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## Keap1/Nrf2 signaling regulates oxidative stress tolerance and lifespan in *Drosophila*

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### SUMMARY

Keap1/Nrf2 signaling defends organisms against the detrimental effects of oxidative stress, and has been suggested to abate its consequences, including aging-associated diseases like neurodegeneration, chronic inflammation, and cancer. Nrf2 is a prominent target for drug discovery, and Nrf2-activating agents are in clinical trials for cancer chemoprevention. However, aberrant activation of Nrf2 by *keap1* somatic mutations may contribute to carcinogenesis and promote resistance to chemotherapy. To evaluate potential functions of Keap1 and Nrf2 for organismal homeostasis, we characterized the pathway in *Drosophila*. We demonstrate that Keap1/Nrf2 signaling in the fruitfly is activated by oxidants, induces antioxidant and detoxification responses, and confers increased tolerance to oxidative stress. Importantly, *keap1* loss-of-function mutations extend the lifespan of *Drosophila* males, supporting a role for Nrf2 signaling in the regulation of longevity. Interestingly, cancer chemopreventive drugs potently stimulate *Drosophila* Nrf2 activity, suggesting the fruitfly as an experimental system to identify and characterize such agents.

### INTRODUCTION

Nrf2 (NF-E2-Related Factor 2) is a transcription factor of the leucine zipper family, and Keap1 (Kelch-like ECH-Associated Protein 1) is its specific repressor. Keap1/Nrf2 signaling mediates cellular responses to oxidative stresses and electrophilic xenobiotics (Motohashi and Yamamoto, 2004). Studies in *keap1* and *nrf2* knockout mice have demonstrated the crucial role of the Keap1/Nrf2 module as a multi-organ protector *in vivo*. Nrf2 signaling defends the organism against the sequelae of oxidative stress, including aging-related diseases like neurodegeneration, chronic inflammation, and cancer (Lee et al., 2005; Motohashi and Yamamoto, 2004). Thus, Nrf2 is a prominent target in the discovery of preventive and therapeutic modalities for such diseases. Importantly, compounds that activate Nrf2 have been shown to prevent cancer in animal models, and they are currently evaluated in human clinical trials for cancer chemoprevention (the prevention of cancer in high risk individuals through the use of non-toxic natural or synthetic agents) (Sporn and Liby, 2005; Yu and Kensler, 2005; Zhang et al., 1997). On the other hand, the recent discovery of *keap1* somatic mutations in cancer cell lines and human cancer samples suggests that the aberrant activation of Nrf2 signaling may also contribute to carcinogenesis and promote resistance to chemotherapy

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(Padmanabhan et al., 2006; Singh et al., 2006). Thus, a better understanding of the Keap1/Nrf2 pathway and of its roles in health and disease is urgently needed.

In unstressed conditions Nrf2 is tethered to its cytoplasmic inhibitor Keap1, an actin-binding protein (Figure 1A). Keap1 suppresses the activity of Nrf2 by sequestering it in the cytoplasm, and also by targeting it for proteasomal degradation. In addition to serving as an inhibitor, Keap1 can function as a sensor of oxidants and electrophiles, which react with its redox-sensitive cysteine residues (Zhang, 2006). Oxidative stresses or electrophilic xenobiotics abolish the inhibition of Nrf2 by Keap1 (Itoh et al., 2004). Nrf2 is then stabilized and accumulates in the nucleus, where it binds to the Antioxidant Response Element (ARE) in the enhancers of its target genes (Jaiswal, 2004). Experiments in vertebrate systems indicate that Nrf2 activates transcription as a dimer with a small Maf (Musculo-Aponeurotic Fibrosarcoma) protein (Itoh et al., 1997). Binding of the small Maf/Nrf2 dimer to ARE sequences results in the coordinated transcriptional up-regulation of a battery of antioxidant enzymes and detoxifying proteins. This regulated adaptive response has been elegantly termed “the electrophile counter-attack” (Prester et al., 1993); it includes thioredoxins and glutathione-synthesizing enzymes (which maintain the redox balance), glutathione S-transferases (which detoxify xenobiotics), molecular chaperones, and proteasome subunits (which remove damaged macromolecules). In addition, the basal Nrf2 activity that is present under non-stressed conditions maintains the housekeeping expression of the same antioxidant and detoxification genes (Lee et al., 2005; Motohashi and Yamamoto, 2004). Thus, Keap1/Nrf2 signaling regulates the basal and inducible activity of a cell defense network.

The oxidative stress theory of aging predicts that bolstering the organism's antioxidant defenses may retard the aging process and extend lifespan (Harman, 1956). This hypothesis has received considerable support in recent years, mainly by studies in model organisms showing that the over-expression of antioxidant genes can augment oxidative stress tolerance and promote longevity (Dugan and Quick, 2005; Orr et al., 2005). By extension, signaling pathways that regulate antioxidant responses are plausible regulators of the aging process (Wang et al., 2003). On the other hand, some of the model organism studies showing lifespan extension by “longevity genes” have been performed using short-lived control strains; this suggests that the observed lifespan extensions may represent a rescue of pathology rather than a genuine effect on lifespan (Sohal et al., 2002). Thus, the oxidative stress theory of aging warrants further experimental tests in model organisms, by using healthy, long-lived control strains, and by exploring the roles of additional antioxidant response pathways.

Model organisms could be employed to test the hypothesis that Nrf2 signaling may regulate lifespan. Keap1, Nrf2, and small Maf homologues are well conserved in *Danio rerio*, and studies in zebrafish have provided important insights into Keap1/Nrf2 signaling (Kobayashi et al., 2002; Takagi et al., 2004); however, zebrafish are not yet broadly developed as a platform for aging studies. *C. elegans*, a well-established model for lifespan experiments, possesses an Nrf2-related protein (SKN-1) which confers resistance against certain pro-oxidant xenobiotics. However, the biochemical mechanisms underlying its regulation and function are somewhat different from those of Nrf2 in vertebrates: most notably, the worm does not possess Keap1 and small Maf homologues. SKN-1 is apparently regulated mainly by phosphorylation, and binds DNA as a monomer (An and Blackwell, 2003; Blackwell et al., 1994; Inoue et al., 2005). Recently, this distant relative of mammalian Nrf2 was found to be required for lifespan extension by caloric restriction in the worm (Bishop and Guarente, 2007). Whether increasing SKN-1 activity extends lifespan in non-restricted conditions was not addressed; also, *C. elegans* cannot be used to test the role of Keap1 in longevity, because it lacks a *keap1* homologue.

Although *Drosophila melanogaster* is a well-established model for aging research, Keap1/Nrf2 signaling has to our knowledge not yet been characterized in this organism. This is surprising, given the fact that Nrf2 belongs to the *cap'n'collar* (*cnc*) subfamily of leucine zippers, named after the *cnc* gene of *Drosophila*, and that Keap1 is a member of the Kelch family of actin-binding proteins, also named after the fruitfly's Kelch protein (a component of the egg chambers). We decided to employ *Drosophila* as a model system to investigate the role of Keap1/Nrf2 signaling in organismal responses to oxidative stress and the regulation of lifespan. We demonstrate that the fruitfly possesses functional homologues of Keap1 and Nrf2. Our data show that the *Drosophila* Keap1 and Nrf2 proteins comprise a cell protective module that responds to oxidants and cancer chemopreventive agents, induces antioxidant and detoxification responses, confers increased tolerance to oxidative stress, and regulates longevity.

## RESULTS

### Nrf2 and Keap1 homologues are conserved in *Drosophila*

Three RNA isoforms are transcribed off the *Drosophila cnc* locus, designated *cncA*, *cncB*, and *cncC* (McGinnis et al., 1998). These isoforms encode three different proteins, which share their C-terminal regions and thus comprise the same DNA-binding domain. However, the three Cnc translation products differ at their N-termini: CncC encompasses CncB, which in turn encompasses CncA (Figure 1B). The CncB isoform is required together with a small Maf subunit for the development of embryonic head structures (Veraksa et al., 2000). The unique N-terminus of CncC predicts a different role for this isoform: it shows similarity to the Neh2 domain of Nrf2, which contains the Keap1-binding ETGE motif and an upstream hydrophobic region (Supplemental Figure 1A). Based on this distinctive homology, it has been suggested that CncC might be the *Drosophila* counterpart of Nrf2 (Kobayashi et al., 2002). As a first step to addressing this idea and the potential role of CncC in antioxidant responses, we examined its expression in *Drosophila*. In larvae, *cncC* mRNA is most abundantly expressed in the alimentary canal, and shows a strong, distinctive staining pattern (Supplemental Figure 2A-D). This is reminiscent of the broad expression of mammalian Nrf2 in the digestive tract (Chan et al., 1996; Wakabayashi et al., 2003), which, like the skin and airways, is a major frontier where the organism comes into direct contact with its environment. *cncC* mRNA expression was also detected in the Malpighian tubules (which are detoxification organs) and in the salivary glands (Supplemental Figure 2C, E); but not in imaginal discs or in the fat body. *cncC* mRNA is also present in adult female and male flies as determined by RT-PCR (not shown), which is consistent with a role in the homeostasis of the mature organism.

The *Drosophila* genome also harbors a likely homologue of vertebrate *keap1* genes. This gene, *CG3962*, and its product have not yet been functionally characterized. Multiple sequence alignments show that the predicted *Drosophila* Keap1 (dKeap1) protein has striking similarity with its mammalian and zebrafish homologues (Supplemental Figure 1B). Similar to *cncC*, *dkeap1* mRNA is expressed in the alimentary canal and in the Malpighian tubules of third instar larvae and adult flies, and it shows a similar distribution as *cncC* but with a weaker, more diffuse staining pattern. *keap1* mRNA is also detected in the salivary glands, the brain, and the ring gland, but not in imaginal discs or in the fat body (Supplemental Figure 2F-I). These findings demonstrate that the candidate *Drosophila* homologues of Nrf2 and Keap1 are expressed in the fly's digestive tract, which represents the first line of defense to ingested environmental stressors, and in the Malpighian tubules, which are major sites of detoxification. Expression of *keap1* and *cncC* in the adult gut and Malpighian tubules was verified by RT-PCR on dissected adult tissues (not shown).

Consistent with the conservation of the Keap1-binding motif in CncC (Supplemental Figure 1A), the physical association between *Drosophila* Keap1 and CncC has previously been

suggested by a genome-wide yeast two-hybrid experiment (Giot et al., 2003). This interaction predicts that Keap1 should act as a negative regulator of CncC *in vivo*. To test this hypothesis, we examined whether these proteins interact genetically when over-expressed in the *Drosophila* eye (Figure 1C). The compound eye of *Drosophila* comprises about 800 ommatidia or facets arranged in a regular hexagonal pattern. Genetic manipulation of cell signaling during eye development often causes distinctive, but generally non-lethal phenotypes, making the eye a convenient system for the analysis of regulatory interaction between different genes. Over-expression of Keap1 in the eye using the eye-specific *sepGal4* driver does not distort the wild-type pattern (Figure 1C). In contrast, over-expression of CncC with the same driver disrupts the ommatidial arrangement, resulting in a rough eye appearance. Co-expression of Keap1 completely suppresses this CncC gain-of-function (g-o-f) phenotype, restoring the eye to a wild-type morphology. Although the basis of the CncC over-expression phenotype is not yet well understood, its rescue by Keap1 co-expression suggests that *Drosophila* Keap1 may inhibit the activity of CncC. This effect requires the CncC-specific N-terminal domain, which includes the predicted Keap1-binding region. The shorter CncB isoform lacks this N-terminal domain, and does not interact with Keap1 in this assay: over-expression of CncB in the eye with the *GMRGal4* driver results in ommatidial loss and a smooth eye appearance, a phenotype that is not modified by the co-expression of Keap1. These data suggest an inhibitory role of Keap1 on CncC, which is reminiscent of the regulatory function of vertebrate Keap1 on Nrf2.

### CncC regulates the stress response gene *gstD1* and the *keap1* gene

Nrf2 regulates the basal and inducible expression of antioxidant and detoxifying genes (Lee et al., 2005; Motohashi and Yamamoto, 2004). To test whether CncC has functional homology to Nrf2, we first examined whether CncC can also regulate the transcription of such genes. *gstD1* is a prototypical oxidative stress response gene; it encodes a well known detoxification enzyme (Sawicki et al., 2003) and its transcription unit is preceded by a consensus ARE sequence (Figure 3A). mRNA *in situ* hybridization was used to examine whether the over-expression of CncC in embryos is sufficient to induce expression of *gstD1* mRNA. CncC was over-expressed using the *engrailedGal4* (*enGal4*) driver, which is active in a distinctive, easily recognizable striped pattern in the epidermis, and also in the dorsal part of the hindgut. Induction of *gstD1* mRNA expression in response to ectopic expression of CncC was readily apparent (Figure 2A). Interestingly, we found that over-expression of CncC can also induce the expression of *dkeap1* mRNA (Figure 2A).

To confirm the identification of *gstD1* and *keap1* as CncC target genes, and to derive quantitative and temporally resolved information about their regulation, we performed real-time RT-PCR experiments in CncC and Keap1 loss-of-function (l-o-f) conditions (Figure 2B). CncC and Keap1 were conditionally knocked-down in adult flies by RNAi (by expressing transgenes with inverted repeats corresponding to their respective coding segments, *UAS cncC<sup>RNAi</sup>* or *UAS keap1<sup>RNAi</sup>*). These genetic manipulations employed the conditional driver *tubulin-GeneSwitch-Gal4* (*tubGSGal4*), which is ubiquitously expressed, but is active only upon the dietary administration of RU486 (Scott Pletcher, personal communication). The efficiency of knockdown was validated by real-time RT-PCR (Supplemental Figure 3). The mRNA levels of *gstD1* and *keap1* were examined by real-time RT-PCR after 4 and 10 days of RU486 administration (Figure 2B). *gstD1* behaved as a *bona fide* Keap1/CncC target gene: its levels were reduced by CncC knockdown and increased by Keap1 knockdown after 4 days of treatment. *gstD1* levels tended to return to baseline after 10 days. Consistent with these data, *gstD1* levels were also increased in flies heterozygous for either of two *keap1* mutations (Figure 6B). Importantly, *keap1* levels were dramatically reduced by CncC knockdown; taken together with the inhibitory function of Keap1 on CncC (Figure 1C), and the induction of *keap1* by CncC (Figure 2A) these findings confirm the presence of an auto-regulatory loop in the pathway. These observations are consistent with the recent finding that *keap1* is a target gene

of Nrf2 in mammalian cell culture systems: stress releases Nrf2 from Keap1 inhibition, Nrf2 trans-activates *keap1*, and Keap1 promotes the degradation of Nrf2 (Lee et al., 2007). Our findings show that this auto-regulatory loop is an ancient mechanism conserved in evolution, further highlighting Keap1 as a crucial node in the pathway.

### An oxidative stress-responsive enhancer is regulated by Keap1/CncC via an ARE

To address the role of Keap1/CncC in antioxidant and detoxification responses of adult flies, we constructed reporter transgenes that express GFP or  $\beta$ -galactosidase (LacZ) under the control of a 2.7 kb genomic sequence upstream of the *gstD1* gene. In flies carrying the *gstD-GFP* reporter, GFP activity can be monitored in adults at basal conditions and after exposure to oxidative stressors (Figure 3). In unstressed conditions, GFP activity was detected mostly in the gut, which is reminiscent of the *cncC* expression pattern. Reporter activity is markedly induced when these flies are exposed to various oxidants, including Paraquat (a free radical generator), arsenic (a heavy metal), diethyl-maleate (DEM, a glutathione-depleting agent), and hydrogen peroxide, a *bona fide* oxidant (Figure 3B, and data not shown). These findings demonstrate that the cloned genomic sequence represents an enhancer responsive to diverse oxidative stressors, and that the *gstD-GFP* reporter fly lines are valuable tools for the live monitoring of antioxidant responses.

To investigate whether the genetic activation of Keap1/CncC signaling elicits a transcriptional effect that mimics gene induction after exposure to oxidants, we analyzed the transcriptional response of the *gstD* enhancer to the over-expression of CncC, or to the knock-down of Keap1 by RNA interference (RNAi) in adult flies. Reporter activity was induced in CncC g-o-f conditions and in Keap1 l-o-f conditions, supporting a role of these proteins as regulators of antioxidant gene expression (Figure 3C). Consistent results were obtained in larvae: the *gstD-GFP* reporter could be induced in the epidermis and dorsal hindgut by knocking down *keap1*, and the *gstD-lacZ* reporter could be induced in brains with *keap1* mutant clones (Supplemental Figure 4). The activation of reporter activity upon suppression of *keap1* expression in several tissues of larvae and adults suggests that Nrf2 function is latently present in a broad range of *Drosophila* cell types and organs.

The finding that Keap1/CncC signaling activates the *gstD-GFP* transgene indicates that reporter activity is regulated by one or more functional ARE sequences in the cloned enhancer. Indeed, the *gstD* enhancer harbors a 9 bp sequence (TGACcggGC) that perfectly matches the consensus core ARE sequence (TGAYnnnGC). Moreover, this 9 bp core element lies at the center of a 20 bp sequence (TCAGcATGACcggGCAaaaa), which also conforms perfectly to the extended ARE consensus sequence (TMAnnRTGAYnnnGCRwww; (Nioi et al., 2003). Since this cloned genomic fragment does not contain any other ARE-like sequences, we hypothesized that this element mediates trans-activation by the Keap1/CncC pathway. To test this idea, we generated transgenic reporter flies bearing a version of the enhancer with mutated ARE (*gstD $\Delta$ ARE-GFP*). In contrast to the wild-type *gstD-GFP* reporter, the ARE-mutated version could not be induced by either CncC over-expression or Keap1 knock-down (Figure 3D). Finally, we showed that a lacZ reporter transgene driven by a synthetic multimer ARE is also activated in CncC g-o-f or Keap1 l-o-f conditions, proving that the ARE is sufficient for transcriptional activation by Keap1/CncC (Supplemental Figure 5). These findings demonstrate that an ARE sequence mediates the trans-activation of the oxidative stress-responsive *gstD* enhancer by Keap1/CncC signaling, supporting the functional conservation of AREs in the *Drosophila* genome.



## The cancer chemopreventive Nrf2 activator oltipraz regulates the *gstD* enhancer via CncC and the ARE

Oltipraz, a synthetic dithiolthione compound, is effective as a cancer chemopreventive agent in rodent models, and it is evaluated in human clinical trials for cancer chemoprevention (Glintborg et al., 2006; Kensler et al., 1999; Zhang et al., 1997). We tested whether dietary administration of oltipraz would activate the *gstD-GFP* reporter in flies, and if such induction would require CncC and the ARE. Adult flies were allowed to feed on food supplemented with oltipraz or on control food, and reporter activity was examined after 48 hours. Oltipraz-treated flies showed marked activation of the *gstD-GFP* reporter in the gut as well as other regions of the body (Figure 4). This induction was reduced when CncC was knocked-down, and it was abolished when the ARE was mutated. Similar results were obtained after exposing reporter flies to tBHQ (another Nrf2 inducer), and to the potent oxidant DEM (not shown). These findings demonstrate that CncC is essential for ARE-mediated transcriptional responses, and thus validate the functional homology of CncC and Nrf2. Moreover, the results prove the feasibility of *in vivo* pharmacological manipulation of the Nrf2 pathway in *Drosophila* using chemical probes like oltipraz. Thus, *Drosophila* could be used as a platform for the discovery or preclinical testing of novel Nrf2 activators.

## CncC regulates the organism's resistance to oxidative stress

Having established that Keap1/Nrf2 signaling elicits transcriptional antioxidant and detoxification responses in the fruitfly, we examined its importance for organismal homeostasis. If CncC initiates an “electrophile counter-attack” in *Drosophila*, then CncC gain-of-function conditions might be expected to confer increased tolerance to oxidative stress; conversely, loss of CncC function should decrease oxidative stress tolerance. To test these hypotheses, we conditionally over-expressed or knocked-down CncC. We then compared the flies' oxidative stress tolerance to that of their sibling controls (Figure 5). Over-expression of CncC significantly increased the survival of both female and male flies after exposure to a semi-lethal dose of Paraquat (94% vs 31% for females,  $p < 0.001$ ; 62% vs 45% for males,  $p = 0.003$ ). Conversely, knocking-down CncC significantly decreased the survival of both sexes after Paraquat exposure (35% vs 65% for females,  $p = 0.008$ ; 53% vs 72% for males,  $p = 0.02$ ). These findings demonstrate that CncC is critical for the defense of *Drosophila* against oxidative stress, and that survival after an oxidative stress challenge can be augmented by the pre-emptive activation of CncC signaling.

## *keap1* heterozygosity confers oxidative stress resistance and lifespan extension

The regulation of *keap1* by CncC (Figure 2) and the inhibitory effect of Keap1 on CncC (Figure 1C) suggested a negative feedback loop in Keap1/CncC signaling, highlighting Keap1 as a crucial node of the pathway. To further investigate the role of Keap1, we used two independent mutant alleles that disrupt coding exons of *keap1*: *keap1<sup>EY1</sup>* and *keap1<sup>EY5</sup>* (shown in Figure 6A and described in detail in the Supplemental Data). Significantly, steady state levels of *gstD1* mRNA are elevated in flies heterozygous for either *keap1* allele (Figure 6B), suggesting that loss of one copy of the *keap1* gene can result in a gain of CncC function *in vivo*. The oxidative stress tolerance and lifespan of *keap1* heterozygotes were compared to those of their otherwise genetically identical wild-type siblings. Male *keap1<sup>EY1</sup>/+* and *keap1<sup>EY5</sup>/+* flies showed significantly increased survival rates after exposure to Paraquat (Figure 6C). Importantly, male *keap1* heterozygotes also lived significantly longer (8-10% extension of median lifespan,  $p < 0.0001$ ) than their otherwise genetically identical siblings (Figure 6D and Supplemental Table 1). Female *keap1* heterozygotes did not show significant differences in either Paraquat resistance or longevity. These findings demonstrate that partial loss of the Nrf2 pathway's negative regulator has significant beneficial effects on the oxidative stress tolerance

and longevity of male *Drosophila*. To our knowledge, this is the first evidence for a role of Keap1 in lifespan regulation.

## DISCUSSION

Here we show that the *Drosophila* CncC and Keap1 proteins are genuine Nrf2 pathway components, regulate ARE-mediated transcription and detoxification gene expression, and are crucial for the organism's defense against oxidative stress. These findings should accelerate genetic approaches to the functional characterization of the Nrf2 pathway aimed at the comprehensive elucidation of its roles in health and disease. In this respect, the lifespan extension of *keap1* heterozygous male flies is an example of new insight derived from *Drosophila*. Very recently, the Nrf2-related SKN-1 protein was found to be required for longevity induced by dietary restriction in *C.elegans* (Bishop and Guarente, 2007). This finding supports the concept that Cnc family members have an evolutionarily conserved function in lifespan regulation. Our results suggest that this role extends beyond calorie restriction, and establish a function for Keap1 (which is not conserved in worms) in this context. It was previously reported that the transcriptional activity of Nrf2 decreases in aging mice (Suh et al., 2004). Based on these findings, it will be very interesting to investigate the role of Keap1/Nrf2 in the aging of vertebrates.

The effect of *keap1* heterozygosity on stress resistance and longevity shows a marked sexual dimorphism, with significant, albeit modest, effects discernible only in males. Although the basis for this sex difference is presently unknown, sexually dimorphic genetic effects on *Drosophila* lifespan are not uncommonly reported, for example in the case of *chico* (Clancy et al., 2001; Tu et al., 2002). Also other genes with effects on aging, such as *p53* or *puc*, affect males and females differently (Bauer et al., 2005; Wang et al., 2005). It is important to note that the sexual dimorphism in the longevity of *keap1* heterozygotes is reflected in their Paraquat sensitivity. In males, removal of one gene copy of *keap1* increases Paraquat resistance, whereas females do not show this effect (Fig. 6C). While these findings imply that females react differently to *keap1* heterozygosity, such a difference is not manifested at the level of *gstD1* expression, which is significantly elevated in *keap1* heterozygotes of both sexes (Fig. 6B). These data suggest that although *keap1* heterozygosity increases stress response gene levels in both sexes, this translates into a benefit for Paraquat tolerance and longevity only in male flies. Importantly, when CncC is over-expressed Paraquat resistance is enhanced in both males and females (Fig. 5). Taken together, the data suggest that females can also benefit from increased CncC activity, but *keap1* heterozygosity is not sufficient to yield stress protection and extend lifespan in females. Wild-type female flies generally live longer and are more stress resistant than males, and this effect is also seen in our study. Figure 6D and Supplemental Table 1 show that the extended lifespan of male *keap1* heterozygotes is very similar to that of female flies. Given the modest, albeit significant, benefits observed in males, we propose that *keap1* heterozygosity is not sufficient to increase stress tolerance and extend lifespan beyond certain biological limits.

The involvement of Nrf2 in both cancer prevention and lifespan regulation adds to the intricate links between cancer and aging. In addition to its role in cancer prevention, the antioxidant and cell protective properties of Nrf2 make it an important target for drug discovery in the prevention and treatment of oxidative stress- and aging-related diseases, including neurodegenerative disorders (van Muiswinkel and Kuiperij, 2005). Many *Drosophila* models of neurodegeneration are available, rendering the fruitfly an excellent system for the preclinical testing of this strategy (Bilen and Bonini, 2005). Thus, our characterization of Keap1/Nrf2 signaling in *Drosophila* facilitates studies that will examine the preventive and/or therapeutic effects of Nrf2-activating cancer chemopreventive agents in neurodegenerative diseases. Studies utilizing oltipraz, in particular, are especially appealing, since it is a relatively safe

agent that has been used for the treatment of schistosomiasis (a parasitic disease) and is tested in human clinical trials for cancer chemoprevention (Kensler et al., 1999; Zhang et al., 1997).

## EXPERIMENTAL PROCEDURES

### *Drosophila* lines

Flies were reared at 25°C. *sepGal4* and *GMRGal4* flies were from Marek Mlodzik. *tubGSGal4* flies express RU486-regulated Gal4 under the control of the tubulin enhancer (Scott Pletcher, personal communication); we used insertions on the second (*tubGS10*) and third chromosome (*tubGS5*). *engrailedGal4* and *EY02632* flies were from the Bloomington Stock Center. 4xARE-lacZ (originally named 4C (Veraksa et al., 2000)) and *UAS cncB* flies were from William McGinnis. To create lines expressing Keap1 and CncC, the coding segments of each gene were amplified by PCR from adult cDNA and cloned into pUAST (Brand and Perrimon, 1993). For RNAi-mediated knock-down of Keap1 and CncC, lines expressing inverted repeats corresponding to the parts of each gene's coding segment were created by PCR amplification from genomic DNA and cloning into pWIZ (Lee and Carthew, 2003). Primers and cloning details are available upon request. Transgenic flies were generated by standard procedures in a *w<sup>1118</sup>* background. Independent insertions of *UAS cncC<sup>RNAi</sup>* or *UAS keap1<sup>RNAi</sup>* transgenes on the major autosomes were combined to increase knock-down efficiency. The *gstD-GFP* and *gstD-lacZ* reporter lines were constructed by amplifying a 2708 bp genomic fragment that lies between the *gstD1* and *gstD2* genes by PCR and cloning it into pGreen H-Pelican and pH-Pelican, respectively (Barolo et al., 2000). To create the ARE-mutant version of the reporter (*gstDΔARE-GFP*), the ARE sequence in the cloned *gstD* enhancer was mutagenized to an *XhoI* restriction site (TGACCG>CTCGAG). Somatic mutant clones were generated by the MARCM system (Wu and Luo, 2006) using the chromosomes *eyFlp*; *actGal4>>UASGFP*; *FRT82B tubGal80*, and *FRT82B* or *FRT82B keap1<sup>036</sup>*.

### X-gal staining, mRNA *in situ* hybridization, and real-time RT-PCR

X-Gal staining of embryos was performed as previously described (Su et al., 1998). Since endogenous CncC is zygotically expressed from late embryonic stages onwards (McGinnis et al., 1998), the Keap1 RNAi experiments were performed in third instar larvae. Dissected larval tissues were fixed in 1% glutaraldehyde in PBS, and stained at 37°C in 10 mM sodium phosphate buffer pH 7.2, 150 mM NaCl, 1 mM MgCl<sub>2</sub>, 10 mM K<sub>4</sub>[Fe(CN)<sub>6</sub>], 10 mM K<sub>3</sub>[Fe(CN)<sub>6</sub>], and 0.1% Triton-X-100.

mRNA *in situ* hybridization was performed as previously described (Jasper et al., 2001). Briefly, genomic DNA fragments corresponding to parts of the coding segments of *cncC* and *keap1* were amplified by PCR and cloned into PCRII-TOPO (Invitrogen, Carlsbad, CA). Digoxigenin-labeled *cncC* and *keap1* -specific RNA probes were prepared by *in vitro* transcription with the DIG RNA labeling kit (Roche, Palo Alto, CA). The hybridization and detection procedure was performed as previously described (Jasper et al., 2001). RNA extraction from whole flies or dissected adult guts and the real-time RT-PCR procedure were performed as previously described (Wang et al., 2003).

### Chemicals and fluorescent reporter assays

Paraquat, DEM, hydrogen peroxide, tBHQ, and RU486 were from Sigma-Aldrich (St. Louis, MO), sodium meta-arsenite from J.T.Baker (Phillipsburg, NJ), and oltipraz from LKT Labs (St. Paul, MN). To examine the effects of oxidative stressors on *gstD* enhancer activity, 1-day old homozygous *gstD-GFP* reporter flies were mated for one day, starved for 3 hours in vials, and then fed a solution of 5% sucrose ± Paraquat (20 mM), sodium meta-arsenite (1 mM), hydrogen peroxide (3%) or DEM (20 mM; the test and control solutions for DEM also contained 10% ethanol). To assess whether Keap1 and CncC regulate the *gstD* enhancer via



the ARE, flies bearing both a *tubGSGal4* driver and a wild-type or mutant reporter transgene were generated by genetic recombination and crossed to the appropriate UAS lines. The genetically identical progeny of such crosses were separated by gender after one day of mating, and each gender was further split into two groups that were maintained for 48 hours on either RU486-containing (320  $\mu$ M) or control food (corresponding amount of ethanol) (McGuire et al., 2004). To assess the effects of oltipraz on reporter activity, 2-day old flies of the appropriate genotypes were maintained for 48 hours on food supplemented with oltipraz. To examine the role of CncC in mediating the effects of oltipraz, oltipraz administration was preceded by a 4-day period of feeding on RU486-containing food to knock-down CncC by RNAi.

### Paraquat resistance assays

Resistance of adult flies to an oxidative challenge was assessed as previously described (Wang et al., 2003). To evaluate the role of CncC in Paraquat tolerance, 1 day old flies of the indicated genotypes were mated for one day, split into females and males, and then kept on RU486-containing (320  $\mu$ M) or control food for 4 days (flipped on fresh food after the first 2 days). Groups of 60 flies were then starved for 3 hours in vials, and then fed a solution of 5% sucrose  $\pm$  a semi-lethal dose of Paraquat (10-20 mM). Survivors were scored after 16 hours. To evaluate the oxidative stress resistance of *keap1* heterozygotes, flies of the appropriate genotypes were treated in the same manner except for the RU486 feeding period. Percentages of surviving flies were compared by t-test using SPSS 9.0 (SPSS Inc., Chicago, IL). From each genotype and gender, more than 1000 flies were scored.

### Lifespan measurements

Flies carrying the *mini-white*-marked *keap1<sup>EY1</sup>* or *keap1<sup>EY5</sup>* mutation were backcrossed more than 12 times into the *w<sup>1118</sup>* background, and then heterozygous mutant males were crossed to *y<sup>1</sup>,w<sup>1118</sup>* virgins. The lifespan (and oxidative stress tolerance) of mutant and wild-type progeny of this cross was compared as previously described (Wang et al., 2003). Briefly, female and male progeny of the cross were collected 1 day after hatching and allowed to mate in bottles for 1 day. Next, females and males were separated and transferred to vials (30 flies per vial). Flies were transferred to fresh vials every other day (daily at old ages), at which time the number of dead flies was recorded. From each genotype and gender, 500-700 flies were scored for longevity. Survival curves were analyzed by the Kaplan-Meier procedure and log-rank test.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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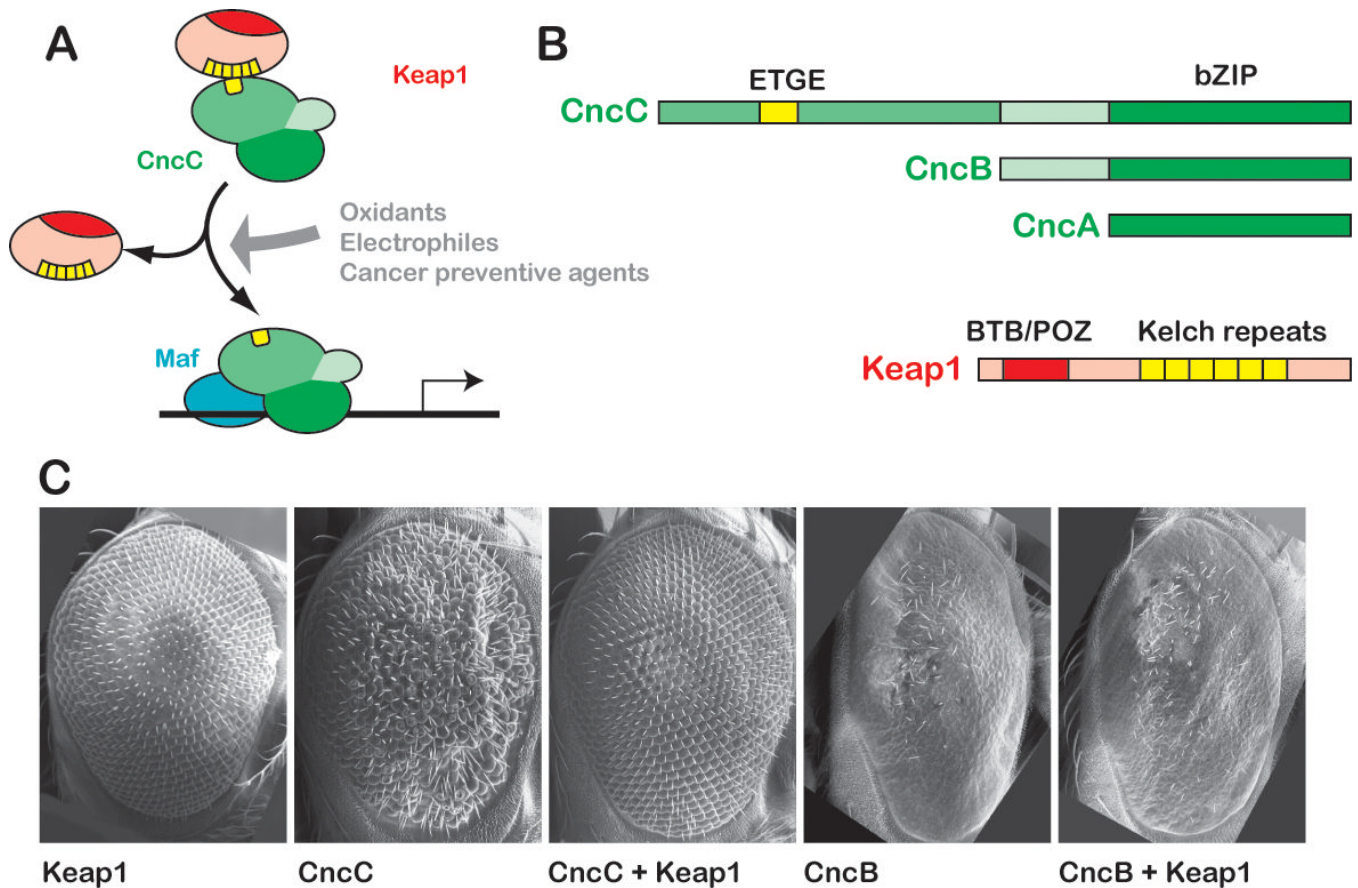
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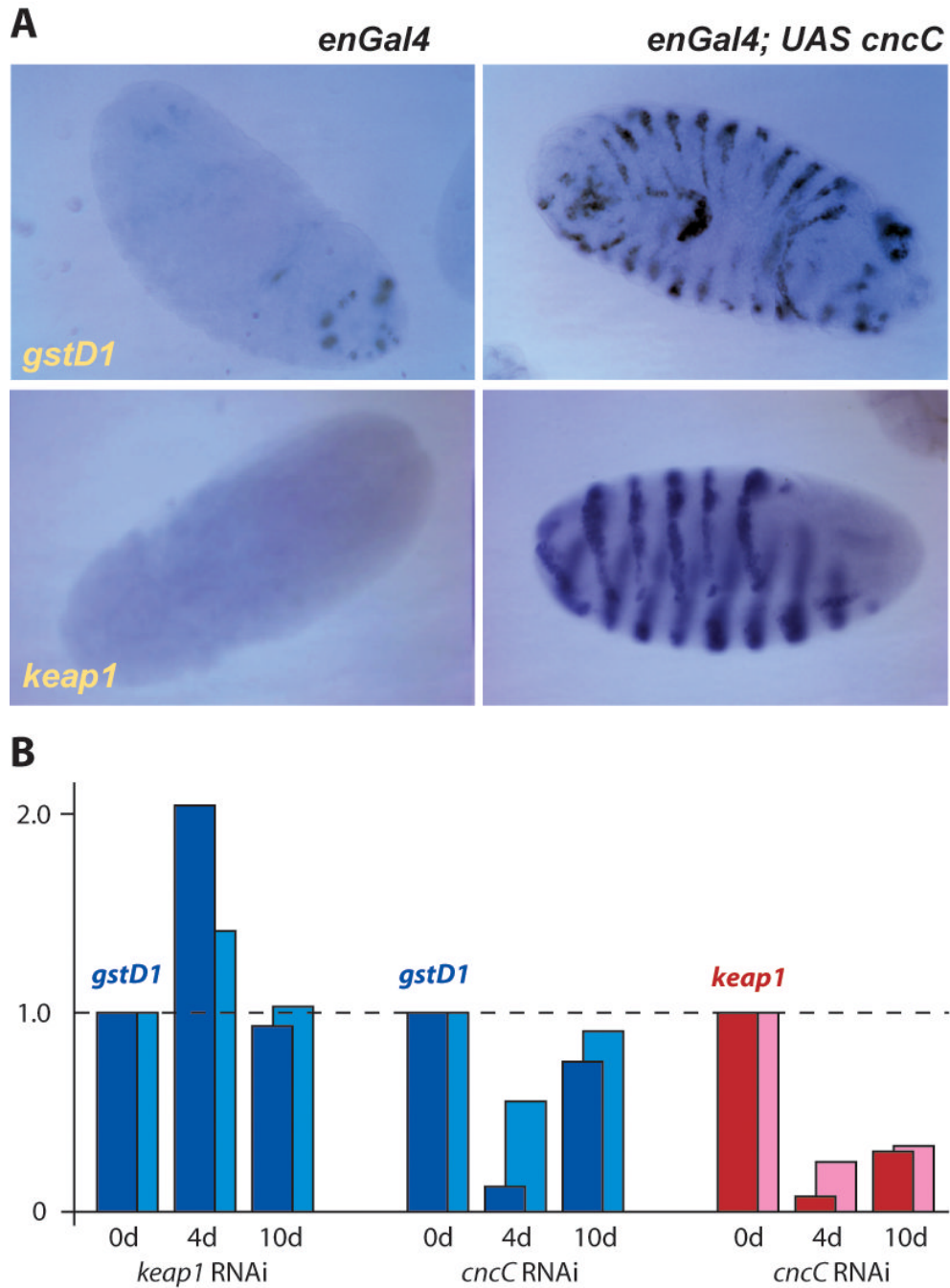
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**Figure 1. Nrf2 and Keap1 homologues are conserved in *Drosophila***

**A.** The Keap1/Nrf2 signaling pathway. In basal conditions, Keap1 binds to Nrf2 and inhibits its activity. Oxidative stressors, electrophilic xenobiotics, and cancer chemopreventive agents release this inhibition. Stabilized Nrf2 then accumulates in the nucleus and dimerizes with a small Maf protein to transcriptionally activate a battery of cell protective genes. **B.** Nrf2 and Keap1 homologues are present in *Drosophila*. The *cnc* locus of *Drosophila* encodes three protein products. All contain the bZIP region that mediates dimerization and DNA binding. Only the longest isoform, CncC, contains domains predicted to bind Keap1, including the ETGE motif, and is thus potentially a Nrf2 homologue. The *Drosophila* Keap1 protein shows a high degree of sequence similarity to its vertebrate Keap1 counterparts (sequence alignments for CncC and Keap1 proteins are shown in Supplemental Fig 1). Conserved domains include the BTB/POZ domain required for dimerization and 6 Kelch repeats for binding to Nrf2 and anchoring to actin. **C.** Over-expression of *Drosophila* Keap1 can inhibit CncC activity *in vivo*. Expression of CncC in the developing *Drosophila* eye from a UAS transgene under the control of *sepGal4* causes a reproducible aberrant phenotype. Over-expression of Keap1 under the same conditions (or with *GMRGal4*, data not shown) has no phenotypic effect, but it can completely suppress the effects of CncC over-expression. The activity of the shorter CncB isoform, which lacks the putative Keap1-interacting domain, is not inhibited by Keap1 co-expression. The *GMRGal4* driver was used to express CncB (and Keap1) in the rightmost two panels of C., because expression by *sepGal4* did not produce a phenotype at 25°C.



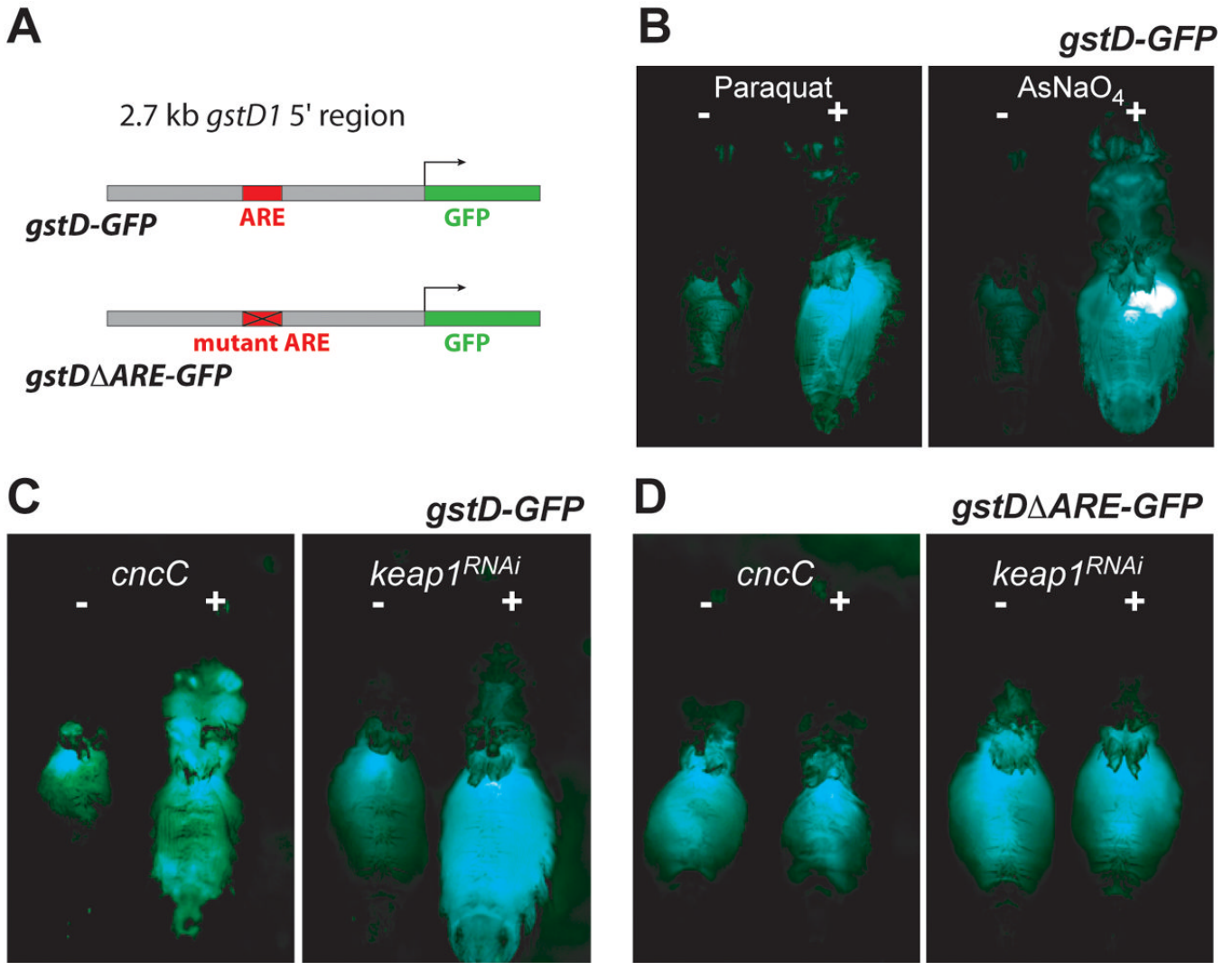


**Figure 2. CncC regulates stress response genes and the *keep1* gene**

**A.** The over-expression of CncC in the epidermis of the embryo using the *enGal4* driver induces the *glutathione S-transferase D1* gene, a well known antioxidant and detoxification gene (detected by mRNA *in situ* hybridization). As negative controls, other members of the *gstD* gene family were not induced in parallel experiments (not shown). The *Drosophila keep1* gene can also be up-regulated by CncC over-expression.

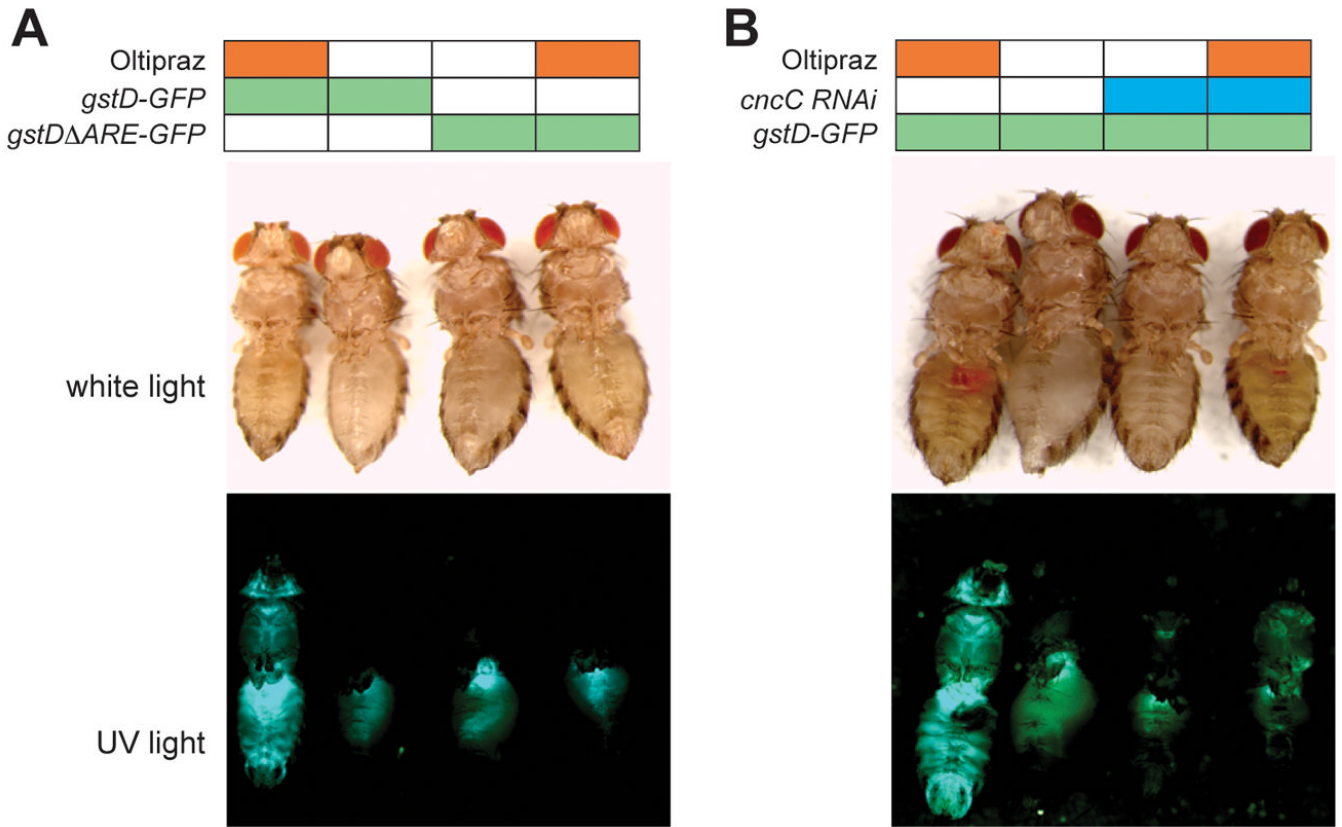
**B.** Validation of *gstD1* and *keep1* as CncC target genes by real-time RT-PCR. These experiments employ transgenic expression of RNAi targeting either *keep1* or *cncC* mRNA under the control of *tubGSGal4*, which permits the conditional knockdown of CncC or Keap1 in adult flies. Two separate driver lines with independent transgene insertions were used;

*tubGS5* (dark blue and dark red bars) consistently yielded slightly stronger effects than *tubGS10* (light blue and light red). The expression of *UAS keep1<sup>RNAi</sup>* or *UAS cnc<sup>RNAi</sup>* (as indicated at the bottom of the histogram) was activated by feeding adults with RU486 as detailed in the methods section. Sibling control flies from the same culture were treated with food containing an equivalent amount of ethanol solvent. The resultant changes in the mRNA levels of *gstD1* and *keep1* were examined by real time RT-PCR after 4 and 10 days of conditional knockdown.



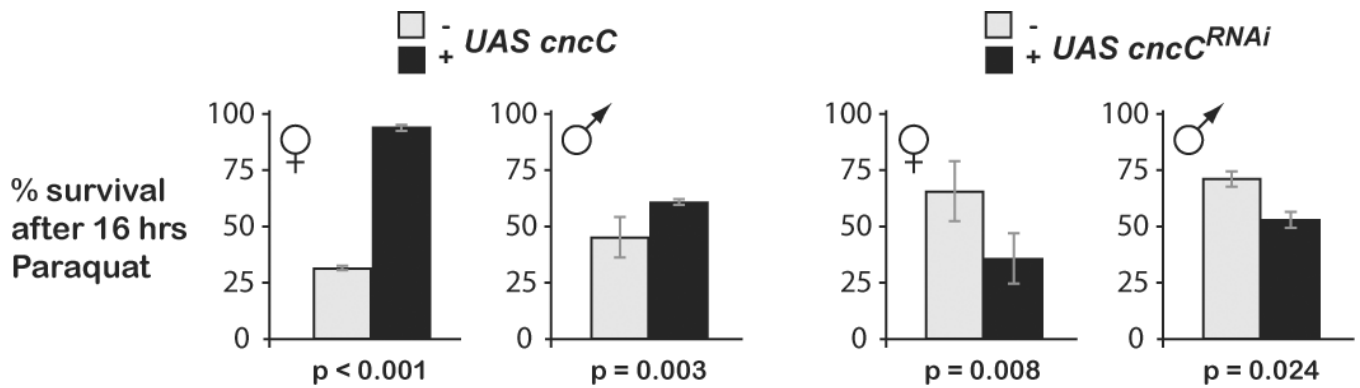
**Figure 3. An oxidative stress-responsive enhancer is regulated by Keap1/CncC**

**A.** Structure of the *gstD-GFP* reporter transgene. The genomic sequence upstream of the *gstD1* gene harbors an ARE sequence, and was used to control the expression of GFP in transgenic reporter flies. The *gstDΔARE-GFP* reporter is identical, except that the ARE consensus sequence has been disrupted by base substitutions (see methods). **B.** The transcriptional activity of the *gstD* enhancer was potently induced in the gut and in other tissues by oxidants. Animals were exposed to 20 mM Paraquat or 1 mM sodium meta-arsenite in sucrose solution, and GFP fluorescence was monitored after 16 hours. The flies' wings and legs were dissected away to facilitate handling and to expose the abdomen. **C.** The *gstD* enhancer responds to Keap1/CncC signaling. The activity of the *gstD* reporter can be induced by over-expression of CncC or by RNAi-mediated knock-down of Keap1. Keap1 RNAi and CncC expression was driven by the ubiquitously expressed RU486-inducible *tubGSGal4* driver; thus the two animals shown in any of the panels are genetically identical and differ only by being fed RU486 or mock treated for 48 hours before analysis. RU486 feeding by itself has no effect on reporter activity (not shown). **D.** The transcriptional activation of the *gstD* enhancer by Keap1/CncC signaling is mediated by an ARE. Mutation of the ARE abolishes enhancer activation by both CncC over-expression and Keap1 knock-down.



**Figure 4. The cancer chemopreventive agent oltipraz regulates the *gstD* enhancer via CncC and the ARE**

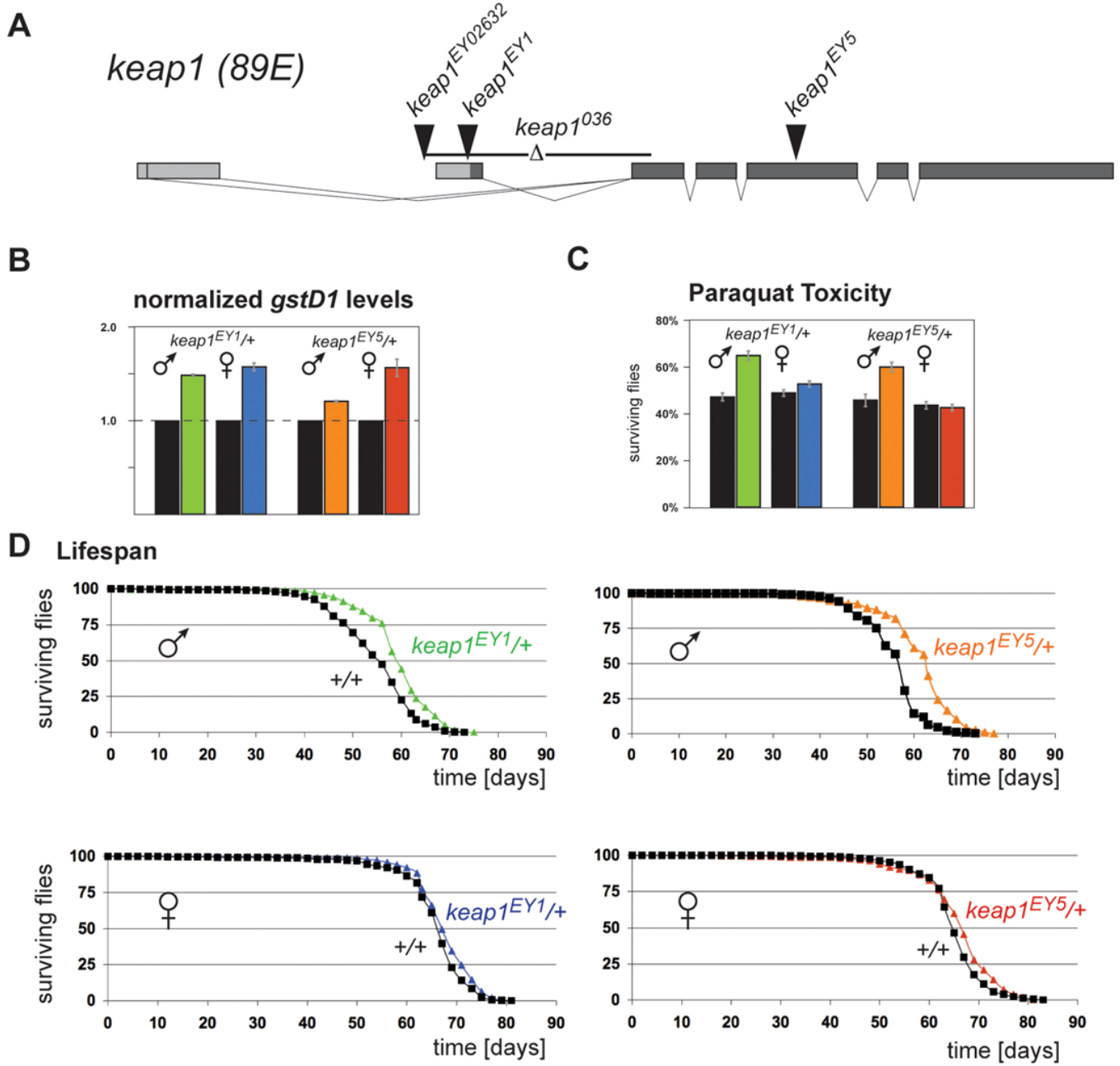
**A.** Activation of the *gstD* enhancer by oltipraz requires the ARE. Feeding oltipraz to flies activates the *gstD-GFP* reporter. However, mutation of the ARE completely abolishes reporter induction by oltipraz. The activity of the *gstD-GFP* reporter was monitored by inspection of the respective animal under UV light (bottom panel). The same animals are also shown in white light illumination. **B.** Activation of the *gstD* enhancer by oltipraz requires CncC. CncC activity was reduced by RU486-inducible knock-down of CncC for 4 days before oltipraz administration. Targeting CncC by RNAi reduced both the basal activity of the *gstD* enhancer, and its activation by oltipraz.



**Figure 5. CncC mediates the resistance of *Drosophila* to oxidative stress**

**A.** Over-expression of CncC before a Paraquat challenge increases the flies' survival rate. Flies bearing *tubGSGal4* and *UAS cncC* transgenes were maintained on RU486-containing or control food for 4 days before being challenged with a semi-lethal dose of Paraquat. The survival rate of RU486-fed female and male flies 16 hours after the start of Paraquat exposure was significantly higher than that of their sibling controls, suggesting that CncC over-expression increases oxidative stress tolerance. **B.** RNAi-mediated knock-down of CncC before a Paraquat challenge reduces the flies' survival rate. Flies bearing *tubGSGal4* and *UAS cncC<sup>RNAi</sup>* transgenes were maintained on RU486-containing or control food for 4 days before being challenged with Paraquat. The survival rate of RU486-fed female and male flies after 16 hours was significantly lower than that of their sibling controls, suggesting that CncC is required for normal oxidative stress tolerance. All data are represented as mean  $\pm$  SEM of three experiments performed in triplicate. p-values are shown below each histogram. The flies' gender and specific genetic background can profoundly influence their tolerance to oxidative stress. Therefore, the dose of Paraquat for each experiment was adjusted to achieve (approximately) a 50% death rate in each control group after 16 hours. Moreover, only flies within a panel are directly comparable, because they are of the same gender and genetically identical, and differ only in having being fed RU486 for 4 days before the stress. RU486 feeding by itself has no effect on the Paraquat resistance of wild-type *w<sup>1118</sup>* flies, or on any of the *tubGSGal4*, *UAS cncC*, and *UAS cncC<sup>RNAi</sup>* stocks when out-crossed to the *w<sup>1118</sup>* background (not shown).





**Figure 6. *keep1* heterozygosity confers oxidative stress resistance and lifespan extension**  
**A.** Structure of the *Drosophila keep1* gene and nature of loss-of-function alleles. Alternative *keep1* transcripts differ at their 5' ends. Exons are depicted as boxes, with coding segments indicated by dark shading. *EY02632* is a homozygous viable allele; *keep1<sup>036</sup>*, *keep1<sup>EY1</sup>*, and *keep1<sup>EY5</sup>* are larval lethal alleles (see Supplemental Method). **B.** Elevated *gstD1* expression levels in *keep1* heterozygotes. mRNA levels of *gstD1* in 1 day old flies were quantified separately for males and females by real-time RT-PCR. Control flies were siblings of the heterozygotes and were homozygous wild-type for *keep1*. Data shown are mean  $\pm$  SEM of three experiments performed in duplicate. **C.** Partial loss of Keap1 increases Paraquat resistance. Male heterozygous *keep1<sup>EY1</sup>/+* and *keep1<sup>EY5</sup>/+* flies show a significantly higher survival rate 16 hours after a Paraquat challenge than their otherwise genetically identical wild-

type siblings. Data are represented as mean  $\pm$  SEM of four experiments performed in triplicate. Females do not display this effect. **D.** *keap1* heterozygosity extends lifespan. Under standard culture conditions, male *keap1<sup>EY1</sup>/+* and *keap1<sup>EY5</sup>/+* flies live significantly longer than their sibling controls. Data are represented as the percentage of flies that are alive at each age. 500-700 flies of each genotype and gender were assayed (see methods for experimental details).