spp. with *Menatostella vectensis* (starlet sea anemone, a nonbilaterian), all metazoan groups show up in the expected places of the tree. In particular, the two species most closely related to vertebrates, *Ciona intestinalis* (vase tunicate, a urocordate) and *Branchiostoma floridae* (lancelet, a cephalocordate) form a sister group with vertebrates. The latter contains four paralogs, while all other species contain just a single gene from the group. It can then be concluded that the ancestral gene containing the conserved domain has been duplicated more than once in the vertebrate lineage, and all four paralogs in vertebrates are equally closely related to, for example, insect Cnc genes. The position of the *Caenorhabditis* spp. sequences could be an artifact caused by sequence divergence (13). Furthemore, SKN sequences for *Caenorhabditis* contain a conserved domain that is shorter than all other sequences in the tree.

membrane anchoring of Nrf1, rather than direct binding of Keap1, is likely to regulate Nrf1's ability to activate transcription.

In support of the idea that *Drosophila* Cnc has multiple transcriptional activities, comparison of conserved functional domains showed that Cnc-C contains both Nrf1 and Nrf2 domains (Fig. 2C). The Cnc-C protein shares sequence similarities with the Nrf1 N terminus and also the Keap1 binding domains present in both Nrf2 and Nrf1. The function of Keap1 binding domains present in Nrf1 is currently unknown. It has been shown that the Nrf1 N terminus is an important functional regulatory element that anchors the transcription factor to the membrane (57). The above analysis supports the idea that the various functions for the Nrf family were present before multiple duplications in modern vertebrates.

While Cnc-C clearly has a role in the antioxidant response (44), phylogenetic analysis did not support Cnc-C as a specific invertebrate homolog of the mammalian Nrf2 (Fig. 3). A com-

prehensive phylogenetic analysis with metazoan cap 'n' collar bZIP sequences from a wide range of metazoan species supported an evolutionary model in which the Nrf2 gene arose after the split between vertebrates and invertebrates. This was also supported by a previous study (25). Interestingly, the phylogenetic analysis also suggested that the duplication of bZIP proteins appears to have occurred in *Chordata* after the divergence of the vertebrates from the primitive chordates, such as the tunicates *Ciona intestinalis*, an urocordate, and the lancelet *Branchiostoma floridae*, a cephalocordate.

RNAi depletion of *cnc-C* mRNA decreases levels of the 26S proteasome. The stabilization of Ub^{G76V}-GFP following gene-specific dsRNA knockdown can be caused by a general decline in the expression of either the whole 26S proteasome, a proteasome subcomplex, or a single critical component. We attempted to identify the type of defects in the *Drosophila* UPS that resulted from the RNAi knockdowns of the top candidate transcription factors, focusing specifically on the roles of Cnc-C. Immunoblot assays of protein isolated from *Drosophila* cells harvested 3 days after treatment with dsRNA to *cnc-C* resulted in a decrease in individual proteasome subunits from different subcomplexes of the proteasome (Fig. 4A). The greatest decline at the protein level was observed for the 20S proteasome subunit alpha 7, followed by the 19S subunit Rpt1. Knockdown of *cnc-C* levels resulted in a modest decline in the p97 ATPase. As has been previously observed for *Drosophila* cells, RNAi knockdown of the ubiquitin receptor S5a resulted in increased levels of proteasome subunits. To examine the abundance of the proteasome complex itself, equivalent amounts of total cellular protein were separated by native gel electrophoresis, and in-gel peptidase assays were carried out by overlaying with the fluorogenic proteasome substrate LLVY-AMC, followed by exposure to UV light (Fig. 4B). After knockdown of *cnc-C* levels, the in-gel peptidase assays showed reductions of 26S proteasome complexes but little or no apparent decreases in the 20S proteasome. The cellular activity for 20S proteasomes was also examined with a more sensitive assay using whole-cell protein extracts following the cleavage of LLVY-AMC in a standard cuvette-based assay (Fig. 4C). The 20S proteasome assays on RNAi-treated cell extracts showed a strong reduction of the 20S proteasome activity when Cnc-C levels were depleted with different dsRNAs that were specific for the *cnc-C* transcripts. This assay measures total 20S proteasome pools of both free 20S and 20S proteasome that are assembled in the 26S proteasome. The apparent discrepancy between the two 20S proteasome assays may be due to changes in the ratios of 20S versus 26S proteasomes. Inhibited assembly of 26S proteasomes would tend to maintain the levels of free 20S proteasomes, as observed with the in-gel peptidase assay, even though the overall level of 20S proteasomes declined, as measured with the cuvette-based assay.

In addition, *cnc* RNAi could prevent the S5a subunit RNAi-related increase in 20S subunit activity. The S5a RNAi knockdown was effective in the presence of dsRNAs to *cnc*, and immunoblot assays against S5a showed apparently equivalent depletion in S5a when treated with both dsRNAs or alone (Fig. 4A).

Depletion of the Keap1 substrate adaptor protein does not induce proteasome increases. In *Drosophila* it has been shown that the expression of Keap1 rescues defects due to overexpression of *cnc*-C in the eye (46). In mammals Keap1 is a BTB-Kelch-type substrate adaptor protein of the Cul3-dependent ubiquitin ligase complex and functions as an adaptor for the cullin 3-based E3 ligase to regulate the stability of the CNC protein Nrf2 (6, 15, 24, 55). As found in mammalian systems with Keap1 and Nrf2, *Drosophila* Keap1 and Cnc-C have been proposed to directly bind to each other and have been identified to interact in two-hybrid experiments (16). In the standard model, Keap1 acts as a negative regulator of Cnc-C, and together they play an important role in the cell as an important sensor for oxidants and toxic agents during antioxidant and detoxification responses (see Fig. 7, below).

To examine the effects on proteasome subunit levels, *Drosophila* Keap1 was knocked down with RNAi (Fig. 4D). Surprisingly, no increases in any proteasome subunit levels were apparent after dsRNA treatment against Keap1, but there was a decrease in each subunit. Quantitative RT-PCR measurements of cells treated with dsRNA to *Keap1* showed that partial depletion of *Keap1* by RNAi treatment significantly increased an oxidative stress response gene, *gstD1*, but no significant increase in mRNA level was observed for a proteasome subunit (see Fig. S3 in the supplemental material).

Cnc-C is essential for the *Drosophila* proteasome recovery pathway. To confirm that RNAi directed to *cnc* led to decreases at the transcriptional level, mRNA levels for several components of the 26S proteasome were measured by quantitative RT-PCR (Fig. 5A). Levels of mRNA for one subunit each from the 20S proteasome (alpha 7), the 19S regulatory base (Rpt3), and the 19S lid (Rpn11) were measured. The levels of the p97 ATPase mRNA were also measured, as they have been shown to be coregulated with the proteasome in *S. cerevisiae* and *Drosophila* (23, 29). The levels were normalized to the *RpL32* mRNA standard and compared to a control RNAi knockdown by using dsRNA to GFP. Knockdown of these transcription factors resulted in modest but significant decreases at the mRNA level for all three classes of proteasome genes, with declines between 20 and 40% (Fig. 5A).

Consistent with the earlier RNAi screening, knocking down a single *cnc* transcript, *cnc-C*, resulted in decreases at the proteasome mRNA level that were equal to or stronger than when targeting all *cnc* transcripts. A significant decline in the level of mRNA for the p97 ATPase was also observed following *cnc-C* knockdown. A TaqMan probe was available to examine the mRNA levels of *cnc* after RNAi treatments and confirmed the specificity of the dsRNA treatments. The commercially available probe was designed to anneal to all the predicted transcript forms of *cnc*. Targeting specifically the *cnc-C* transcript lowered the mRNA levels of the overall *cnc* mRNA to a lesser extent (*cnc-C* dsRNA1 to 36% and dsRNA2 to 29%) than targeting with one designed to target all *cnc* transcripts (cnc-All; to 42%).

A number of ubiquitin binding proteins have been characterized, and several others have been proposed to assist in the proteasomal degradation of ubiquitylated substrates. One of these facilitator proteins is the proteasome subunit S5a. Depletion of the ubiquitin binding subunit S5a increased the protein levels of *Drosophila* proteasome subunits (Fig. 4) but required the presence of Cnc-C. Quantitative PCR measurements showed that this regulation occurs at the mRNA level (Fig. 5B). As was seen at the protein level, the induction of

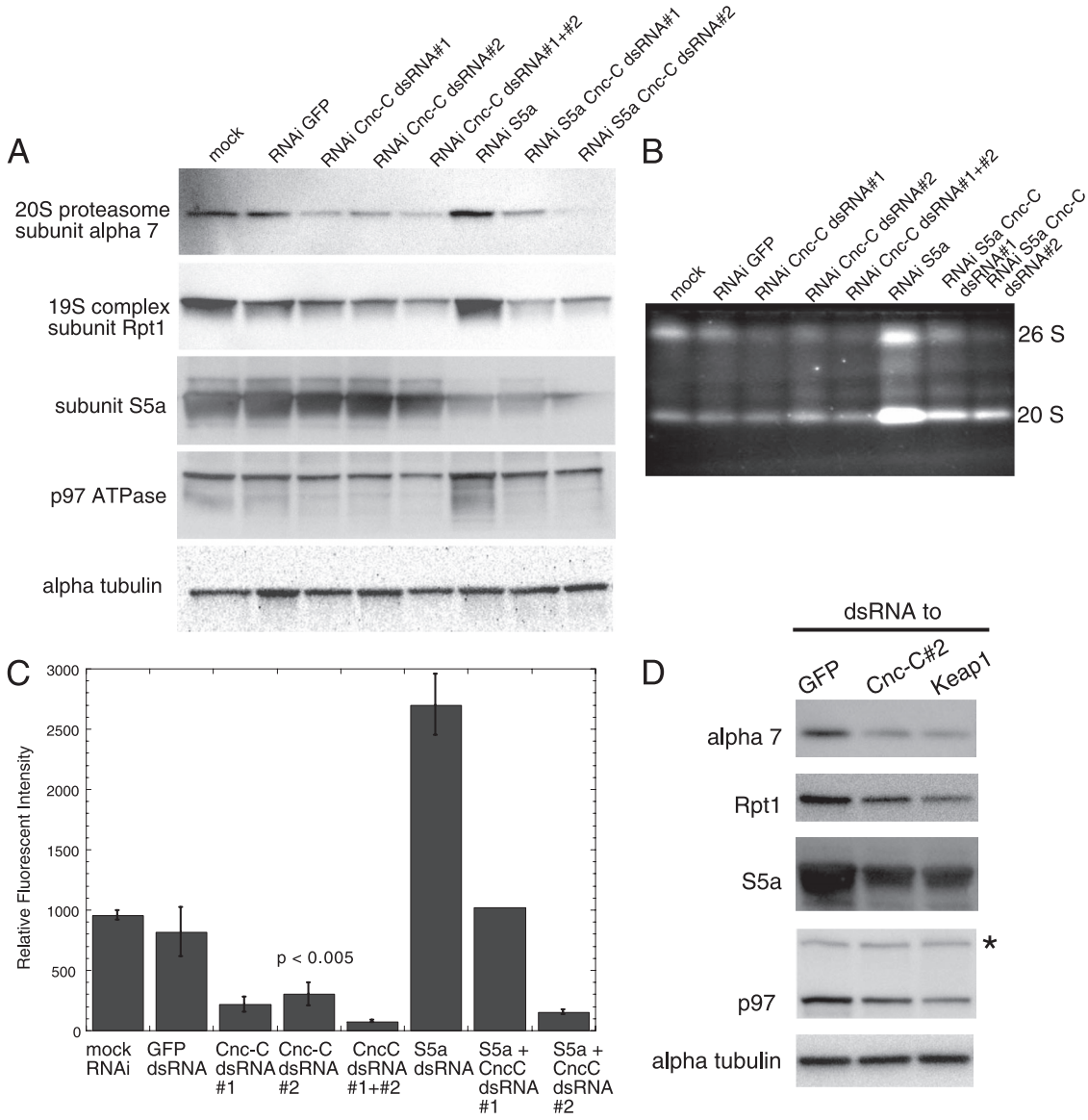

FIG. 4. Decreases in the expression of proteasome subunits after knockdown of Cnc-C. (A) Immunoblots to subunits from different subcomplexes of the 26S proteasome and p97 ATPase. RNAi knockdown of ubiquitin receptor S5a was previously shown to increase overall 26S proteasome levels and the p97 ATPase (29). Co-RNAi depletions of S5a and Cnc blocked S5a-induced increases in proteasome levels. (B) Results from a native gel proteasome assay in which 26S and 20S proteasomes were separated by electrophoresis and identified by in-gel hydrolysis of the LLVY-AMC proteasome fluorogenic substrate. (C) 20S proteasome assay of Cnc-C RNAi-depleted *Drosophila* extracts. After RNAi treatments the cell extracts were measured for total protein concentrations and assayed for 20S proteasome levels. Assays were carried out in the absence of ATP and with the addition of dilute SDS (0.03%) to activate the 20S proteasomes. Activity was determined by hydrolysis of Suc-LLVY-AMC (final concentration, 200 μM) for 1 h at 37°C. (D) Immunoblots to 26S proteasome subunits and p97 ATPase subunits after knockdown of the Keap1 E3 ligase. Knockdown of Keap1 did not increase proteasome subunit levels.

proteasome mRNA after depletion of the ubiquitin receptor S5a was blocked in the absence of Cnc-C, indicating that the Cnc-C transcription factor is responsible for the increased levels of proteasome and p97 mRNAs after loss of the S5a proteasome subunit.

The recovery of proteasome levels after proteasome inhibitor treatment was also examined. After treatment with the proteasome inhibitor MG132, mammalian and *Drosophila* cells increase their proteasome component mRNA levels (29, 34). This has been termed the proteasome recovery pathway (41), and these increases in proteasome and p97 mRNA levels were blocked by RNAi against *cnc-C* (Fig. 5C). Overall, these results showed that Cnc-C is essential for a cell's ability to restore proteasome levels after proteasome inhibitor treatment, and it contributes partially to maintaining basal levels of the proteasome mRNAs under steady-state conditions.

Cnc-C is a proteasome substrate and is stabilized when the ubiquitin receptor S5a is depleted. To understand the role of the proteasome in maintenance of Cnc-C protein levels, a V5-His-tagged recombinant Cnc-C containing 576 amino acid

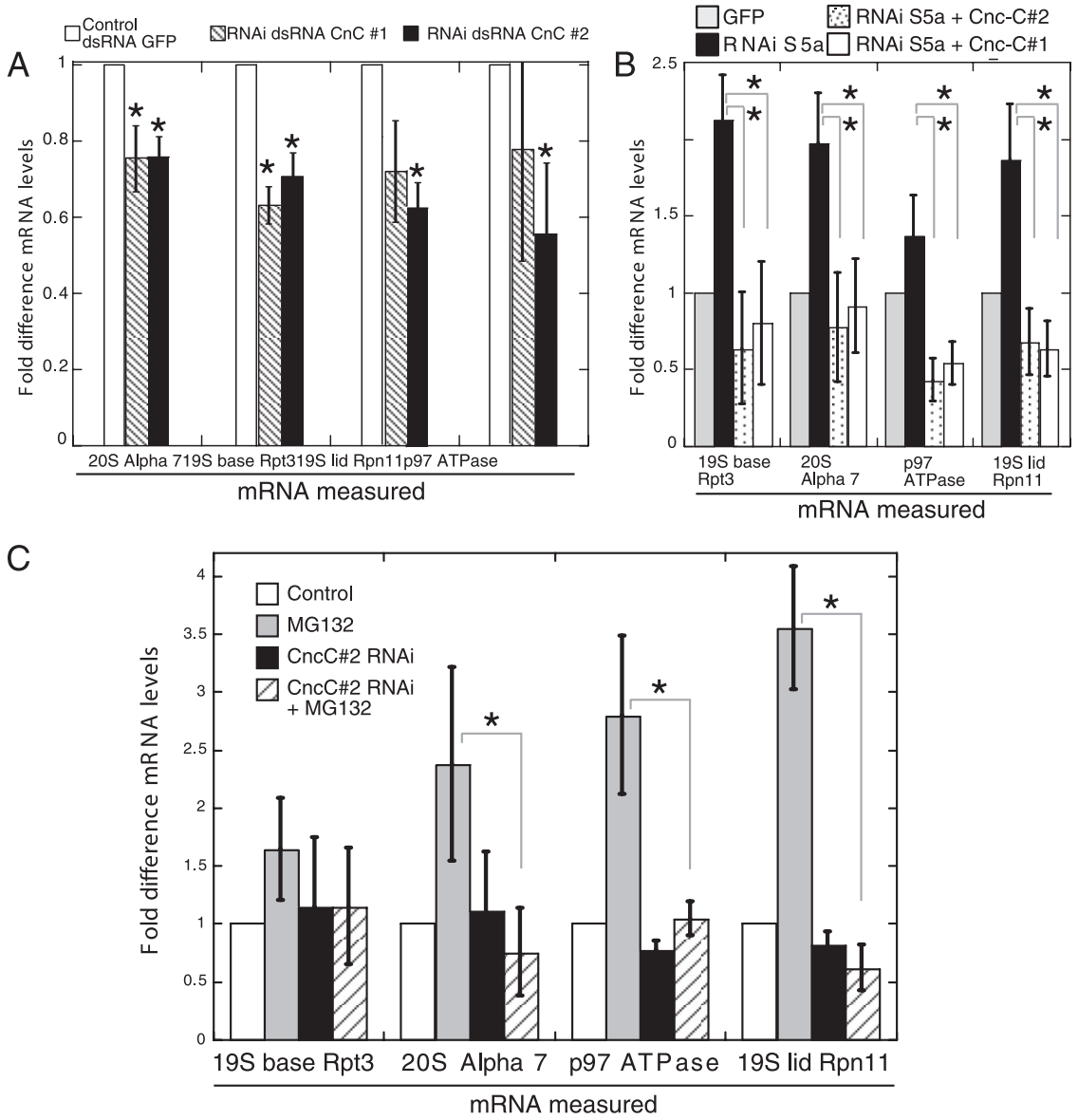

FIG. 5. The Cnc-C transcription factor contributes to maintaining basal levels of proteasome mRNAs and is essential for proteasome recovery after inhibition. For each quantitative PCR, triplicate knockdown measurements for each target were carried out independently and the mRNA levels were determined using real-time PCR and TaqMan probes for *Drosophila* proteasome subunits and the p97 ATPase. A control RNAi treatment used dsRNA to the GFP sequence and was used for normalization of levels. (A) Effects of knockdown of Cnc-C on a 20S proteasome subunit mRNA (alpha 7), on a 19S lid subunit (Rpt3), a 19S lid subunit (Rpn11), and, finally, the mRNA levels for p97 ATPase. The Cnc was depleted with either a dsRNA that was specific for only the Cnc-C alternative transcript or for all Cnc transcripts, Cnc-All. Cnc-C RNAi depletion resulted in significant decreases in all measured proteasome mRNAs. Student's *t* test was used to analyze differences in proteasome mRNAs ($P < 0.0065$) and for p97 ATPase ($P = 0.037$). (B) RNAi knockdown of the proteasome subunit S5a and increased levels of proteasome mRNA levels. Coknockdown of S5a and Cnc-C blocked the induction of proteasome and p97 mRNA levels. Depletion of Cnc-C with either dsRNA significantly blocked mRNA proteasome induction after loss of S5a. (C) Proteasome recovery after inhibition with MG132 was blocked in cells previously RNAi depleted of the transcription factor Cnc-C. RNAi-treated cells were treated for 7 h with MG132 (10 μM). Student's *t* test was used to compare control cell recovery after MG132 treatment versus Cnc-C-depleted MG132-treated cells: $P < 0.02$ for alpha 7 mRNA levels; $P < 0.007$ for p97 ATPase mRNA levels; $P < 0.0005$ for Rpn11.

residues of the unique Cnc-C protein was expressed in *Drosophila* S2 cells. Since multiple attempts to generate polyclonal Cnc-C-specific antibodies were unsuccessful, *cnc-C* was cloned from a *Drosophila* cDNA library and a C-terminal V5-His tag was added in place of the bZIP DNA binding domain, whose sequence was not present in the expressed sequence tag transcript. A transfection mixture of *Drosophila* S2 cells was divided and treated with either proteasome inhibitor or dsRNA and compared with untreated controls (Fig. 6A). Transfected control cells showed little or no detectable Cnc-C V5-His-tagged protein; however, after epoxomicin treatment, the same transfection showed a high Cnc-C V5-His level. Interestingly, RNAi knockdown of the S5a proteasome subunit, but not a GFP dsRNA-treated control, also stabilized the Cnc-C V5-His

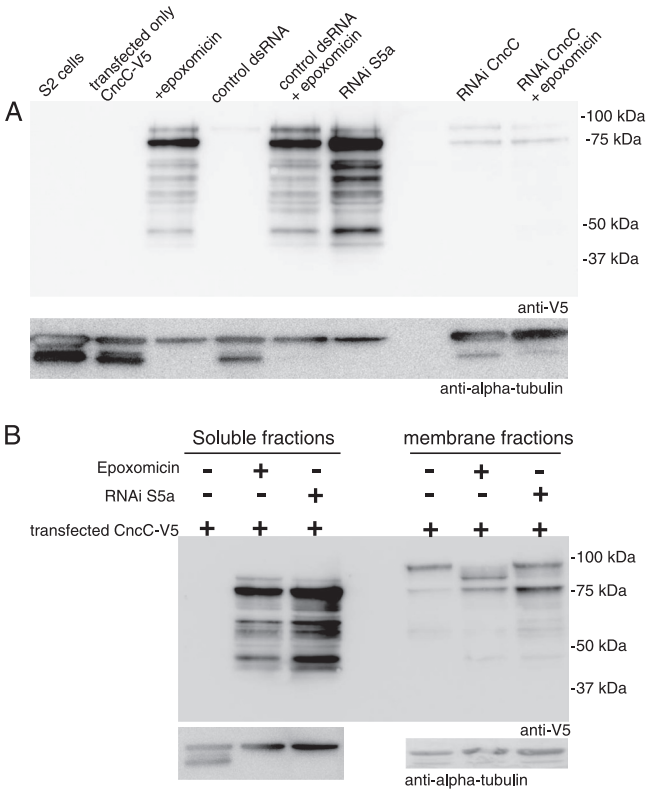

FIG. 6. (A) Soluble *Drosophila* Cnc-C is a proteasome substrate and is stabilized by RNAi depletion of proteasome subunit S5a. A C-terminal V5 epitope tagged Cnc-C construct was transfected into *Drosophila* S2 cells, and cells were divided into different treatment groups. Collected cells were freeze-thawed, and the soluble protein fractions were immunoblotted and developed with a V5 antibody. RNAi depletion of S5a stabilized soluble Cnc-C, but levels were not stablilized with a control dsRNA to CG10372. RNAi using dsRNA that was employed in the original screen knocked down CncC-V5 in epoxomicin-treated cells. (B) Soluble Cnc-C-transfected *Drosophila* S2 cells expressing CncC-V5 were treated, and soluble and membrane fractions were immunoblotted against the C-terminal V5 epitope. The faster migration for soluble Cnc-C suggests cleavage at the N-terminal end of the CncC-V5 compared to the slower-migrating membrane-associated CncC-V5. Both soluble and membrane-bound Cnc-C bands migrated more slowly than the expected molecular mass for the Cnc-V5 construct, 66 kDa.

protein. As expected, RNAi treatment using the *cnc-C* dsRNA blocked the appearance of Cnc-C V5-His after proteasome inhibition.

It has been observed that the mammalian Nrf1 transcription factor is a membrane/endoplasmic reticulum (ER)-bound protein and is proposed to be proteolytically cleaved for activation and transport to the nucleus independently of Keap1 (56). To determine if this is the case with *Drosophila* Cnc-C, *cnc-C* V5-His-transfected cells were harvested, and membrane and soluble fractions were prepared and immunoblotted for the presence of the Cnc-C construct (Fig. 6B). A membrane-bound fraction of the Cnc-C with V5-His had a significantly slower migration rate through the SDS-PAGE gel than the soluble Cnc-C. Both the soluble and membrane-bound Cnc-C V5-His had significantly slower migrations through the SDS-PAGE gel than expected for the size of the construct. Previously, both human Nrf1 and Nrf2 transcription factors also have been observed to show abnormally slow migration in SDS-PAGE gels (56). The V5 antibody can only recognize a Cnc-C protein that contains an intact C terminus, and this suggests that the small soluble form of Cnc-C present after proteasome inhibition may be caused by an N-terminal truncation. Posttranslation modification, such as glycosylation of the membrane form of Cnc-C, cannot be ruled out at this time to explain the migration differences. Assuming that the protease activity of the proteasome is inhibited after epoxomicin treatment, the proposed cleavage of the Cnc-C V5-His would require a second protease for the generation of the soluble form. Additional studies are required to confirm and map the potential cleavage site(s) apparently present in the soluble Cnc-C V5-His.

A number of bZIP transcription factors have been shown to be constitutive substrates for the 26S proteasome, including Nrf2 (6). Our current results support a model where decreased proteasome activity or the loss of *Drosophila* S5a prevents the degradation of the Cnc-C transcription factor. Under conditions of low cellular proteasome activity, the membrane-bound Cnc-C protein is cleaved by a currently unknown proteolytic system, and the liberation of an N-terminal truncated soluble form of Cnc-C promotes increased transcription of proteasome genes in the nucleus (see Fig. 7, below).

DISCUSSION

Targeted inhibition of 20S proteasome proteolytic activity has become a new and unique avenue for treating multiple myeloma and other cancers (44). While the understanding of how to inhibit the *in vivo* levels of the proteasome has greatly advanced in the past decade, less is known about the basic cellular mechanisms that increase or decrease overall proteasome levels, such as upregulation of proteasome activity observed in muscle wasting conditions or the poorly understood age-related decline in proteasomes (7). To identify disease states that would be likely candidates for the use of proteasome inhibitors, it is important to understand the mechanisms and unique features of maintaining adequate proteasome levels in metazoan cells.

A widely used model substrate for measuring the UPS within living cells is the ubiquitin fusion UFD substrate Ub^{G76V}-GFP (8), and this was chosen as the reporter in our RNAi screen. An advantage of using the Ub^{G76}-GFP reporter is that the cell assay requires the basic transcriptional machinery to still be functional, i.e., that transcription off the actin promoter of the Ub^{G76V}-GFP reporter has successfully occurred. Specific loss of transcription of UPS mRNAs is measured by the stabilization of Ub^{G76V}-GFP but does not create false positives for general transcription defects that would typically also prevent Ub^{G76V}-GFP mRNA transcription.

Our current results indicate a role for Cnc-C in maintaining 26S proteasome levels and the p97 ATPase. The *Drosophila cnc* gene was the first identified member of the extensive CNC family of basic leucine zipper proteins (36). The Cnc-bZIP factors are believed to function as obligate heterodimers with small Maf proteins (21). In *Drosophila* there is a single *cnc* gene that has a number of distinct transcript forms (33). The *cnc-C* transcript is expressed ubiquitously and is essential for

life, while the *cnc-B* transcript is expressed in an embryonic pattern that includes the labral, intercalary, and mandibular segments and is required for the proper development of these structures (33, 35, 51). A much more complex gene arrangement is present in mammalian cells, represented by a family of bZIP factors related to the *Drosophila* Cnc protein, and includes the Nrf1, Nrf2, Nrf3, p45NFE2, and Bach1 and Bach2 transcription factors (47).

At both the protein and mRNA levels, proteasome components in this study were found to decrease when *cnc-C* transcripts were depleted in *Drosophila* S2 cells. Overall, the decreases observed for the proteasome subunits mRNA levels were modest, causing 20 to 40% declines, when Cnc-C was depleted. The partial depletion of proteasome mRNA may indicate that transcription of proteasome mRNAs is regulated by multiple pathways and Cnc-C may only contribute a fractional role in the basal transcription of proteasome genes. Other candidates identified from our screen may also contribute to basal maintenance of proteasome mRNA levels. Surprisingly, large declines in the level of proteasome subunits were present in Cnc-C-depleted cells that showed only a modest decline at the mRNA level (Fig. 4C compared to 5A). Over an extended period of time, continuous suboptimal mRNA levels may drain proteasome levels if proteasome turnover is significantly faster than replenishment.

On the other hand, the Cnc-C transcription factor does appear to have a dominant role in the reestablishment of proteasome levels after proteasome inhibitor treatment. A significant loss of proteasome mRNA induction was seen in cells depleted of Cnc-C. Also, the increase of proteasome mRNA levels caused by the depletion of the ubiquitin binding S5a protein was blocked when Cnc-C was depleted.

The *cnc* gene with its alternative transcripts may serve multiple roles in *Drosophila*, similar to the multigene *nrf* transcription factor family in mammals. Treatment with dithiolethiones leads to increased proteasome levels in mammalian cells through the oxidative stress pathway Nrf2-Keap1 due to increased transcription of genes for proteasome 20S and 19S subunits (27). However, it is unlikely that Nrf2 plays a major role for the basal maintenance of 26S proteasome levels. Knockout of the *nrf2* gene results in mice that are developmentally normal but are more sensitive to chemical or environmental stress (5). In contrast with the knockout of *nrf2*, knockout of the *nrf1* gene is embryonic lethal (4, 12). Nrf1 is essential for normal liver function in mice; liver-specific disruption of *nrf1* in mice results in increased inflammation and apoptosis, and with additional time these mice spontaneously develop cancer (54).

Drosophila Cnc-C and mammalian Nrf1 N-terminal regions share sequence similarities, and this suggests possible similar regulation of the transcription factors. Nrf1 is not believed to be regulated by Keap1, but instead is proteolytically cleaved, liberating it from its membrane anchor, in a poorly understood process (57). Through the expression of a tagged form of a V5-His Cnc-C, we also observed possible proteolytic cleavage of a soluble form of the *Drosophila* transcription factor. Based on the retention of the C-terminal tag present in the expressed transgenic Cnc-C sequence, the cleavage likely occurs N-terminally. During the revision of our manuscript, the long isoform of human Nrf1, TCF11, was shown to be an essential regulator for 26S proteasome formation via an ER-associated protein degradation membrane feedback loop (45). For human TCF11 to upregulate proteasome subunit genes, both deglycosylation and cleavage of TCF11 are required for translocation from the ER membrane to the nucleus.

Cnc-C may control proteasome levels independently of Keap1. Previous *Drosophila* genomic transcriptional profiling experiments showed that S5a depletion results in a specificity for inducing proteasome genes (29). Second, Keap1, which binds to and prevents Cnc-C from entering the nucleus and upregulating antioxidant genes, is surprisingly one of the few nonproteasome genes transcriptionally upregulated after S5a depletion, when transcription of proteasome components is induced. Finally, RNAi depletion of *Keap1* does not increase proteasome levels, as would be expected if Keap1 functioned to downregulate proteasome transcription in *Drosophila* cells.

If *Drosophila* proteasome genes were under the control of the transcription factor Cnc-C, it would be expected that proteasome genes share related promoter elements, as found in other known genes that are transcriptionally activated by Cnc-C. Antioxidant response elements (ARE) are enhancer sequences that allow Nrf transcription factors to bind at the promoter region of regulated genes (22, 50). Recently, an ARE sequence was identified as a regulatory element near the start of transcription for the mammalian proteasome gene PSMB6 (41). For *Drosophila* Cnc-C, an ARE enhancer element was identified for oxidative stress regulation and Cnc-C binding upstream of the glutathione *S*-transferase gene *gstD* (46). This *Drosophila gstD* regulatory sequence, TCAgcATGACcggGCA aaaa, shows clear similarity (uppercase letters) with the extended consensus of the mammalian ARE sequence motif (37). We have previously shown that increased levels of proteasome gene expression are regulated by small regions that overlap the transcriptional start regions of proteasome genes. For the proteasome genes Rpn1 and Beta-2, the transcriptional start regions contain ARE-like sequences, GCAgtGTG ACcgcGCGgcga and GAGcgATGACaaaCAAaatt, centered within previously mapped locations essential for induced transcription after proteasome inhibition (29). However, these sequences are found directly after the transcription start site and are present on the antisense strand of the DNA (see Fig. S4 in the supplemental material). Future studies will be required to understand the function of Cnc-C binding in both oxidative stress-regulated genes and proteasome genes.

Our recent results have led us to propose a speculative model for *Drosophila* proteasome regulation (Fig. 7). The model proposes that RNAi knockdown of the ubiquitin receptor protein S5a stabilizes Cnc-C, which is posttranslationally modified and cleaved to specifically serve in transcribing proteasome and proteasome-related genes. It was previously shown that loss of S5a stabilizes a subset of important cellular proteasome substrates. In *Saccharomyces cerevisiae*, deletion of S5a (Rpn10) stabilizes a range of ubiquitin proteasome substrates, including the bZIP transcription factor Gcn4 (32, 52).

We propose that for *Drosophila*, a single protein, Cnc-C, is important for transcription of two large pathways of genes, antioxidant genes and proteasome genes, and our findings support the idea that transcriptional specificity is occurring even though a single transcription factor is involved. Our model does not rule out the possibility that both pathways can be

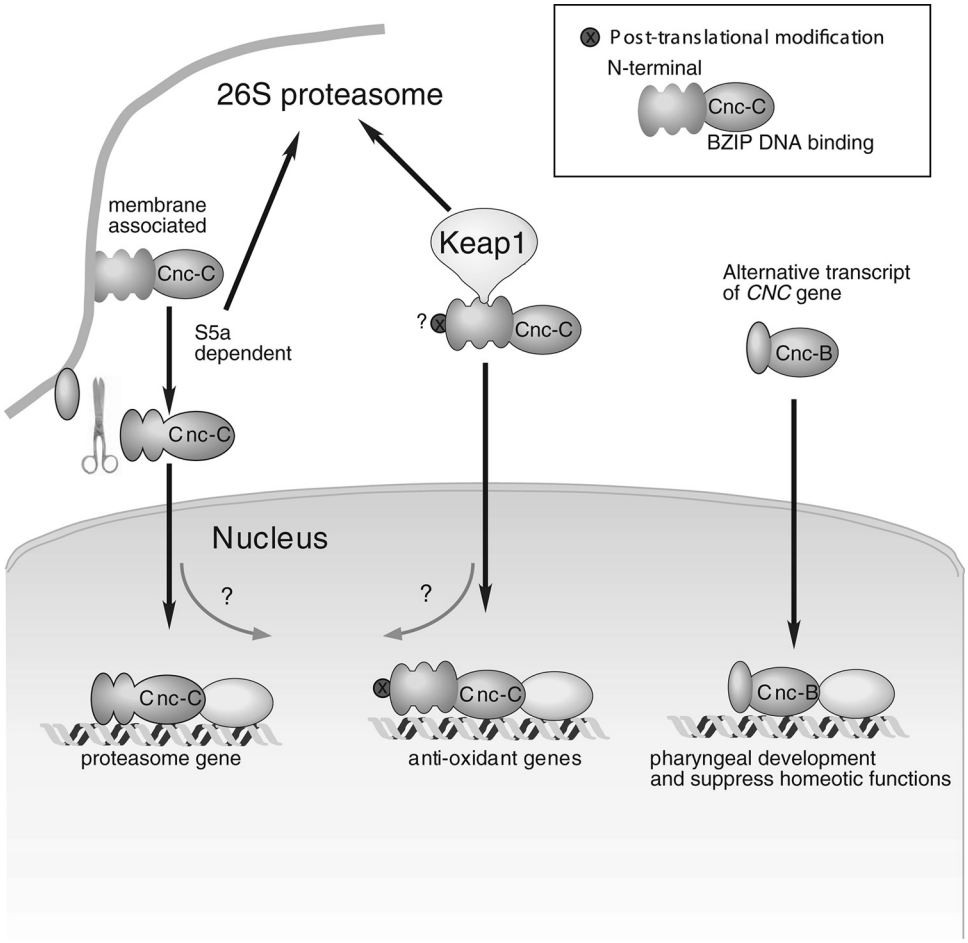

FIG. 7. Speculative model for a *Drosophila* transcription feedback control mechanism for 26S proteasome levels. Unlike the mammalian system, only a single locus exists in *Drosophila* for a CNC bZIP transcription factor. An alternative short transcript, Cnc-B, is identical in sequence to the longer Cnc-C transcript, but the shorter form has been previously shown to transcribe a specific developmental gene class (51). Past and current evidence suggests a model in which Cnc-C is able to regulate the two gene classes independently and that posttranscriptional modifications of the Cnc-C protein could generate two forms of Cnc-C with distinct activities. The model proposes that a Cnc-C form that does not bind Keap1 functions similarly to the mammalian Nrf-1 protein and uses the Nrf-1-like N-terminal region to become membrane bound to the ER or other cell membrane surfaces. Depletion of the S5a shuttle protein activates this form of Cnc-C by preventing the degradation of the proteasome transcription factor. Regulation of oxidative stress genes is controlled by the level of a posttranscriptionally modified Cnc-C that functions like the mammalian Nrf-2 transcription factor and is controlled by polyubiquitylation through the Keap1 E3 ligase system. After entry into the nucleus, the Cnc-C protein binds additional proteins and functions as a transcription factor for either proteasome degradation or an antioxidative stress class of genes.

cotranscriptionally upregulated by Cnc-C. In fact, previous transcriptional array profiling of *Drosophila* cells recovering from proteasome inhibitor treatment showed upregulation of a number of antioxidant gene and xenobiotic-metabolizing gene mRNAs (29), including a 62-fold increase of a cytochrome P450, CYP6A20, and a 2-fold increase for the known Cnc-C-regulated glutathione *S*-transferase D1(46). Additional work is required to understand the role of Cnc-C in *Drosophila* cells and the possible mechanisms that allow specific induction of the proteasome pathway. In conclusion, *Drosophila* Cnc appears to share an evolutionarily conserved proteasome regulation pathway with the human Nrf transcription factors. Future examination of this regulatory transcription pathway should help explain how metazoan cells maintain proteasome activity and also identify additional targets for future therapeutic inhibitors of the proteasome system.

ACKNOWLEDGMENTS

This work was supported by grants from the Swedish Research Council and Swedish Cancer Society.

REFERENCES

1. **Anisimova, M., and O. Gascuel.** 2006. Approximate likelihood-ratio test for branches: a fast, accurate, and powerful alternative. Syst. Biol. **55:**539–552.
2. **Beedholm, R., B. F. Clark, and S. I. Rattan.** 2004. Mild heat stress stimulates 20S proteasome and its 11S activator in human fibroblasts undergoing aging in vitro. Cell Stress Chaperones **9:**49–57.
3. **Beskow, A., et al.** 2009. A conserved unfoldase activity for the p97 AAA-ATPase in proteasomal degradation. J. Mol. Biol. **394:**732–746.
4. **Chan, J. Y., et al.** 1998. Targeted disruption of the ubiquitous CNC-bZIP transcription factor, Nrf-1, results in anemia and embryonic lethality in mice. EMBO J. **17:**1779–1787.
5. **Chan, K., R. Lu, J. C. Chang, and Y. W. Kan.** 1996. NRF2, a member of the NFE2 family of transcription factors, is not essential for murine erythropoiesis, growth, and development. Proc. Natl. Acad. Sci. U. S. A. **93:**13943–13948.
6. **Cullinan, S. B., J. D. Gordan, J. Jin, J. W. Harper, and J. A. Diehl.** 2004. The

Keap1-BTB protein is an adaptor that bridges Nrf2 to a Cul3-based E3 ligase: oxidative stress sensing by a Cul3-Keap1 ligase. Mol. Cell. Biol. **24:**8477–8486.
7. **Dahlmann, B.** 2007. Role of proteasomes in disease. BMC Biochem. **8**(Suppl. 1)**:**S3.
8. **Dantuma, N. P., K. Lindsten, R. Glas, M. Jellne, and M. G. Masucci.** 2000. Short-lived green fluorescent proteins for quantifying ubiquitin/proteasome-dependent proteolysis in living cells. Nat. Biotechnol. **18:**538–543.
9. **DasGupta, R., and F. C. Gonsalves.** 2008. High-throughput RNAi screen in Drosophila. Methods Mol. Biol. **469:**163–184.
10. **Do, C. B., M. S. Mahabhashyam, M. Brudno, and S. Batzoglou.** 2005. ProbCons: probabilistic consistency-based multiple sequence alignment. Genome Res. **15:**330–340.
11. **Dohmen, R. J., I. Willers, and A. J. Marques.** 2007. Biting the hand that feeds: Rpn4-dependent feedback regulation of proteasome function. Biochim. Biophys. Acta **1773:**1599–1604.
12. **Farmer, S. C., C. W. Sun, G. E. Winnier, B. L. Hogan, and T. M. Townes.** 1997. The bZIP transcription factor LCR-F1 is essential for mesoderm formation in mouse development. Genes Dev. **11:**786–798.
13. **Felsenstein, J.** 1978. Cases in which parsimony or compatibility methods will be positively misleading. Syst. Zool. **27:**401–410.
14. **Fuchs, D., C. Berges, G. Opelz, V. Daniel, and C. Naujokat.** 2008. Increased expression and altered subunit composition of proteasomes induced by continuous proteasome inhibition establish apoptosis resistance and hyperproliferation of Burkitt lymphoma cells. J. Cell. Biochem. **103:**270–283.
15. **Furukawa, M., and Y. Xiong.** 2005. BTB protein Keap1 targets antioxidant transcription factor Nrf2 for ubiquitination by the Cullin 3-Roc1 ligase. Mol. Cell. Biol. **25:**162–171.
16. **Giot, L., et al.** 2003. A protein interaction map of Drosophila melanogaster. Science **302:**1727–1736.
17. **Glickman, M. H., and A. Ciechanover.** 2002. The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. Physiol. Rev. **82:**373–428.
18. **Groll, M., et al.** 2000. A gated channel into the proteasome core particle. Nat. Struct. Biol. **7:**1062–1067.
19. **Guindon, S., and O. Gascuel.** 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst. Biol. **52:**696–704.
20. **Hordijk, W., and O. Gascuel.** 2005. Improving the efficiency of SPR moves in phylogenetic tree search methods based on maximum likelihood. Bioinformatics **21:**4338–4347.
21. **Itoh, K., K. Igarashi, N. Hayashi, M. Nishizawa, and M. Yamamoto.** 1995. Cloning and characterization of a novel erythroid cell-derived CNC family transcription factor heterodimerizing with the small Maf family proteins. Mol. Cell. Biol. **15:**4184–4193.
22. **Johnsen, O., P. Murphy, H. Prydz, and A. B. Kolsto.** 1998. Interaction of the CNC-bZIP factor TCF11/LCR-F1/Nrf1 with MafG: binding-site selection and regulation of transcription. Nucleic Acids Res. **26:**512–520.
23. **Kapranov, A. B., et al.** 2001. Isolation and identification of PACE-binding protein rpn4: a new transcription activator, participating in regulation of 26S proteosome and other genes. Mol. Biol. (Mosk.) **35:**420–431. (In Russian.)
24. **Kobayashi, A., et al.** 2004. Oxidative stress sensor Keap1 functions as an adaptor for Cul3-based E3 ligase to regulate proteasomal degradation of Nrf2. Mol. Cell. Biol. **24:**7130–7139.
25. **Kobayashi, M., et al.** 2002. Identification of the interactive interface and phylogenic conservation of the Nrf2-Keap1 system. Genes Cells **7:**807–820.
26. **Kumatori, A., et al.** 1990. Abnormally high expression of proteasomes in human leukemic cells. Proc. Natl. Acad. Sci. U. S. A. **87:**7071–7075.
27. **Kwak, M. K., N. Wakabayashi, J. L. Greenlaw, M. Yamamoto, and T. W. Kensler.** 2003. Antioxidants enhance mammalian proteasome expression through the Keap1-Nrf2 signaling pathway. Mol. Cell. Biol. **23:**8786–8794.
28. **Le, S. Q., and O. Gascuel.** 2008. An improved general amino acid replacement matrix. Mol. Biol. Evol. **25:**1307–1320.
29. **Lundgren, J., P. Masson, Z. Mirzaei, and P. Young.** 2005. Identification and characterization of a Drosophila proteasome regulatory network. Mol. Cell. Biol. **25:**4662–4675.
30. **Mannhaupt, G., and H. Feldmann.** 2007. Genomic evolution of the proteasome system among hemiascomycetous yeasts. J. Mol. Evol. **65:**529–540.
31. **Mannhaupt, G., R. Schnall, V. Karpov, I. Vetter, and H. Feldmann.** 1999. Rpn4p acts as a transcription factor by binding to PACE, a nonamer box found upstream of 26S proteasomal and other genes in yeast. FEBS Lett. **450:**27–34.
32. **Mayor, T., J. Graumann, J. Bryan, M. J. MacCoss, and R. J. Deshaies.** 2007. Quantitative profiling of ubiquitylated proteins reveals proteasome substrates and the substrate repertoire influenced by the Rpn10 receptor pathway. Mol. Cell. Proteomics **6:**1885–1895.
33. **McGinnis, N., E. Ragnhildstveit, A. Veraksa, and W. McGinnis.** 1998. A cap 'n' collar protein isoform contains a selective Hox repressor function. Development **125:**4553–4564.
34. **Meiners, S., et al.** 2003. Inhibition of proteasome activity induces concerted expression of proteasome genes and de novo formation of mammalian proteasomes. J. Biol. Chem. **278:**21517–21525.
35. **Mohler, J., J. W. Mahaffey, E. Deutsch, and K. Vani.** 1995. Control of Drosophila head segment identity by the bZIP homeotic gene cnc. Development **121:**237–247.
36. **Mohler, J., K. Vani, S. Leung, and A. Epstein.** 1991. Segmentally restricted, cephalic expression of a leucine zipper gene during Drosophila embryogenesis. Mech. Dev. **34:**3–9.
37. **Nioi, P., M. McMahon, K. Itoh, M. Yamamoto, and J. D. Hayes.** 2003. Identification of a novel Nrf2-regulated antioxidant response element (ARE) in the mouse NAD(P)H:quinone oxidoreductase 1 gene: reassessment of the ARE consensus sequence. Biochem. J. **374:**337–348.
38. **Nouhi, Z., G. Chevillard, A. Derjuga, and V. Blank.** 2007. Endoplasmic reticulum association and N-linked glycosylation of the human Nrf3 transcription factor. FEBS Lett. **581:**5401–5406.
39. **Oyake, T., et al.** 1996. Bach proteins belong to a novel family of BTB-basic leucine zipper transcription factors that interact with MafK and regulate transcription through the NF-E2 site. Mol. Cell. Biol. **16:**6083–6095.
40. **Rabl, J., et al.** 2008. Mechanism of gate opening in the 20S proteasome by the proteasomal ATPases. Mol. Cell **30:**360–368.
41. **Radhakrishnan, S. K., et al.** 2010. Transcription factor Nrf1 mediates the proteasome recovery pathway after proteasome inhibition in mammalian cells. Mol. Cell **38:**17–28.
42. **Ramadan, N., I. Flockhart, M. Booker, N. Perrimon, and B. Mathey-Prevot.** 2007. Design and implementation of high-throughput RNAi screens in cultured Drosophila cells. Nat. Protoc. **2:**2245–2264.
43. **Richardson, P. G., T. Hideshima, and K. C. Anderson.** 2003. Bortezomib (PS-341): a novel, first-in-class proteasome inhibitor for the treatment of multiple myeloma and other cancers. Cancer Control **10:**361–369.
44. **Shah, J. J., and R. Z. Orlowski.** 2009. Proteasome inhibitors in the treatment of multiple myeloma. Leukemia **23:**1964–1979.
45. **Steffen, J., M. Seeger, A. Koch, and E. Kruger.** 2010. Proteasomal degradation is transcriptionally controlled by TCF11 via an ERAD-dependent feedback loop. Mol. Cell **40:**147–158.
46. **Sykiotis, G. P., and D. Bohmann.** 2008. Keap1/Nrf2 signaling regulates oxidative stress tolerance and lifespan in Drosophila. Dev. Cell **14:**76–85.
47. **Sykiotis, G. P., and D. Bohmann.** 2010. Stress-activated cap'n'collar transcription factors in aging and human disease. Sci. Signal. **3:**re3.
48. **Szlanka, T., et al.** 2003. Deletion of proteasomal subunit S5a/Rpn10/p54 causes lethality, multiple mitotic defects and overexpression of proteasomal genes in Drosophila melanogaster. J. Cell Sci. **116:**1023–1033.
49. **Taylor, D. M., E. Kabashi, J. N. Agar, S. Minotti, and H. D. Durham.** 2005. Proteasome activity or expression is not altered by activation of the heat shock transcription factor Hsf1 in cultured fibroblasts or myoblasts. Cell Stress Chaperones **10:**230–241.
50. **Venugopal, R., and A. K. Jaiswal.** 1996. Nrf1 and Nrf2 positively and c-Fos and Fra1 negatively regulate the human antioxidant response element-mediated expression of NAD(P)H:quinone oxidoreductase1 gene. Proc. Natl. Acad. Sci. U. S. A. **93:**14960–14965.
51. **Veraksa, A., N. McGinnis, X. Li, J. Mohler, and W. McGinnis.** 2000. Cap 'n' collar B cooperates with a small Maf subunit to specify pharyngeal development and suppress deformed homeotic function in the Drosophila head. Development **127:**4023–4037.
52. **Verma, R., R. Oania, J. Graumann, and R. J. Deshaies.** 2004. Multiubiquitin chain receptors define a layer of substrate selectivity in the ubiquitin-proteasome system. Cell **118:**99–110.
53. **Xie, Y., and A. Varshavsky.** 2001. RPN4 is a ligand, substrate, and transcriptional regulator of the 26S proteasome: a negative feedback circuit. Proc. Natl. Acad. Sci. U. S. A. **98:**3056–3061.
54. **Xu, Z., et al.** 2005. Liver-specific inactivation of the Nrf1 gene in adult mouse leads to nonalcoholic steatohepatitis and hepatic neoplasia. Proc. Natl. Acad. Sci. U. S. A. **102:**4120–4125.
55. **Zhang, D. D., S. C. Lo, J. V. Cross, D. J. Templeton, and M. Hannink.** 2004. Keap1 is a redox-regulated substrate adaptor protein for a Cul3-dependent ubiquitin ligase complex. Mol. Cell. Biol. **24:**10941–10953.
56. **Zhang, Y., D. H. Crouch, M. Yamamoto, and J. D. Hayes.** 2006. Negative regulation of the Nrf1 transcription factor by its N-terminal domain is independent of Keap1: Nrf1, but not Nrf2, is targeted to the endoplasmic reticulum. Biochem. J. **399:**373–385.
57. **Zhang, Y., J. M. Lucocq, M. Yamamoto, and J. D. Hayes.** 2007. The NHB1 (N-terminal homology box 1) sequence in transcription factor Nrf1 is required to anchor it to the endoplasmic reticulum and also to enable its asparagine-glycosylation. Biochem. J. **408:**161–172.