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A Conserved Role for the 20S Proteasome and Nrf2 Transcription Factor in Oxidative-Stress Adaptation in Mammals, C. elegans and D. melanogaster.

Andrew M. Pickering*,†, Trisha A. Staab‡, John Tower†, Derek S. Sieburth‡, and Kelvin. J. A. Davies*†§

*Ethel Percy Andrus Gerontology Center of the Davis School of Gerontology;
†Molecular and Computational Biology Program, Department of Biological Sciences, Dornsife College of Letters, Arts & Sciences;
‡Department of Cell & Neurobiology & Zilkha Neurogenetic Institute of the Keck School of Medicine;
§Senior author to whom correspondence should be addressed as follows:
Prof. Kelvin J. A. Davies, Ethel Percy Andrus Gerontology Center,
The University of Southern California, 3715 McClintock Avenue, Los Angeles, CA 90089-0191, U.S.A.
Telephone: (213)740-8959, Fax number: (213)740-6462, e-mail: kelvin@usc.edu

Short title: Oxidative Stress Adaptation in Worms and Flies

ABBREVIATIONS

H2O2, hydrogen peroxide; Caenorhabditis elegans, C. elegans; Drosophila melanogaster, D. melanogaster or just Drosophila; Nrf2, Nuclear factor (erythroid-derived 2)-like 2; PA28αβ, SKN-1, the C. elegans SKiNhead-1 transcription factor (an ortholog of mammalian Nrf2); CNC-C, D. melanogaster Cap'n'collar transcription factor (an ortholog of mammalian Nrf2); Proteasome Activator 28αβ, ARE, Antioxidant response element; AMC, 7-Amino-4-Methylcoumarin; Suc-LLVY-AMC, the succinylated peptide N-Succinyl-Leucine-Leucine-Valine-Tyrosine-7-Amino-4-Methylcoumarin (used as a peptide substrate to measure proteolytic capacity); SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PAS-7, Proteasome Alpha Subunit 7; PAS-5, Proteasome Alpha Subunit 5; PBS-3, Proteasome Beta Subunit 3; PBS-5, Proteasome Beta Subunit 5; PBS-6, proteasome beta subunit 6; RPN-10, Proteasome Regulatory particle Non-ATPase like 10.
SUMMARY

In mammalian cells H$_2$O$_2$ induced adaptation to oxidative stress is strongly dependent on an Nrf2 mediated increase in 20S proteasome. Here we report that both *Caenorhabditis elegans* nematode worms and *Drosophila melanogaster* fruit-flies are also capable of adapting to oxidative stress with H$_2$O$_2$ pretreatment. As in mammalian cells, this adaptive response in worms and flies involved an increase in proteolytic activity and increased expression of 20S proteasome, but not of 26S proteasome. It was also found that the increase in 20S proteasome expression in both worms and flies, as in mammalian cells, is important for the adaptive response, and that it is mediated by the SKN-1 and CNC-C orthologs of the mammalian Nrf2 transcription factor, respectively. These studies demonstrate that stress mechanisms operative in cell culture also apply in disparate intact organisms across a wide range of biological diversity.

Keywords: 20S Proteasome, Nrf2, *Drosophila melanogaster*, *Caenorhabditis elegans*, Oxidative Stress Adapation, Proteolysis

INTRODUCTION

Cells, small organisms, and animals frequently experience protein damage due to oxidative stress. The levels of oxidative stress to which all are exposed is not static, but varies based on both external factors, such as air pollution (Halliwell et al., 1992; Menzel, 1994), pesticides (Abdollahi et al., 2004), ozone (Cross et al., 1992a; Cross et al., 1992b), exposure to radiation including both UV (Hu and Tappel, 1992) and ionizing radiation (Leach et al., 2001); as well as internal factors such as mitochondrial activity (Kappus, 1987) and immune responses (Baueuerle et al., 1996). Although oxidative stress exposure is variable, the ability of cells and organisms to cope with such stress can also be transiently varied to meet changing needs; this process is called oxidative stress adaptation (Davies, 2000; Pickering et al., 2010; Pickering et al., 2012; Wiese et al., 1995), or conditioning hormesis (Gems and Partridge, 2008; Ristow and Zarse, 2010). In previous studies using cultured mammalian cells, oxidative stress adaptation has typically been assayed using a pretreatment and challenge model; in this model, exposing cells to a mild oxidative stress produces a transient increase in tolerance to a subsequent higher (and normally toxic) stress (Davies, 2000; Pickering et al., 2010; Pickering et al., 2012; Wiese et al., 1995). Such oxidative stress adaptation lasts for no more than 48 hours (if the stimulating oxidant is removed or metabolized) and involves direct enzyme activation,
up-regulated expression of more than 50 protective and transient growth-arrest genes, and down-
regulation of a similar number of housekeeping and proliferative genes.

In mammalian cell culture (murine embryonic fibroblasts), an important part of this adaptive
response is an increase in 20S proteasome. Blocking the induction of 20S proteasome expression
(with siRNA or antisense RNA) significantly limits the effectiveness of the adaptive response
(Pickering et al., 2010). The induction of 20S proteasome is regulated by the stress-responsive
transcription factor Nrf2 (Pickering et al., 2012). In mammalian cells, adaptive increases in the levels
and activity of the 20S proteasome, as well as the Pa28αβ (or 11S) proteasome regulator, and the
immunoproteasome, play a vital role in the proteolytic removal of oxidatively damaged proteins. This
enables cells to cope with oxidative stress, and permits normal cell function to continue (Crawford et
al., 1996; Davies, 1993, 2000; Pickering et al., 2010; Wiese et al., 1995).

Whole organismal adaptation to oxidative stress has been documented in the nematode worm, C.
 elegans. Exposure of the worm to a mild heat shock, or a mild dose of an oxidant (such as brief
exposure to 100% oxygen or the quinone juglone), results in increased tolerance to a future, more
severe, heat or oxidant assault. Interestingly, such adaptation can also result in increased lifespan
(Cypher and Johnson, 2002; Lithgow et al., 1995; Przybysz et al., 2009). Similarly, in the common
fruit fly, D. melanogaster, a mild oxidant pretreatment, or irradiation, increases the tolerance of the
fly to a future, normally toxic oxidant challenge (Moskalov et al., 2009). Experiments were
conducted to test whether transient adaptation to hydrogen peroxide (H2O2) in C. elegans and D.
 melanogaster follows the same precepts that operate in mammalian cells in culture, and whether
adaptation in worms and flies also depends on induction of 20S proteasome, and if 20S proteasome
induction is regulated by (orthologs of) the Nrf2 transcription factor. Although Pa28αβ and
immunoproteasome are important to adaptation in mammalian cells (Pickering et al., 2010), neither is
present in C. elegans or D. melanogaster, and while both organisms have a ortholog of the Pa28γ
regulator, its function remains largely unclear (Rechsteiner and Hill, 2005). For these reasons
attention was focused on the 20S proteasome and Nrf2 in worms and flies.

MATERIALS AND METHODS

C. elegans culture

Strains were maintained at 20°C as described by Brenner et al (Brenner, 1974). A bacterial lawn of
OP50 Escherichia coli was spread on the plates for feeding. The N2 Bristol strain was used as the
control for all experiments. *skn-1(zu67)* was obtained from the Caenorhabditis Genetics Center, University of Minnesota. For experiments investigating SKN-1::GFP localization, the strain LD1 containing the integrant idIs7, generously provided by the Blackwell lab, was crossed into *glo-l(zu391)* to minimize auto-fluorescence. M9 solution (35mM Na₂HPO₄, 22mM KH₂PO₄, 85mM NaCl, 19mM NH₄Cl) was used as a media for washing worms and for treating worms with H₂O₂.

**Preparation of age-matched *C. elegans* cultures**

In all experiments age matched worms cultures were used. It was found that egg preparation using an NaOH/bleach solution, as is commonly used for age synchronization (Stiernagle, 2006), itself produced a stress response. Therefore, eggs layed over a 24h period were used for synchronization.

**C. elegans H₂O₂ adaptation**

2-3 day old (juvenile/young adult) worms were collected and washed with M9 medium; worms were washed 3 times to remove any contamination from the bacterial lawn. Worms were then transferred to Eppendorf tubes containing 1ml of M9 medium, plus 0μM, 0.1μM, 1μM, or 10μM H₂O₂. Worms were incubated in this medium with mild shaking for 1 h then returned to normal plates for 24 h to permit adaptation to occur. Unless otherwise stated 1μM H₂O₂ was used as an adaptive pre-treatment for *C. elegans*.

**C. elegans H₂O₂ challenge**

3-4 day old adult worms were collected using washing with M9 medium; worms were washed 3 times to remove any contamination from the bacterial lawn. Worms were then transferred to Eppendorf tubes containing 1ml of M9 medium plus 100μM – 160mM H₂O₂. Worms were incubated in this medium, with mild shaking, for 1 h then returned to normal plates. Survival was either scored instantly, or 24 h later, by response to prodding with a pick. This experiment was typically performed blind, by another member of the group encoding and randomizing the plates immediately before they were scored. Where pre-treatment challenge assays were performed, pre-treatment was typically performed 24 h prior to challenge and 80mM H₂O₂ was used for the challenge dose unless otherwise stated.

**SKN-1 GFP worm imaging**

Age-matched idIs7;*glo-l(zu391)* worms were prepared as described above. L4 animals were used for imaging experiments since they have lower levels of intestinal auto-fluorescence. Animals were collected 48 h after egg lay and washed 3 times with M9 medium to remove any bacterial contamination. Worms were then transferred to Eppendorf tubes containing 1ml of M9 medium.
±1μM H₂O₂ or ±10mM H₂O₂. Worms were incubated ± H₂O₂ for 20 minutes and then paralyzed by addition of 2,3, Butanedione monoxime. Worms were then mounted on slides containing an agarose pad. SKN-1::GFP nuclear localization was scored by microscopic analysis using a 500X – 1000X magnification with GFP illumination (An and Blackwell, 2003; An et al., 2005).

**Drosophila melanogaster culture**

*Drosophila melanogaster* were cultured on a standard agar/dextrose/corn meal/yeast media (Ren et al., 2009) at 25°C. Unless otherwise stated, w[1118] flies were used in all assays. Flies were collected over a 48 h period from pre-cleared bottles, and allowed 4 days to mature so that at initiation of assays, flies were 4-6 days old.

**Drosophila H₂O₂ challenge assays**

Samples of 20 flies were transferred to vials containing ½ a Kim-wipe© soaked in 1ml of 5% sucrose and 4.4M H₂O₂. Survival was then scored ever 6 or 8 h following initiation of challenge. Flies were scored as dead once they became completely immobile.

**Drosophila H₂O₂ adaptation**

Samples of 20 flies were transferred to vials containing ½ a Kim-wipe© soaked in 1ml of 5% sucrose plus 0μM, 10μM, 100μM or 1mM H₂O₂ for 8 h. Flies were then returned to normal vials for 16 h to permit adaptation to occur. Flies were then challenged with a toxic dose of H₂O₂. In the case of experiments using *cnc-C* RNAi flies we found that, for effective adaptation to occur, flies needed to be incubated on H₂O₂ soaked Kim-wipes© for 24 h instead of 8 h. In addition, because of the longer incubation time in the experiments with *cnc-C* RNAi flies, 10μl of either ethanol or RU486 was added to the Kim-wipes© during this treatment.

**Western blot assays**

**Preparation of C. elegans**: ≈1000-2000, 3-4 day old adult worms were collected per sample and washed with M9 medium. Worms were washed 3 times with M9 medium to remove any bacterial contamination. Worms were then transferred into a solution containing RIPA buffer, (catalog # 89901) from Thermo Fisher (Waltham, MA, USA), supplemented with protease inhibitor cocktail (catalog #11836170001) from Roche (Nutley, NJ, USA). We then lysed the worms by freezing them once, subjecting them to 3x10s bursts of sonicication, followed by 3 cycles of incubation on ice for 5 min, and vortexing. Lysates were centrifuged at 10,000g to remove un-lysed fragments. Protein content was quantified with a BCA protein assay kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions for Western analysis.
Preparation of Drosophila: 20 flies were collected per sample. Flies were then transferred into a solution containing RIPA buffer, (catalog # 89901) from Thermo Fisher (Waltham, MA, USA), supplemented with protease inhibitor cocktail (catalog #11836170001) from Roche (Nutley, NJ, USA). Flies were frozen, then homogenized using an electronic pestle after which lysis was performed by three 5 min incubations on ice, followed by vortexing. Samples were then centrifuged at 10,000g to remove cuticle fragments and unlysed cells. Protein content was next quantified with the BCA Protein Assay Kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions.

Western blot analysis: 40 μg of protein was run on an SDS–PAGE gel and transferred to a PVDF membrane using standard Western blot techniques.

Fluorpeptide proteolytic assays

Preparation of C. elegans: 200 adult worms were collected per sample and washed 3 times with M9 medium to remove bacterial contamination. Worms were then transferred into a solution containing proteolysis buffer (50 mM Tris, 25 mM KCl, 10 mM NaCl, 1 mM MgCl2, 1 mM DTT (pH 7.5). Worms were frozen once, then homogenized, after which cell lysis was achieved by 3 freeze-thaw cycles in dry ice for 5 min, followed by a room temperature water bath for 5 mins. After this samples were centrifuged at 10,000g to remove cuticle fragments and unlysed cells. Protein content was quantified with the BCA Protein Assay Kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Samples were then diluted to the appropriate concentration based on BCA assays results.

Preparation of Drosophila: 10 flies were collected per sample. Flies were then transferred into a solution containing proteolysis buffer (50 mM Tris, 25 mM KCl, 10 mM NaCl, 1 mM MgCl2, 1 mM DTT, at pH 7.5). flies were frozen once, then homogenized using a pestle after which lysis was performed by 3 freeze-thaw cycles performed through incubation in dry ice for 5 mins followed by a room temperature water bath for 5 mins. After this samples were centrifuged at 10,000g to remove cuticle fragments and unlysed cells. Protein content was quantified with the BCA protein assay kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Samples were then diluted to the appropriate concentration based on BCA assays results.

Fluorpeptide proteolytic analysis: 5.0 μg – 1mg aliquots (depending on assay) were transferred, in triplicate, to 96 well plates, and 2 μM of N-succinyl-Leu-Leu-Val-Tyr-AMC (catalog # 80053-860) purchased from VWR (Chester, PA, USA) was added to each well. Plates were incubated at 37°C and mixed at 300 rpm for 4 h. Fluorescence readings were taken at 10 minute intervals using
an excitation wavelength of 355 nm and an emission of 444 nm. Fluorescence units were converted to moles of free AMC, with reference to an AMC standard curve of known amounts of AMC (catalog #164545) purchased from Merck (Whitehouse Station, NJ, USA), following subtraction of background fluorescence. In some experiments, cells were treated with 20 µM of the proteasome inhibitor lactacystin (catalog #80052-806) from VWR (Chester, PA, USA), 30 min prior to incubation and addition of substrates. Lactacystin was dissolved in DMSO at a 100X concentration and combined with samples at a concentration of 0.1%.

**C. elegans RNAi treatment**

2-3 day old (juvenile/young adult) worms were cultured on plates containing a bacterial lawn expressing RNAi against the proteasome subunits *pas-5, pbs-3, pbs-5* and *pbs-6*. In addition, some plates contained bacteria expressing RNAi against *skn-1* or an empty vector control (*L4444*). Worms were incubated on these plates for 6 h, and then collected and washed 3 times with M9 medium to remove any contamination from the bacterial lawn. Worms were then transferred to Epindorf tubes containing 1ml of M9 medium ±1 mM H$_2$O$_2$. Worms were incubated, with mild shaking, for 1 h then returned to RNAi plates for 24 h. Worms were collected and washed 3 times with M9 medium to remove any bacterial contamination. Worms were then transferred to 48 well plates containing 500µl of M9 medium + 8mM H$_2$O$_2$. Worms were incubated, with mild shaking, for 1 h, then survival was scored immediately (as above).

**Drosophila RNAi experiments**

Flies expressing RNAi against two proteasome subunits were purchased from the Vienna *Drosophila* RNAi center (VDRC, Vienna, Austria). These were *prosβ1*$_{RNAi}$ (w[1118]; P[GD13913];v35923), and *prosβ2*$_{RNAi}$ (w[1118]; P[GD10938];v24749). In addition we received the kind donation of flies expressing RNAi against the *cnc-C* and *dkeap-1* from Dr Dirk Bohman (Sykiotis and Bohmann, 2008, 2011). Males from these lines (or w[1118] as a control) were crossed with virgin females containing the Act-GS-255B driver (Fischer et al., 1988; Ford et al., 2007). Parents were removed 4 days after initiation of cross. Progeny were then collected over a 48 h period after eclosion. The exception to this was the *cnc-C*$_{RNAi}$ line where a low birthrate required collections over a 72 h period. The Act-GS-255B driver is activated by feeding flies RU486. Flies were cultured in normal vials containing either 50µl of stock RU486 (20mg/ml) or ethanol which had been added to vials and air dried 24 h prior to the assay. Flies were incubated in these vials for 5-7 days. In experiments involving H$_2$O$_2$ adaptation, flies were removed from ± RU486 vials 24 h before the end of RU486
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1 treatment. Flies were then transferred to vials containing a ½ Kim-wipe© soaked in 1ml of 5%
2 sucrose ± 100uM H₂O₂ for 8 h. The flies were then returned to RU486 or ethanol vials for the
3 remaining 16 h of RU486 treatment.

RESULTS

H₂O₂ induced adaptation to oxidative stress in C. elegans

To determine if C. elegans are capable of H₂O₂ oxidative stress adaptation, a pretreatment/challenge
assay, similar to that developed in mammalian culture was employed (Pickering et al., 2010;
Pickering et al., 2012; Wiese et al., 1995). In this assay age matched young adult worms were
exposed to low adaptive doses of H₂O₂ for 1 hour and 1 hour later were assayed for survival in
response to toxic doses of H₂O₂ (40mM – 160mM) using standard survival assays (See materials and
methods) (Larsen, 1993). Non-pretreated worms exhibited a progressive decline in survival with
increasing H₂O₂ challenge (Fig. 1A). A challenge dose of 80mM produced a 70% loss (only 30%
survival) of non-pretreated worms. This survival was increased to 45-50% with H₂O₂ pretreatment
(Fig. 1B). From the above results it appeared that mild H₂O₂ pre-treatment produces the expected
enhanced resistance to oxidative stress in C. elegans.

The challenge doses used in the above experiment, however, were highly toxic and produced 0%
survival 24 h after pretreatment, even in pretreated samples (Fig. 1A and data not shown). To better
test oxidative stress adaptation, it seemed reasonable to examine longer-term survival with less severe
H₂O₂ challenges. To test this, the assays in Fig. 1A were repeated, using milder H₂O₂ challenge doses
(200μM-3.2mM). A 24 h recovery period was then permitted after H₂O₂ challenge before scoring
survival. Using this protocol, a similar pattern of declining survival with increasing H₂O₂ challenge
was observed (Fig. 1C). It was found that a 1.6mM H₂O₂ pre-treatment would produce a 16%
survival increase upon subsequent challenge, and survival upon challenge was increased to over 30%
with a 10 μM H₂O₂ pretreatment (Fig. 1D).

It is difficult to directly measure the activity of individual proteases in an extract, so instead their
activity is approximated though the capacity of the extract to degrade peptide substrates (“proteolytic
capacity”). It has been previously shown that an increase in proteolytic capacity is an important
component of the adaptive response to oxidative stress in mammalian cell culture. (Grune et al.,
2011; Pickering et al., 2010; Pickering et al., 2012). To determine whether a similar increase in proteolytic capacity occurs in *C. elegans*, the proteolytic activities of whole worm extracts were examined 24 h after pretreating worms with H$_2$O$_2$ (as per Figs. 1B and 1D), by measuring degradation of the short peptide substrate, Suc-LLVY-AMC. H$_2$O$_2$ pretreatment produced a five-fold adaptive increase in proteolytic capacity in extracts from animals that were pretreated with mild H$_2$O$_2$ compared to non-pretreated extracts (Fig. 1E). Interestingly, pretreatment with either 1μM or 10μM H$_2$O$_2$ increased both survival and proteolytic activity, whereas 0.1μM pretreatment did not significantly increase survival or proteolytic activity. This appears to be a non-linear response in which a mild H$_2$O$_2$ (0.1μM) pretreatment yields a weak increase in proteolytic activity but a much stronger increase in oxidative stress tolerance.

**The role of 20S proteasome in H$_2$O$_2$ induced adaptation in *C. elegans***

These results suggest that as an overall phenomenon, oxidative stress adaptation and the corresponding increase in proteolytic activity appear similar in *C. elegans* to that observed in mammalian cells (Grune et al., 2011; Pickering et al., 2010; Wiese et al., 1995). In the mammalian system up-regulation of 20S proteasome has been shown to play an important role in this response through an increase in the capacity of cells to degrade oxidized proteins (Pickering et al., 2010; Pickering et al., 2012). To see if this was also the case in *C. elegans* the level of 20S proteasome protein under H$_2$O$_2$ adaptation was measured. To do this, animals were pretreated with a variety of mild H$_2$O$_2$ doses. Subsequently worm lysates from these pretreated animals were analyzed for the abundance of either the 20S proteasome subunit PAS-7, or the 19S regulator subunit RPN-10 (a key component of the 26S proteasome) using Western Blotting (Figs. 2A and 2B). This H$_2$O$_2$ pretreatment produced a 2-3 fold increase in 20S proteasome (PAS-7) levels with no significant change in the levels of the 19S regulator of 26S proteasome (RPN-10). These results suggest that the 20S but not the 26S proteasome may be involved in oxidative stress adaptation.

Because 20S proteasome was induced by H$_2$O$_2$ treatment it was next determined whether 20S proteasome induction was important for the adaptive response. To test this conditioning hormesis was assayed in animals in which the 20S proteasome was first knocked-down by RNA interference. The effects of knockdown of four 20S subunit genes were tested. These corresponding RNAi clones were available from an RNAi library (Kamath et al., 2003): *pas-5, pbs-3, pbs-5*, or *pbs-6*. In this
experiment worms were cultured with RNAi directed against the 20S proteasome subunits *pas-5*, *pbs-3*, *pbs-5*, or *pbs-6* or the empty vector (*L4440*) for 6 h to block new synthesis of proteasome genes without significantly reducing baselines levels. Worms were then transferred into ± 1μM H₂O₂ for 1 h to induce adaptation. Worms were then returned to their RNAi plates for a further 24 h, and then collected for adaption experiments, performed as in Fig. 1B. RNAi knock-down of the four proteasomal subunits did not significantly alter survival on 80mM H₂O₂ compared to control RNAi treatments, however, RNAi knockdown of any one of these proteasome subunits severely blunted the enhanced survival of animals that were pretreated with adaptive doses of H₂O₂ (Fig. 2C). RNAi knockdown of *pbs-5* appeared to be the most effective, whereas *pas-5*, *pbs-3* and *pbs-6* appeared to have partial effects. This may be due to differences in the efficacy of RNAi in these experiments or this may reflect differences in the functions of these subunits in the proteasome. These results indicate that the 20S proteasome plays an important role in the adaptive increase in oxidative stress tolerance exhibited by H₂O₂ pre-treated *C. elegans*.

**The role of SKN-1 in oxidative stress adaptation in *C. elegans***

Nrf2 has previously been found to be an important regulator of oxidative stress adaptation in mammalian cell culture (Pickering et al., 2012). At least part of the reason for this is the Nrf2-dependent increase in 20S proteasome synthesis during mammalian cell oxidative stress adaptation (Pickering et al., 2012). Studies by the Blackwell lab have demonstrated that SKN-1 is a functional ortholog of Nrf2. Like Nrf2, SKN-1 is normally maintained at low levels in the cytoplasm, but under conditions of oxidative stress it translocates to the nucleus (An and Blackwell, 2003; An et al., 2005; Kahn et al., 2008; Oliveira et al., 2009). It has been shown that high doses of H₂O₂ treatment (10mM) can cause the cytoplasmic to nuclear translocation of SKN-1 fused to GFP (SKN-1::GFP) in *C. elegans* intestinal cells (An and Blackwell, 2003; An et al., 2005). Also a report by Przybysz et al has shown that with depletion of SKN-1, worms become more susceptible to the oxidant juglone (Przybysz et al., 2009). The SKN-1::GFP line (An and Blackwell, 2003), was crossed with *glo-1* mutant strain to produced worms with reduced non-specific fluorescence. Using transgenic animals expressing the SKN-1::GFP reporter, the number of fluorescent SKN-1::GFP intestinal nuclei in H₂O₂ treated animals was compared to untreated controls. First it was confirmed that exposure to 10mM H₂O₂ caused a three-fold increase in the number of SKN-1::GFP fluorescent nuclei (Fig. 3A, B). Treated worms had an average of 10.0 ± 1.4 nuclei exhibiting expression of GFP, compared with
3.7 ± 1.2 in untreated worms (Fig. 3B). It was next tested whether lower adaptive treatment concentrations could also produce an increase in SKN-1::GFP nuclear localization. The experiments of Figs. 3A and 3B were repeated, using an adaptive dose of 1μM of H2O2, and a smaller but still significant increase in the number of SKN-1::GFP fluorescent nuclei was observed relative to untreated controls (Fig. 3C, D). Treated worms had an average of 6.1 ± 1.1 nuclei exhibiting expression of GFP, compared with 2.1 ± 0.6 in untreated worms (Fig. 3B). These results suggest that low adaptive doses of H2O2 that confer enhanced survival may activate SKN-1 by promoting its translocation to nuclei.

To determine whether SKN-1 is important in H2O2 induced oxidative stress adaptation, experiments were performed to test if enhanced survival following pretreatment through H2O2 adaptation is dependent on the presence of SKN-1. Knock-down of SKN-1 by RNAi did not alter survival of non-pre-treated animals compared to control RNAi treated animals upon challenge with 80mM H2O2. However, the adaptive response with 1μM H2O2 pre-treatment was blunted following treatment with RNAi against skn-1 (Fig. 3E). Thus, not only does the adaptive response in C. elegans involve an increase in SKN-1 nuclear localization, but SKN-1 activity is important for oxidative stress adaptation, implicating SKN-1 nuclear translocation as a critical event in oxidative stress adaptation.

However as it was unclear how effective the SKN-1 RNAi was at causing depletion of skn-1 the experiment was repeated using a skn-1(zu67) mutant (Bowerman et al., 1992). The skn-1(zu67) mutant encodes an early stop codon that is predicted to eliminate activity of the two SKN-1 isoforms (SKN-1a and SKN-1c) that are expressed in the intestine and are required for stress responses (An and Blackwell, 2003). Despite pretreating this mutant line with a range of doses of H2O2 no adaptive increase in oxidative stress tolerance was observed upon H2O2 challenge (Fig. 3F). This result is therefore highly supportive of an important role for skn-1 in regulating the adaptive response.

Given the role of mammalian Nrf2 in regulating proteasome function (Pickering et al., 2012), it is possible that SKN-1 may also regulate proteasome activity in response to adaptive doses of H2O2. This idea is consistent with the findings in (Oliveira et al., 2009), that at least half of the proteasome subunit genes in the C. elegans genome contain SKN-1 binding sites and are inducible by SKN-1. Furthermore it has been demonstrated that RNAi depletion of SKN-1 causes a marked reduction in the proteolytic capacity of the worm (Kirkwood, 2005). To determine if SKN-1 regulates the
oxidative-stress induced adaptive increase in 20S proteasome, Western blotting was used to measure the levels of a 20S proteasome subunit, PAS-7, following 1µM H₂O₂ treatment, in wild-type (N2) worms and skn-1(zu67) mutant worms. H₂O₂ treatment of control animals caused a 60% increase in the level of PAS-7 from whole worm lysates (Fig. 3G). In contrast only a 30% increase in PAS-7 levels was observed following H₂O₂ treatment in lysates from the skn-1 mutant animals (Fig. 3G), and PAS-7 is one of the subunits previously shown to be inducible by skn-1 (Oliveira et al., 2009). These results suggest that skn-1 regulates the oxidative stress-induced increase in 20S proteasome in C. elegans, similar to the effect of Nrf2 in mammalian cell cultures,

H₂O₂ induced increase in proteolytic capacity in D. melanogaster

Previous studies have shown that pre-treatment of D. melanogaster with mild doses of oxidants or radiation can result in a subsequent increase in tolerance to oxidative damage (Moskalev et al., 2009). First, it was investigated if H₂O₂ pretreatment in D. melanogaster could be used to induce an increase in proteolytic capacity as has been seen in the other model systems. Because of the greater size and complexity of the fly compared to mammalian cell cultures, and even nematode worms, it was not possible to simply incubate flies in H₂O₂. Instead, flies were maintained in vials without food, and containing a Kim-wipe© soaked in 5% sucrose ± an adaptive dose of H₂O₂ (Grover et al., 2009). For these experiments, flies were collected 0-48 h after eclosion. Flies were then allowed to mature for 4 days, after which they were transferred into vials containing Kim-wipes© soaked in 5% sucrose ± 10-1000 µM H₂O₂ for 8 h. It was confirmed that the sucrose/H₂O₂ solution was ingested by the flies over the 8 h incubation period by uptake of a visible dye (10% blue food color from Amerifoods, Los Angeles, CA, USA) (Fig. S1). The flies were returned to normal vials for 16 h to permit adaptation, and then homogenized. The proteolytic capacity of the fly lysates was then measured by degradation of the fluorogenic peptide model substrate, Suc-LLVY-AMC. As shown in Fig. 4A, a progressive increase in proteolytic capacity was observed in females following H₂O₂ pretreatment. In contrast, no increase in proteolytic capacity was observed when male flies were pretreated with H₂O₂: instead, 100 and 1,000 µM H₂O₂ pretreatments were found to decrease the proteolytic capacity of lysates from male flies (Fig. 4A).

This difference in adaptive response between male and female flies might be due to different food consumption rates. Female Drosophila consume approximately three times as much food as their
male counterparts, have a much larger gut, and have more stem cells present in their digestive tract (Wong et al., 2009). It was confirmed that during the 8 h H$_2$O$_2$ pre-treatment male flies consumed considerably less of the sucrose/H$_2$O$_2$ solution than did female flies, and this might mean that male flies receive less of an exposure to the adaptive dose of H$_2$O$_2$ and so are less responsive to H$_2$O$_2$ pretreatment (Fig. S1). Although it is clear from Fig. S1 that male flies consumed less than half as much of the sucrose/H$_2$O$_2$ solution as did females, we can think of no reason why this difference in ingestion would negatively affect proteolytic capacity. An alternative hypothesis is that the observed differences are due to sexual dimorphism in stress responses between male and female flies, and consistent with this idea, sexual dimorphism has previously been observed in for thermotolerance and oxidative stress resistance in Drosophila (Sorensen et al., 2007; Waskar et al., 2009; Weber et al., 2012).

To determine if the increased proteolytic capacity of female flies (Fig. 4A) might be due to increased proteasome activity, selected samples were pre-exposed to the proteasome-selective inhibitor lactacystin 30 minutes before proteolytic capacity was measured (Pickering et al., 2010). No increase in proteolytic capacity was evident in lactacystin-treated lysates, indicating that increased proteasome activity underlies the elevated proteolytic capacity (Fig. 4B).

**H$_2$O$_2$ induced adaptation to oxidative stress in D. melanogaster**

Because H$_2$O$_2$ treatment increased proteolytic capacity in female fly extracts, but not male fly extracts, it was next tested whether stress-resistance and proteasome levels would correlate with tolerance to oxidative stress in an adaptation experiment. The first step was to develop an appropriately toxic challenge treatment for the flies. Instead of subjecting flies to a single challenge as in mammalian cell culture and *C. elegans*, the assay employed for *D. melanogaster* involved incubating the flies in vials containing the toxic dose of H$_2$O$_2$ and scoring survival over time, as previously described (Grover et al., 2009; Sykiotis and Bohmann, 2008, 2011). Survival was scored every 8 h, for up to a 104 h total period. The survival curves for male and female flies cultured in vials containing Kim-wipes© with 0, 1, 2, 4, or 8 M H$_2$O$_2$ are shown in Supplemental Fig. S2.

Having determined an optimal H$_2$O$_2$ challenge concentration of 4.4M (15%) (Fig. S2), adaptation experiments were performed using flies prepared and pre-treated as in Fig. 4. Then 24 h after
initiating pretreatment (16 h after terminating pretreatment), flies were transferred to vials containing Kim-wipes® soaked in 5% sucrose and a toxic challenge dose of 4.4 M (15%) H₂O₂. Survival was scored every 8 h, for up to 80 h. Non-pretreated female flies survived an average of 48 h following initiation of challenge. By comparison female flies pretreated with 100μM H₂O₂ (which was found to be the optimum pretreatment) survived an average of 64 h. This represented a significant increase in survival time (Figs. 5A and 5B). In contrast, no change in survival time was seen following pretreatment of male flies; both pretreated and non-pretreated male flies survived an average of 40 h following initiation of challenge (Figs 5A and 5B), thereby supporting a functional correlation between proteolytic activity and adaptation.

**Role of 20S proteasome in oxidative stress adaptation in D.melanogaster**

Next it was tested whether 20S proteasome expression is induced by H₂O₂ treatment in female flies. To do this female flies were pretreated for 8 h with 100μM H₂O₂, which appeared to be the optimum treatment regime. Then, 24 h after the start of pretreatment, female flies were homogenized and the lysates were analyzed by Western blotting. The Western blots were incubated with antibodies directed against α subunits of the 20S proteasome, the CG13349 subunit of the 19S regulator of the 26S proteasome, and β-tubulin as a loading control. As presented in Fig. 5C, a 50-60% increase in the expression 20S proteasome α subunits was observed (reflecting increased overall 20S proteasome levels) but no change in the 19S regulator (indicating no increase in 26S proteasome). Having seen an increase in female fly 20S proteasome as a product of oxidative stress adaptation, it was next asked if this induction was required for adaptation to occur. To test this two RNAi lines were used that are directed against the prosβ1 and prosβ2 20S proteasome subunits). These lines were crossed to the Act-GS-255B strain (Ford et al., 2007) enabling the RNAi to be conditionally expressed in the presence of the drug RU486 in all the somatic tissues of the adult fly (Shen et al., 2009). Additionally, the Act-GS-255B strain was crossed with w[1118] flies as controls for potential effects of RU486. The female progeny of these three crosses were then cultured ± RU486 for 7 days. RU486 activates the Gene-Switch transcription factor, thereby inducing expression of the appropriate RNAi. One day prior to the end of the 7 day incubation, the female flies were transferred to vials containing Kim-wipes® soaked in 5% sucrose ± 100μM of H₂O₂ for 8 h, and then returned to vials containing ± RU486 for the remaining 16 h. At the start of the assay the flies were transferred to vials containing 5% sucrose and a toxic dose of 4.4M H₂O₂. Fly survival was then scored every 8 h for the next 96 h.
As a minor confounding issue it was observed that RU486 induced a small increase in tolerance to H$_2$O$_2$ challenge. This was controlled for by including ± RU486 controls in all experiments. In w[1118] x Act-GS-255B flies an H$_2$O$_2$ pretreatment-induced increase in tolerance to oxidative stress was observed, both with and without RU486 treatment (Figs 6A and 6B). The experiment was then repeated using the progeny from the two RNAi lines crossed with Act-GS-255B. In the prosβ2 RNAi flies it was found that non-RU486 treated flies adapted to an extent similar to that seen for control female flies following H$_2$O$_2$ pretreatment. In contrast, H$_2$O$_2$ pretreatment in female flies which were also treated with RU486 failed to increase tolerance to oxidative stress. In fact, H$_2$O$_2$ pretreatment actually reduced oxidative stress tolerance in RU486 treated females (Fig.s 6C and 6D). Similarly for the prosβ1 RNAi flies, non-RU486 treated flies adapted to H$_2$O$_2$ pretreatment to a similar extent to control flies. When flies were treated with both RU486 and H$_2$O$_2$, however, not only did they lose the ability to adapt, but tolerance to oxidative stress was severely reduced (Fig.s 6E and 6F).

**Role of CNC-C in oxidative stress adaptation in D.melanogaster**

In both mammalian cells (Pickering et al., 2012), and *C. elegans* (Fig. 3) it was observed that Nrf2/SKN-1 is important for oxidative stress adaptation, and that it regulates the H$_2$O$_2$ induced increase in 20S proteasome. We wished to test if this was also the case in *D. melanogaster*. The *D. melanogaster* orthologue of Nrf2/SKN-1 is CNC-C, which has also been shown to be an important regulator of oxidative stress tolerance (Sykiotis and Bohmann, 2008, 2011). First, to test if CNC-C is involved in adaptation, a line expressing RNAi against *cnc-C* (Sykiotis and Bohmann, 2008, 2011) was crossed, with Act-GS-255B, and adaption experiments were performed on the progeny as with the proteasome RNAi lines. As before, an increase in oxidative stress tolerance was observed with H$_2$O$_2$ treatment in non-RU486 treated flies (Fig. 7A), while no adaptive increase in oxidative stress tolerance was observed in RU486 treated flies (Fig. 7B). In fact, H$_2$O$_2$ treatment caused a reduction in oxidative stress tolerance (Fig. 7A-C) in RU486 treated female flies.

DKEAP-1 is a repressor of CNC-C and has a similar function to that of Keap-1, which functions as a repressor of Nrf2 in mammalian cells (Sykiotis and Bohmann, 2008). As before, the *dkeap-1* RNAi line was crossed with Act-GS-255B and the progeny were then treated with ± RU486 for 5 days. The flies were subsequently challenged with a toxic dose of H$_2$O$_2$ and survival was measured as before. It was found that knock-down of *dkeap-1* caused a significant increase in resistance to oxidative stress
(Fig. 7D-G). We were next interested to determine whether the induction of 20S proteasome is dependent on CNC-C/Nrf2. To test this cnc-CRNAi x ACT-GS-255B flies were prepared and pretreated as in Fig. 7A. The flies were then lysed and run on Western blots, which were incubated with antibodies directed against α subunits of the 20S proteasome, or β-tubulin as a loading control. It was observed that H2O2 treatment produced an increase in the expression 20S proteasome subunits that was suppressed in flies treated with RU486. These results are supportive of a role for cnc-C in regulating the H2O2 induced increase in 20S proteasome (Fig. 7H and 7I).

DISCUSSION

In mammalian cells, it has been shown that H2O2 induced adaptation to oxidative stress occurs through a pathway that is strongly dependent on an Nrf2 mediated increase in 20S proteasome (Pickering et al., 2010; Pickering et al., 2012). The model organisms Caenorhabditis elegans and Drosophila melanogaster have also been shown to be capable of adaptation to oxidative stresses through mild exposure to heat-shock, 100% oxygen or the redox quinone juglone in worms and heat-shock or irradiation in flies (Cypser and Johnson, 2002; Lithgow et al., 1995; Moskalev et al., 2009; Przybysz et al., 2009). The present results demonstrate that both model organisms are capable of such adaptation with H2O2 pretreatment. As in mammalian cells (Pickering et al., 2010), it was found that this adaptive response involves an increase in proteolytic activity and increased expression of 20S proteasome, but not of 26S proteasome. Moreover, it was found that the increase in 20S proteasome expression in both worms and flies, as in mammalian cells, is important for the adaptive response, and that this increase is mediated by the SKN-1 and CNC-C orthologs of the mammalian Nrf2 transcription factor respectively. These studies indicate conservation of the adaptive response to oxidative stress across a wide range of biological diversity, and demonstrate that stress mechanisms operative in cell culture also apply in disparate intact organisms.

Stress-adaption, or conditioning hormesis, has been widely reported in C. elegans, but it is typically studied in terms of changes in lifespan, with only a few examples of changes in oxidative stress tolerance (Cypser and Johnson, 2002; Lithgow et al., 1995). It was shown in previous reports that exposure to high (10mM) concentrations of H2O2 induces SKN-1 and causes its translocation to the nucleus (An and Blackwell, 2003; An et al., 2005; Oliveira et al., 2009). It appears that much lower (1μM) adaptive concentrations of H2O2 also induce SKN-1 and cause its translocation to the nuclei in
cells throughout the intestines of *C. elegans*. In addition, using *skn-1* RNAi and *skn-1* mutants it was shown that this induction and translocation is important for the adaptive increases in proteasome expression and oxidative stress resistance.

Oxidant or radiation-induced adaptation to oxidative stress in *Drosophila melanogaster* has been previously reported (Moskalev et al., 2009). Here it was confirmed that upon H$_2$O$_2$ pretreatment flies become more resistant to oxidative stress and that their cellular proteolytic capacity increases. This increase in proteolytic capacity was shown to be largely dependent on the 20S proteasome, which is consistent with mammalian cell culture studies (Pickering et al., 2010). Finally, these data indicate that CNC-C, the *Drosophila* orthologue of mammalian Nrf2 and *C. elegans* SKN-1, mediates adaptive increases in stress-resistance, at least in part, through induction of 20S proteasome expression.

An important aspect of this study is the use of short-term RNAi treatments as a means of blocking new protein synthesis without significantly altering the baseline expression level of the target protein. This was achieved in *C. elegans* by feeding worms RNAi for 24 h and in flies by feeding RU486 for 5-7 days in adulthood. One concern in the use of RNAi to deplete a protein from an animal is that this approach may cause additional unwanted physiological changes such as either inhibiting normal cell function or causing a stress response. Here such unwanted physiological changes were reduced or avoided by employing short-term RNAi treatments.

One long-term goal for the present work was to develop viable animal models for aging, consisting of short-lived organisms that recapitulate major stress-adaptive mechanisms that operate in mammals. The present data demonstrate that *C. elegans* and *D. melanogaster*, both of which have long been among the most important organismal aging models, share with mammalian cells a capacity for oxidative stress adaptation that relies on increased 20S proteasome synthesis, mediated by well-conserved (Nrf2/SKN-1/CNC-C) signal transduction pathways.

**FUNDING**

This research was supported by grant #RO1-ES003598, and by American Recovery and Reinvestment Act (ARRA) Supplement 3RO1-ES 003598-22S2, both from the NIH/NIEHS to
KJAD; as well as a grant from the Department of Health and Human Services to JT (AG011833) and a grant from the NIH/NINDS to DSS (NS071085-02).

ACKNOWLEDGEMENTS

We thank Dr Keith Blackwell, from Harvard Medical School, Boston, Massachusetts, Dr Dirk Bohmann from the University of Rochester Medical Center, Rochester and Dr Bruce Bowerman from the Fred Hutchinson Cancer Research Center Seattle, Washington for their kind donation of C. elegans stains and Drosophila lines used in this paper.

REFERENCES


FIGURE CAPTIONS

Fig. 1: (A) H₂O₂ challenge causes a decline in worm survival 1 h after challenge. Triplicate samples of 35 - 100 (typically ≈70) age matched, adult, N2 worms were challenged with the indicated concentrations of H₂O₂ for 1 h. Survival was scored immediately after challenge. (B) H₂O₂ pretreatment reduces H₂O₂ challenge induced mortality in the first hour after challenge. Worms were pretreated with the indicated concentrations of H₂O₂ then, 24 h later, challenged with 80mM H₂O₂. Survival was scored immediately after challenge (C) H₂O₂ challenge causes a decline in worm survival 24 h after challenge. Triplicate samples of 40 - 195 (typically ≈100) age matched, adult, N2 worms were prepared and pretreated as in A, using the indicated concentrations of H₂O₂, survival was scored 24h after challenge. (D) H₂O₂ pretreatment reduces H₂O₂ challenge induced mortality in the 24h after challenge. Worms were pretreated with the indicated concentrations of H₂O₂ then challenged, 24h later, with 1.6mM H₂O₂. Survival was scored 24 h after challenge. (E) H₂O₂ pretreatment increases proteolytic capacity. Worms were treated with the indicated concentrations of
H₂O₂ and lysed 24 h after H₂O₂ treatment. Proteolytic activity assays were performed on these samples as described in materials & methods. Values are shown as nM of AMC released per min per mg of lysate. In all cases values are plotted as means ± s.e.m, where n= 3. Values marked with an * indicate statistically significant differences at p ≤ 0.05 using Student’s t-test.

Fig. 2: (A) H₂O₂ pretreatment causes an adaptive increase in expression of 20S proteasome but not 26S proteasome. Triplicate samples of N2 worms were pretreated with the indicated concentrations of H₂O₂. Worms were then lysed 24h later and the lysate was analyzed by Western blot analysis. (B) Samples were prepared as in A, in triplicate. Values are plotted as means normalized to tubulin ± s.e.m where n = 3. (C) Blocking the H₂O₂ induced adaptive increase in 20S proteasome blunts the pretreatment induced increase in oxidative stress tolerance. Worms were cultured on the indicated RNAi plates for 6 h, then pretreated with 1μM H₂O₂ for 1 h, after which worms were returned to RNAi plates for 24 h. Worms were then H₂O₂ challenged and survival was scored. All values are means ± s.e.m, n = 3. Values marked with an * indicate statistically significant differences at p ≤ 0.05 using Student’s t-test.

Fig. 3: (A) Pretreatment with 10mM H₂O₂ causes an adaptive increase in SKN-1 nuclear localization. Representative images of L4 stage worms expressing a SKN-1::GFP transgene (idIS7) in intestinal nuclei following treatment with10mM H₂O₂. (B) Quantification of the number of fluorescent nuclei observed per worm following 10mM H₂O₂ treatment (n= 20-30 worms). (C) Treatment with 1μM H₂O₂ also causes an adaptive increase in SKN-1::GFP nuclear fluorescence. Worms were prepared as in A, except worms were treated with 1μM H₂O₂. (D) Quantification of the number of fluorescent nuclei observed per worm following 1μM H₂O₂ treatment (n= 50-60 worms). (E) RNAi knock-down of SKN-1 blunts the pre-treatment-induced adaptive increase in oxidative stress tolerance. Percent survival of either control or skn-1 RNAi treated worms was measured following H₂O₂ pretreatment and challenge as described in materials & methods. (F) skn-1(zu67) mutants do not appear to have an H₂O₂ pretreatment induced adaptive increase in oxidative stress tolerance. Adult skn-1 mutant worms were pretreated and subsequently challenged with H₂O₂ as described in materials & methods (G) H₂O₂ pretreatment causes a skn-1 dependent adaptive increase in 20S subunit protein abundance which is blunted in skn-1(zu67) mutants. Shown are representative Western blots and quantification of PAS-7 protein abundance in wild-type or skn-1 mutants 24 hours following pretreatment with ±
1μM H₂O₂. Values are plotted as means normalized to tubulin ± s.e.m where n = 3. Values marked
with * indicate statistically significant differences (p ≤ 0.05) compared to controls using Students t-

Fig. 4: (A) H₂O₂ treatment of D. melanogaster increases proteolytic capacity in females but not in
males. Triplicate vials of w[1118] flies were pre-treated with the indicated concentrations of H₂O₂.
proteolytic activity assays were performed on flies 24 h later as described in materials & methods.
Values are shown as nmol of AMC released per min per mg lysate. (B) The H₂O₂ induced adaptive
increase in female fly proteolytic capacity is proteasome dependent. The female samples prepared in
A, that were pretreated with ± 100μM H₂O₂ were also treated with ± lactasystin, 30 minutes prior to
addition of Suc-LLVY-AMC. Values are shown as nmol of AMC released per min per mg lysate. In
all cases values are plotted as means ± s.e.m. where n = 3. Values marked with an * indicate
statistically significant differences at p ≤ 0.05 using Students t-test.

Fig. 5: (A) H₂O₂ pretreatment enhances resistance to oxidative stress challenge. Triplicate vials of
w[1118] flies were pretreated with the indicated concentrations of H₂O₂ then challenged with 4.4M
H₂O₂, 24h later. Percent survival was scored every 8 h based on complete loss of motion. (B) Median
survival time of A where n = 60 flies. (C) H₂O₂ pretreatment induces increased expression of 20S
proteasome, but not of 26S proteasome. Triplicate vials of 20, w[1118] flies were pre-treated with ±
100μM of H₂O₂ then Western blots were run with samples 24 h later. Representative blots are
included as an inset. In all cases values are plotted as means ± s.e.m. where n = 3, values marked with
an * indicate statistically significant differences at p ≤ 0.05 using a log-rank test in B and Students t-
test in C.

Fig. 6: Knock-down of proteasome subunits prosβ1 or prosβ2 blocks the adaptive increase in
oxidative stress tolerance of female progeny from: (A,B) (♂) w[1118] x (♀) Act-GS-255B (C,D)
(♂)Prosβ2RNAi x (♀) Act-GS-255B and (E,F) (♂) Prosβ1RNAi x (♀) Act-GS-255B. In all cases flies
were cultured in ± RU486 for 6 days then pre-treated with ± 100μM H₂O₂ (±RU486) for 24 h. After
pre-treatment Flies were returned to ± RU486 vials for 16 h then challenged with 4.4M H₂O₂. Values
are plotted as means ± s.e.m. where n = 3. (A,C,E) Show % survival. Values are plotted as means ±
s.e.m. were n = 3. (B,D,F) Shows Median survival time where n = 60. Values marked with an * indicate statistically significant differences at p < 0.05 using a log-rank test.

Fig. 7: (A, B) Knock-down of cnc-C blocks the adaptive increase in female fly oxidative stress tolerance. The female progeny of (♂) cnc-C\textsuperscript{RNAi} x (♀) Act-GS-255B, were cultured in ± RU486 for 3 days. After this flies were then pre-treated with ± 100μM H\textsubscript{2}O\textsubscript{2} (±RU486) for 24 h. Flies were then allowed to adapt in ± RU486 for 24 h then challenged with 4.4M H\textsubscript{2}O\textsubscript{2}. Values are plotted as means ± s.e.m. where n = 3. (C) Shows Median survival time of A and B where n = 60. (D) Knock-down of dkeap-1 increases oxidative stress tolerance. The female progeny of (♂) Dkeap1\textsuperscript{RNAi} x (♀) Act-GS-255B were cultured in ± RU486 for 5 days in. Flies were then challenged with 4.4M H\textsubscript{2}O\textsubscript{2}. Values are plotted as means ± s.e.m. where n = 3. (E) Shows Median survival time of C where n = 60. (F) RU486 treatment alone does not causes the increase in oxidative stress tolerance observed in D. The female progeny of (♂) w[1118] x (♀) Act-GS-255B were cultured in ± RU486 for 5 days. Flies were then challenged with 4.4M H\textsubscript{2}O\textsubscript{2}. Values are plotted as means ± s.e.m. where n = 3. (G) Median survival time of F where n = 60. (H) RNAi knock-down of cnc-C blocks the H\textsubscript{2}O\textsubscript{2} induced increase in 20S proteasome. The female progeny of (♂) cnc-C\textsuperscript{RNAi} x (♀) Act-GS-255B, were cultured in ± RU486 for 3 days. After this flies were pre-treated with ± 100μM H\textsubscript{2}O\textsubscript{2} (±RU486) for 24 h. Flies were then allowed to adapt in ± RU486 for 24 h analyzed by Western blot analysis. (I) The gels run in H were run in triplicate and plotted as band density plots normalized to β-tubulin. Values are means ± s.e.m. (n = 3), shown as a percent of - RU486 controls. Values marked with an * indicate statistically significant differences at p ≤ 0.05 using a log-rank test.
Fig. 1
Fig. 2
Fig. 4
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Fig. 5

A

B

C

% survival following H₂O₂ challenge

Median survival time following challenge (h)

Relative protein content (adjusted by β-tubulin)

H₂O₂ pretreatment (µM)

H₂O₂ pretreatment

Control H₂O₂

β-tubulin

CG13349

19S Regulator (CG13349)

20S Proteasome (α subunits)

0 µM

100 µM

0

10

100

1000

0

20

40

60

80

100

0

20

40

60

80

100

0 24 48 72

0 10 100 1000

0 10 100 1000
Fig. 6
Fig. 7