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MODULATION OF OXIDATIVE STRESS BY KEAP1/NRF2 SIGNALING IN *DROSOPHILA*: IMPLICATIONS FOR HUMAN DISEASES

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22.1 INTRODUCTION

The signaling module comprising the transcription factor Nrf2 (NFE2-related factor 2) and its cytoplasmic inhibitor Keap1 (Kelch ECH-associated protein 1) is a master mediator of ubiquitous antioxidant and detoxification responses to cellular stressors as well as of various cell type-specific homeostatic functions. Nrf2 belongs to the cap'n'collar (cnc) family of leucine zipper transcription factors named after the *cnc* locus of *Drosophila melanogaster*, which was originally characterized as a regulator of fly development. Even though the sequence similarity between Nrf2 and Cnc was evident early on, it took no less than a decade after Nrf2 was cloned to demonstrate experimentally its functional homology with a protein product of *cnc* (isoform C, CncC). This chapter recounts the story of these recent discoveries and discusses ongoing and future studies thereby made possible in flies on the Keap1/Nrf2 system as well as their implications for understanding and combating human diseases.

22.1.1 The Keap1/Nrf2 System Safeguards Homeostasis and Mediates Antioxidant Defense with Implications for Preventing and Treating Human Diseases

To defend themselves against the deleterious effects of oxidative stress, cells possess antioxidant defense networks that can sense prooxidant and electrophilic reactive species and launch adaptive responses [1]. Since it was cloned in the mid-1990s [2], the vertebrate transcription factor Nrf2 has been established as a ubiquitously expressed master regulator of the cellular redox status and mediator of cell-protective antioxidant and detoxification responses [3–8]. Numerous studies have shown that in mice and in human cultured cells the expression of many protective genes increases in an Nrf2-dependent manner in response to oxidative and electrophilic chemical challenges [reviewed in 3, 4]. Target genes of Nrf2 include those encoding a broad range of redox regulators and so-called phase II detoxification enzymes, such as glutathione *S*-transferases, glutathione-synthesizing enzymes, thioredoxins, peroxyredoxins, NAD(P)H quinone oxidase 1 (NQO1), heme oxygenase 1 (HO1), and many others [9–12]. This battery of antioxidant and detoxifying genes are transcriptionally induced in a coordinated manner through the binding of Nrf2 to antioxidant response element (ARE) sequences in their regulatory regions (Fig. 22.1). The predominant dimerization partners of ARE-bound Nrf2 are the members of the small Maf (musculo-aponeurotic fibrosarcoma oncogene) family of proteins, which are themselves devoid of transcriptional activation potential [13–15].

The abundance and transcriptional activity of Nrf2 increase markedly when cells are exposed to oxidative stressors or electrophilic chemicals, and several of the mechanisms involved have been elucidated over the last

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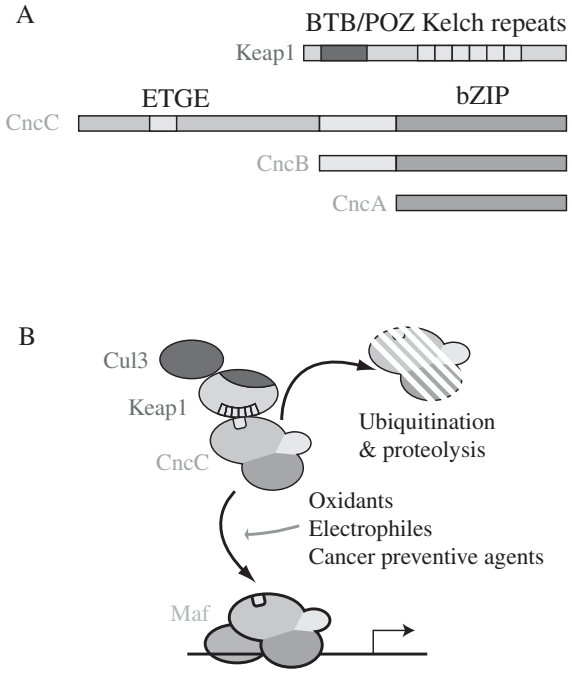

Fig. 22.1 Conservation and simplified illustration of the Keap1/Nrf2 pathway in *Drosophila*. (A) Nrf2 and Keap1 homologs are present in *Drosophila*. The fly *keap1* gene is predicted to encode a protein with high sequence similarity to its vertebrate Keap1 counterparts. Conserved domains include the BTB/POZ domain required for dimerization and 6 Kelch repeats for binding to Nrf2 and anchoring to actin. The *cnc* locus encodes three protein products, which all contain the bZIP region that mediates dimerization and DNA binding. The Nrf2 homolog is the longest isoform, CncC, which contains domains predicted to bind Keap1 such as the ETGE motif. (B) In basal conditions, Keap1 binds to CncC and inhibits its activity, likely through Cul3-mediated ubiquitination and proteasomal degradation. Oxidative stressors, electrophilic xenobiotics, and cancer chemopreventive agents relieve this inhibition. Stabilized CncC then accumulates in the nucleus and transcriptionally activates a battery of cell-protective genes, likely in a dimer with the single small Maf protein of *Drosophila*. This figure is adapted from Sykiotis GP and Bohmann D, Keap1/Nrf2 signaling regulates oxidative stress tolerance and lifespan in *Drosophila*, *Dev Cell*, vol. 14, p. 76–85, Copyright 2008, with permission from Elsevier. *(See color insert.)*

decade [reviewed in 16–19]. Notably, the activation of Nrf2 requires its posttranslational stabilization, because in nonstressed conditions Nrf2 undergoes rapid proteasomal degradation in the cytoplasm. This proteolysis is effected by a Cul3-based ubiquitin ligase system and facilitated by an interaction between Nrf2 and its cognate substrate adaptor, Keap1 (Kelch-like ECH-associating protein 1), a protein tethered to the actin cytoskeleton (Fig. 22.1) [3, 20–26]. The inhibitory effect on Nrf2 is abolished when specific redox-sensitive cysteine side chains of Keap1 undergo oxidative modifications in response to oxidants and electrophiles, which likely triggers a conformational change in Keap1 structure that interferes with Nrf2 ubiquitination [27–32]. Additional mechanisms involved in Nrf2 activation include its phosphorylation by stress-activated kinases and the redox-sensitive regulation of its nuclear import and export [reviewed in 8, 16–8]. In addition to regulating adaptation to acute cellular stress via the induction of ubiquitous antioxidant and detoxification genes, the Keap1/Nrf2 system contributes to the homeostasis of diverse tissues by regulating the expression of corresponding sets of tissue-specific target genes with distinct roles in the specialized function of the respective cell types [reviewed in 4]. Its generic and tissue-specific homeostatic properties have established Nrf2 as an important "multiorgan protector" [4] and a mediator of "programmed cell life" [33].

By damaging proteins, lipids, and DNA, oxidative stress causes or exacerbates various diseases in model organisms and in humans [34, 35]. Conversely, by ameliorating oxidative stress or reversing its sequelae, Nrf2 protects against such pathologies. In mice, a protective role of Nrf2 has been demonstrated in diverse models of disease, including chemically induced cancers [3, 4, 6, 8, 36–39]; pulmonary diseases, such as asthma [40], emphysema [9], pulmonary fibrosis [41], hyperoxia [42], and acute lung injury [43]; neurodegenerative disorders, such as Parkinson disease [44], Alzheimer disease [45], and amyotrophic lateral sclerosis [46]; inflammatory disorders, such as inflammatory bowel disease [47]; liver toxicity, including toxin- and alcohol-induced liver damage [48, 49]; atherosclerosis [50]; and insulin resistance [51], among others [4, 6]. Importantly, compounds that activate Nrf2 have been shown to be effective in several of these disease models, highlighting Nrf2 as a promising target in preventing and treating various disorders [36, 52–57]. In humans, inherited DNA polymorphisms that reduce the expression of Nrf2 [58, 59] have been associated with diseases such as skin vitiligo [60], chronic gastritis [61], peptic ulcer [62], ulcerative colitis [63], and adult respiratory distress syndrome [59]. Moreover, impaired Nrf2 signaling has been implicated in human respiratory and neurodegenerative disorders [64–69]. Thus there is intense interest in elucidating the mechanisms by which Nrf2 activity is induced or impaired during the pathogenesis and progression of various human diseases, with the goal of identifying pharmacological or nutritional strategies to safely activate Nrf2 signaling for disease prevention and treatment. Although the model organism *Drosophila melanogaster* (commonly known as fruit fly) could help to obtain new insights relevant to these pursuits, it had not been utilized in the study of Nrf2 signaling until quite recently.

22.1.2 Cnc as a Developmental Locus of Drosophila melanogaster

Nrf2 is a member of the cap'n'collar (cnc) family of transcription factors, which are conserved from worms to mammals. Cnc factors are defined by the presence of a conserved 43-amino acid Cnc domain located amino-terminally to the DNA binding domain. In addition to Nrf2, the Cnc family comprises the vertebrate p45 NFE2 (nuclear factor erythroid-derived 2), which is present only in hematopoietic progenitor, erythroid, megakaryocytic, and mast cells and is required for proper development of platelets [70–72]; the NFE2-related factors Nrf1 and Nrf3 [73, 74], which have broad expression patterns partly overlapping that of Nrf2 and also function as stress-activated transcription factors [2, 73–75]; the *Caenorhabditis elegans* SKN-1 (Skinhead family member 1) [76], which is critical for the formation of the digestive system during worm embryogenesis [76] and also regulates a phase 2 detoxification response in the digestive tract [77]; and the *Drosophila* Cnc [78], after which the family was named.

The *Drosophila cnc* locus was originally identified 20 years ago as a gene expressed in the primordia of three segments of the developing fly embryo's head: the labral segment, where *cnc* expression is detectable as an anterior "cap," and the intercalary and mandibular primordia, where *cnc* expression is detectable as a more posterior 3-4 cell-wide "collar"—hence the name of the locus and of the gene family [78]. The anterior domain of *cnc* expression (corresponding to the labral segment primordium) was found to be activated by the *bicoid* and *torso* maternal pathways, independently of known zygotic gap genes, and sequentially constricted to its final size by repression from neighboring region-specific genes [79]. Control of the posterior domain (corresponding to the intercalary and mandibular segment primordia) involved combinatorial regulation by zygotic gap genes: activation by *buttonhead* and repression by *orthodenticle* anteriorly and *snail* ventrally [79]. Thus *cnc* is expressed and regulated as is typical for a second-tier region-specific gene. It was further shown that *cnc* is a homeotic gene that defines labral and mandibular segment identity: *cnc* mutants show absence of labral and mandibular head structures, and their mandibular tissues are transformed to a maxillary fate, which is a clear example of classical homeosis [80].

The cnc locus was subsequently found to give rise to three RNA isoforms, designated *cncA*, *cncB*, and *cncC*, each of which encodes a different protein [81]. CncA, CncB, and CncC share their carboxy-terminal regions, which include the DNA-binding domain, but have distinct amino-termini. CncC is the longest protein and encompasses CncB, which in turn encompasses the shortest, CncA [81]. The isoform that mediates the role of *cnc* in embryonic head development was shown to be CncB: In mandibular cells, CncB antagonizes the ability of Deformed (Dfd), another homeotic transcription factor, to transcriptionally activate response elements of its downstream genes [81]. In the absence of CncB, unchecked Dfd activity transforms mandibular segment cells to cells of a maxillary fate [81]. A genetic interaction between *dfd* and *cnc* had been previously discovered in a genetic screen for modifiers of Dfd function [82], and these two genes were also found later to jointly regulate the initial embryonic expression pattern of the gene *proboscipedia*, a member of the Antennapedia complex that is required for the proper specification of the adult mouthparts of *Drosophila* [83].

In the specification of embryonic head structures, the CncB-mediated suppression of Dfd was found to require the *Drosophila* homolog of the mammalian small musculo-aponeurotic fibrosarcoma (Maf) proteins, named Maf-S [84]. In fly embryos, multimers of simple CncB/Maf-S heterodimer sites were transcriptionally activated in response to CncB, and in tissue culture cells the amino-terminal domain of CncB showed properties of a strong transcriptional activation domain [84]. Because the known elements that are activated in response to Dfd and are repressed in a CncB-dependent fashion do not match the CncB/Maf binding consensus sites, the suppressive effect of CncB/Maf-S proteins on Dfd activity must be exerted either indirectly or via direct binding to as yet uncharacterized Dfd response elements [84]. Similar to *Drosophila* CncB, the worm SKN-1 is required for the developmental specification of the pharynx, indicating phylogenetic conservation of this role of cnc transcription factors [76]. In contrast to *cncB* mutants, flies harboring mutations that selectively affect the *cncC* isoform develop normal larval head structures, indicating that CncC is not required for this developmental process [84]. However, *cncC* mutant flies are unable to complete larval development, suggesting a later developmental requirement for CncC [84]. The precise developmental role of CncC is an issue of substantial biological interest that awaits elucidation.

In addition to its role in head development, *cnc* is important for establishing the dorsoventral (DV) polarity of the oocyte's follicular epithelium during oogenesis [85]. Body axis establishment in *Drosophila* requires an ordered progression of events with determination of oocyte polarity, establishment of the anterior-posterior (AP) axis through localization of *bicoid (bcd)* and *oskar* (*osk*) mRNA at the anterior and posterior poles, respectively, and asymmetric movement and positioning of the oocyte nucleus in the anterior cortex, which defines the dorsal side of the egg chamber and initiates the DV patterning of the eggshell and the embryo. In developing

cnc mutant oocytes the nucleus is initially localized correctly at the anterior cortex. Progressively, however, the nucleus loses its proper localization; *osk* and *bcd* mRNA are mislocalized; microtubule arrangement is abnormal; and defects in DV polarity ensue [85]. Thus, whereas the early migration of the nucleus and organization of the oocyte microenvironment are independent of *cnc*, the stable anchoring of the nucleus to the anterior cortex of the oocyte and the proper specification of DV polarity require *cnc* function [85]. Because CncB is not expressed during oogenesis [81], the mutant phenotypes observed must be due to a lack of CncA, CncC, or both isoforms. Identifying the cnc factor(s) mediating establishment of DV polarity, and deciphering the underlying molecular mechanisms would contribute substantially to our understanding of the *cnc* locus and its functions.

22.1.3 Functional Conservation of Keap1/Nrf2 Signaling in *Drosophila*

Until a few years ago it had not been investigated whether Nrf2 shared its stress defense function with the founding member of the cnc family, *Drosophila* Cnc. In light of the intense interest in Nrf2 as a cell-protective factor in vertebrates and the fact that its *C. elegans* relative in the cnc family, SKN-1, had already been shown to have roles in antioxidant defense, the gap in knowledge regarding the family's namesake factor in *Drosophila* was perplexing. Nevertheless, even though no formal studies had been undertaken to evaluate the functional conservation of Nrf2 signaling in flies, both drosophilists and non-drosophilists had noticed that the *Drosophila* genome contained plausible homologs for *nrf2* and *keap1*. The CncC isoform was originally suggested as the likely *Drosophila* counterpart of Nrf2 because its unique amino-terminus showed sequence similarity to the Nrf2 domain that is required for binding to Keap1 [86]. The *Drosophila* genome was also found to harbor a likely homolog of vertebrate *keap1* genes known as *CG3962*, which had not been functionally characterized [86]. Other groups further documented the sequence conservation of putative Keap1-binding and transcription-activating domains in CncC, as well as the presence of ARE sequences in the upstream regions of *Drosophila* stress response genes [1, 87]. On the basis of these observations, it was proposed that CncC/Keap1 signaling is conserved in flies, with likely roles in the oxidative stress response and potentially also in development. As sometimes happens with good ideas in science, this one lingered for a few years without follow-up research studies to substantiate it. Thus the functional correspondence of *Drosophila* Keap1 and CncC to vertebrate Keap1 and Nrf2, respectively, remained an untested albeit plausible hypothesis.

Such was the state of the field at the time when we set out to formally test the idea that the *Drosophila* Keap1 and CncC comprise the fly Keap1/Nrf2 system. First, we found that *cncC* mRNA was most abundantly expressed in the alimentary canal of flies [88], which was reminiscent of the broad expression of Nrf2 in the digestive tract of mammals [89, 90]. *keap1* mRNA was also expressed in the alimentary canal, and both genes were also highly expressed in the Malpighian tubules, which are major sites of detoxification in flies corresponding functionally to vertebrate kidneys [88]. The expression of the candidate *Drosophila* homologs of Nrf2 and Keap1 in the digestive tract, which represents the first line of defense against ingested environmental stressors, and in the major detoxification organs supported the notion that *Drosophila* might employ Keap1/Nrf2 for stress defense, resembling vertebrates.

To test whether *Drosophila* Keap1 and CncC have functional homology to vertebrate Keap1 and Nrf2, we examined whether they can regulate the transcription of genes homologous to those regulated by Nrf2 in mammals. For this purpose we selected as candidate target genes *keap1* itself, which in vertebrates is regulated by Nrf2 in an autoregulatory loop [91], and *gstD1*, a prototypical oxidative stress response gene encoding a well-characterized detoxification enzyme [92], in whose upstream region we identified a sequence matching the ARE consensus [93]. Overexpression of CncC in flies was sufficient to substantially elevate the expression of *keap1* and *gstD1* mRNA [88]. Furthermore, *gstD1* and *keap1* levels were reduced by the conditional knockdown of CncC via RNA interference, and *gstD1* levels were increased by the conditional knockdown of Keap1. Consistently, *gstD1* levels were increased in flies heterozygous for either of the two *keap1* null (i.e., complete loss-of-function) mutations that we generated [88]. Taken together, these findings established *gstD1* and *keap1* as CncC-regulated genes, indicating functional homology of CncC with Nrf2.

To further address whether Keap1 and CncC mediate antioxidant and detoxification responses, we constructed transgenic reporter flies expressing green fluorescent protein (GFP) or β-galactosidase (lacZ) under the control of the ARE-containing genomic sequence upstream of the *gstD1* gene. In unstressed conditions fluorescence was detected most readily in the gut of *gstD-GFP* flies, consistent with the expression of *cncC* and *keap1* in this tissue [88]. Reporter activity was induced by CncC overexpression and by Keap1 knockdown in several tissues of flies at various stages of development. Using a genetic technique that is readily available in flies, we generated clones of cells in the brain that were homozygous for a *keap1* null allele, and found that the *gstD-lacZ* reporter was induced in these tissues. Moreover,

gstD-GFP reporter activity was induced when the flies were exposed to various oxidants, including hydrogen peroxide, the free radical generator Paraquat, the glutathione-depleting agent diethyl-maleate, and the heavy metal arsenic. In addition, the reporter was activated by the known Nrf2-activating compounds oltipraz and *tert*-butylhydroquinone. The knockdown of CncC suppressed *gstD-GFP* reporter inducibility by such conditions, and no induction was detected in transgenic reporter flies bearing a version of the enhancer in which the ARE had been mutated (*gstDΔARE-GFP*) [88].

To test whether an ARE sequence is sufficient to mediate transcriptional activation downstream of Keap1/CncC, we employed reporter flies containing a lacZ transgene driven by a synthetic sequence comprising a concatamer of binding sites that conformed to the consensus ARE and had been shown to respond to CncB (which has the same DNA binding site as CncC) [84]. Indeed, lacZ was markedly induced in CncC gain-of-function or Keap1 loss-of-function conditions, further supporting the functional conservation of ARE-mediated transcriptional responses in *Drosophila* [88]. Finally, we examined the contribution of Keap1 and CncC to oxidative stress resistance at the level of the intact organism. The conditional overexpression of CncC significantly increased the survival of flies that had been exposed to a semilethal dose of Paraquat; in contrast, CncC knockdown significantly decreased survival (Fig. 22.2) [88]. Taken together, these results demonstrated the functional homology of *Drosophila* Keap1 and CncC to vertebrate Keap1 and Nrf2, respectively. Much like their mammalian counterparts, *Drosophila* CncC and Keap1 regulate ARE-mediated transcription and detoxification gene expression and are crucial for the organism's defense against oxidative stress. Thus this work established *Drosophila* as a new genetically accessible model system for the study of this important homeostatic and cell survival pathway.

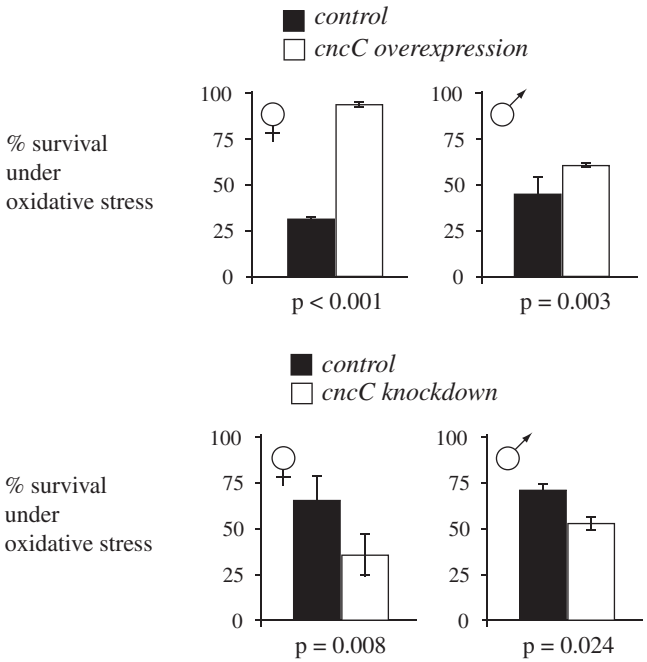

Fig. 22.2 CncC regulates oxidative stress resistance in *Drosophila*. (Top) Conditional ubiquitous overexpression of CncC before exposure to the free radical generator Paraquat significantly increases the flies' survival rate compared to genetically identical controls. Data are shown as means ± SE of three experiments performed in triplicate. (Bottom) RNAi-mediated conditional ubiquitous knockdown of CncC before exposure to Paraquat significantly decreases the flies' survival rate. This figure is adapted from Sykiotis GP and Bohmann D, Keap1/Nrf2 signaling regulates oxidative stress tolerance and lifespan in *Drosophila*, *Dev Cell*, vol. 14, p. 76–85, Copyright 2008, with permission from Elsevier.

22.1.4 Advantages and Limitations of Drosophila as a Model Organism to Study the Nrf2 Pathway

22.1.4.1 Advantages For a century *Drosophila* has been one of the workhorses of genetic research [94, 95]. Therefore, the characterization of Keap1 and Nrf2 in this organism offers the possibility to use flies as a genetic platform for deriving new insights on the regulation and functions of this pathway. Before specific opportunities for such endeavors are presented, it is important to note that as a model organism *Drosophila* has several advantages over other models like worms, zebrafish, and mice. Naturally, it also has certain limitations. Although a general comparison of flies to other model systems is beyond the scope of this chapter, it is useful to highlight those advantages and limitations of flies that are relevant for the study of the Nrf2 pathway. Some of these features may be specific to the study subject. For example, the relatively short life span of the fly compared to mice makes it a very useful model for investigations of the genetic mechanisms that regulate aging and longevity, especially since such mechanisms are known to involve increased resistance to oxidative stress (see next section).

Other features of *Drosophila* that impact on its utility as a model organism for studies on Nrf2 signaling are directly related to its genetic properties. One major genetic feature of *Drosophila* is its low degree of genetic redundancy. Whereas higher organisms can have multiple genes encoding functionally related and even redundant proteins, this is rather uncommon in flies. At the same time, the large majority of human genes have known or presumed homologs in the fly genome. Thus flies strike a very useful balance of good conservation and low redundancy. This is true also in the case of the

Nrf2 pathway: Since CncB and CncA lack the Keap1-binding domain, CncC is assumed to be the only Nrf2 homolog. Flies, like mice and humans, have a single locus encoding Keap1, whereas worms have none and zebrafish have two [96]. Notably, the fly genome also carries only a single gene encoding a small Maf (*CG9954*), while none has been identified in worms; and anyway Skn-1 lacks the leucine zipper domain that mediates the dimerization of Nr2f homologs in higher organisms with Mafs, and thus this aspect of Nrf2 function does not appear to be conserved in the worm [97]. Conversely, the small maf gene family is represented by no fewer than three members in vertebrates [98]. The genes encoding components of the Nrf2 ubiquitination and degradation machinery (Cul3, Rbx1, Cand1, and others) are also well conserved in *Drosophila*. This combination of conservation with little genetic redundancy greatly facilitates investigations of Nrf2 signaling in flies, including studies that might be impossible or very difficult in organisms with either an incomplete Nrf2 module or a high degree of redundancy. At the same time, this property of *Drosophila* suggests that the results of fly studies might be readily extrapolated to higher organisms, in which the pathway is similarly constructed, to form the basis for experimental validation of insights derived from flies.

Another major advantage of *Drosophila* is its exquisite amenability to genetic manipulation. Various genetic technologies have been developed that allow the up- or downregulation, inactivation, or ectopic expression of a chosen gene either ubiquitously or in a tissue-specific and/or temporally controlled manner. With these methods, *Drosophila* offers increased efficiency, higher speed, and lower cost of experimentation compared to vertebrates. Thus, now that the principal role of CncC in oxidative stress protection of *Drosophila* has been established, flies can be used to investigate the tissue-specific requirement for Nrf2 signaling in defending various organs and sustaining their specialized functions [4]. Moreover, the phylogenetic conservation of Nrf2 signaling in flies offers the opportunity to identify new genes that regulate the pathway through unbiased genetic screens. The critical requirement for conducting such screens is to establish an appropriately sensitive in vivo or cell-based assay reflecting the activity status of the pathway, such as the one shown in Fig. 22.3. It then becomes possible to identify pathway components and modulators either in vivo—by utilizing available large collections of flies harboring chemically induced mutations, transposable elements disrupting gene function, or transgenes expressing gene-specific double-stranded RNAs (dsRNAs) that trigger gene knockdown via

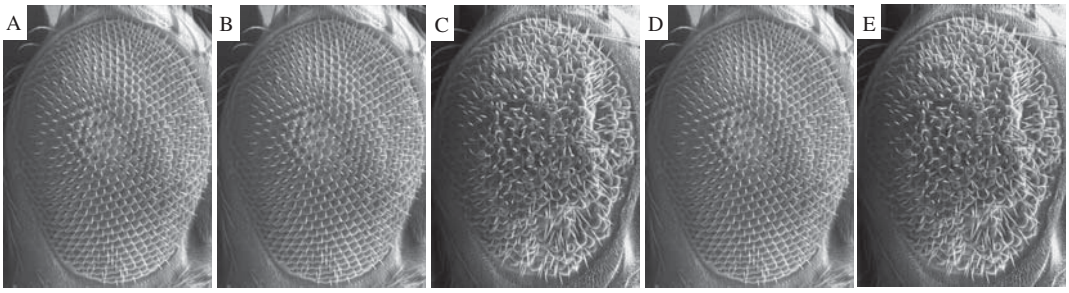

Fig. 22.3 A genetically sensitized in vivo *Drosophila* assay of Keap1/Nrf2 system activity. (A) The compound eye of *Drosophila* comprises about 800 individual ommatidia (meaning "small eyes") arranged in a regular pattern. This pattern can be disrupted by the genetic manipulation of cell signaling during eye development, generally without causing lethality. Thus the *Drosophila* eye is a convenient system for the analysis of regulatory interactions between genes. (B) The wild-type pattern is unaffected by the eye-specific overexpression of Keap1. (C) In contrast, eye-specific overexpression of CncC results in a rough-appearing eye due to loss and disrupted arrangement of ommatidia. (D) Coexpression of Keap1 and CncC results in wild-type eye appearance. The complete suppression of the CncC-induced phenotype by Keap1 is consistent with its role as an inhibitor of Nrf2 signaling. Thus the interaction of overexpressed Keap1 and CncC in the *Drosophila* eye is an in vivo assay reflecting Nrf2 pathway activity. (E) The eye interaction of Keap1 and CncC is sensitive to genetic manipulation, as demonstrated by the reemergence of the rough eye phenotype in the presence of either one of two *cul3* alleles (the chemically induced mutation $cul3^{2}$, and the transposable element-induced mutation $cul3^{06430}$). Therefore, this eye interaction assay, or other similarly sensitized in vivo or cell-based readouts, could be used to test whether specific candidate genes are regulators or components of the Nrf2 pathway, as well as to identify new such genes through unbiased genetic screens. This figure is adapted from Sykiotis GP and Bohmann D, Keap1/Nrf2 signaling regulates oxidative stress tolerance and lifespan in *Drosophila*, *Dev Cell*, vol. 14, p. 76–85, Copyright 2008, with permission from Elsevier.

RNA interference (RNAi)—or in cultured cells—by screening whole-genome collections of gene-specific dsRNAs. Such screens are, of course, also feasible in *C. elegans* and in cultured mammalian cells, and they are currently being pursued in those systems as well to dissect the Nrf2 pathway. The experimental facility of *Drosophila* stems again from its combination of good conservation and low redundancy. This increases the power to identify as screen "hits" genes that may be absent in worms and/or may escape detection in mammalian cells because of mutual compensation among redundant members of the same gene family. The relative ease with which secondary screens can be conducted to validate and characterize such hits is another advantage of the *Drosophila* system. Thus fly screens aiming to expand the CncC pathway should be vigorously pursued and should be cross-referenced to the few worm RNAi screens on SKN-1 that have been published [99–101], as well as to future worm and mammalian screens. Previously unknown pathway modulators discovered in this manner may represent favorable targets for the ubiquitous or tissue-specific pharmacological modulation of Nrf2.

22.1.4.2 Limitations Before embarking on fly studies focused on the Nrf2 pathway, one should also bear in mind certain limitations of the experimental system. One such feature is that null mutations in each of the core pathway components lead to lethality during development. Not only *cnc* isoform-nonspecific but also *cncC*-specific null mutations are lethal [84]. This is in contrast to mice, where $nrf2^{-/-}$ animals are viable and fertile [89]. Lethality can be a useful phenotype for developmental studies, where it can facilitate the discovery of novel molecular mechanisms as well as interacting genes. However, it is an obvious disadvantage for studies of homeostasis and responses to external challenges in the mature organism. In our studies, we circumvented this problem by using a conditional RNAi-mediated knockdown of CncC in adult flies to test for effects on oxidative stress tolerance [88]. Nevertheless, such methods are unlikely to completely eliminate gene expression and to phenocopy null mutations, and thus probably underestimate the magnitude of CncC-mediated effects. The drosophilist's quiver holds several other arrows to hit these targets, including the isolation of temperature-sensitive mutations that behave as nulls, hypomorphs, or amorphs depending on the temperature at which the flies are reared, or the rescue of the developmental lethality of a null allele by tissue-specific transgenic expression of a corresponding wild-type cDNA construct. The application of such methods, however, would be substantially informed by first elucidating the reasons for the developmental lethality of *cncC*-specific alleles. Since SKN-1 is critical to the formation of the digestive system during worm embryogenesis [76], the developmental lethality of CncC during the larval stage might also be related to a requirement for proper development or function of the gut, where *cncC* mRNA is highly expressed [88]. Similarly to *cncC*, loss of both *keap1* alleles leads to lethality during larval development [88]. The lethality of *keap1* null alleles may be due to unchecked induction of CncC, because, whereas conditional moderate overexpression of CncC can confer oxidative stress resistance, its constitutive high overexpression can be lethal to both individual cells as well as the organism [88]. Unfortunately, the developmental lethality of *cncC* mutations [84] precludes a straightforward genetic test of this hypothesis.

In addition to its genetics, the anatomy and biology of *Drosophila* each place limitations on the use of flies to study the Nrf2 system. Even though the ability to manipulate gene expression in flies in a tissue-specific manner could help to investigate some of the tissue-specific functions of Nrf2 signaling, the small size of flies can make it technically challenging, though not necessarily impossible, to evaluate some of these potential functions. Such difficulties may be encountered when examining, for example, dopaminergic neurons in studies on neuronal degeneration (see next section) or oenocytes, the equivalent of vertebrate liver cells, in potential studies on metabolism. For studies that involve the administration of Nrf2-activating compounds (or, potentially, Nrf2-inhibiting compounds), the choice of an appropriate solvent can become a challenging problem. The most commonly used solvent both for maintaining large-scale compound libraries and for dissolving individual compounds is dimethyl sulfoxide (DMSO). Unfortunately, DMSO is an oxidant [102] and may be deleterious in high concentrations. Intact flies are generally assumed to tolerate concentrations of DMSO in their diet of up to 1%. However, it has been shown that even 0.5% of DMSO in the food may lead to developmental, reproductive, and cellular toxicity [103]. This observation cautions against the assumption that "low" concentrations of DMSO are innocuous. Consistently, we have found that DMSO concentrations as low as 0.1% can interfere in vivo with readouts that depend on CncC activity (Fig. 22.4). Thus, if DMSO is used as a solvent in experiments focused on the Nrf2 pathway in flies, caution must be exercised in the interpretation of the experimental results. Specifically, if the administration of CncC-activating compounds dissolved in DMSO is observed to exert beneficial effects against a toxic insult or other detrimental condition compared to the administration of the DMSO solvent alone, this could in principle reflect (partly or wholly) a rescue of DMSO's toxicity or of an additive/synergistic toxic effect of the two oxidants (DMSO and the insult under study).

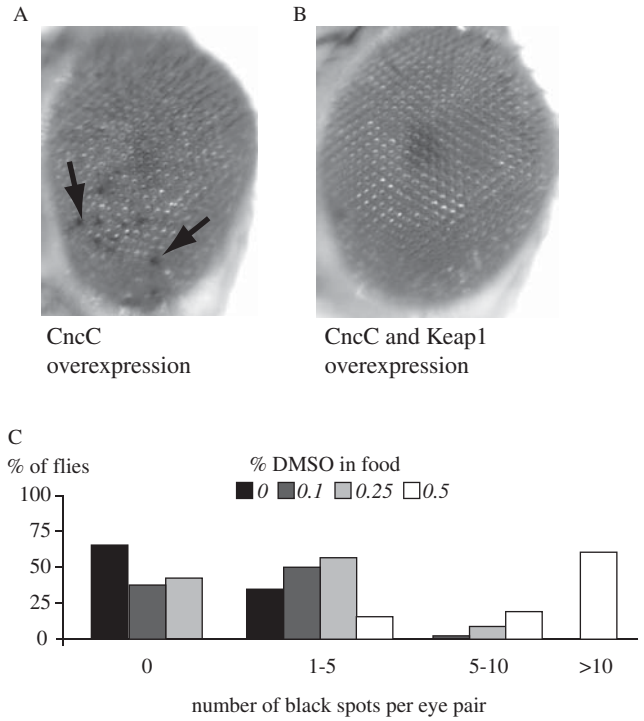

Fig. 22.4 The commonly used solvent DMSO can affect Nrf2 pathway activity in *Drosophila*. (A) In addition to missing and disarranged ommatidia, the eyes of flies overexpressing CncC show black spots (indicated by arrows) that probably represent necrotic and/or melanotic ommatidia. The number of these spots per eye pair correlates with the severity of the phenotype. (B) When Keap1 is coexpressed with CncC in the eye and the flies develop in normal food, the presence of black spots in the eye is suppressed, consistent with the inhibitory function of Keap1. (C) When flies coexpressing CncC and Keap1 in the eye develop in food containing DMSO, which is an oxidant, black spots are detected, and their number correlates positively with the concentration of DMSO. More than 300 flies were scored at each DMSO concentration.

Ethanol may be a more appropriate solvent in such cases, at least in cultured cells, as it most likely evaporates from the medium that flies grow in/on. We have also found lipid-based solvents to be problematic because of their high viscosity: As they are excreted from the flies' guts on the container walls, they tend to trap adult flies—such solvents may, however, be suitable for studies on larvae.

Finally, there is no escaping the fact that *Drosophila* is a latecomer to the Nrf2 arena, in which mammalian studies have been ongoing for more than a decade and worm studies for several years. Thus the cost-benefit ratio of investing in flies to address a research question on the Nrf2 system should be carefully weighed. The study topics ought to be carefully selected to take advantage of *Drosophila*'s unique assets and relative strengths, so as to facilitate real breakthroughs and not mere replications or extensions of mammalian studies. Thus a high degree of strategic planning will be required over the short term to "catch up" with worms and vertebrates in characterizing the pathway, while at the same time proving the value of the fly system for Nrf2 research by deriving unique insights not readily available from these historically leading experimental platforms. Table 22.1 lists some of the fundamental questions that should be addressed as soon as possible to better characterize the pathway in flies, and highlights research directions that should be pursued in parallel because they have the potential to lead to contributions of major impact in the field of Nrf2 biology.

22.1.5 New Insights Derived from *Drosophila* on the Functions of the Nrf2 System

By establishing the phylogenetic conservation of Nrf2 signaling in *Drosophila* and exploiting the experimental

TABLE 22.1 Prospects for Nrf2-related research in the *Drosophila system*.

Fundamental questions that need to be addressed to better characterize the Keap1/Nrf2 system in *Drosophila*	Questions of Nrf2 biology for which *Drosophila* could be used to derive novel insights of potentially major impact
• Is the single small Maf of *Drosophila* the interaction partner of CncC in activating transcription via the ARE?	• Which genes are required for the activation of Nrf2 signaling by oxidants and other inducers?
• Do CncA and CncB have roles in regulating ARE-mediated protective responses? Is CncA, which lacks transcriptional activity, an ARE repressor? Is CncB, which lacks a Keap1-binding domain, a constitutive ARE activator?	• Which genes encode targets for the pharmacological activation or inhibition of Nrf2 signaling?
• Is CncC degraded via the action of Cul3, similarly to vertebrate Nrf2?	• By which mechanism(s) does the relative abundance of ARE-binding factors determine the transcriptional status of Nrf2 target genes?
• Which is the set of CncC target genes, and does it include genes with tissue-specific homeostatic roles like vertebrate Nrf2?	• How is Nrf2 signaling involved in promoting longevity and extending the healthy life span?
• Which are the naturally-occurring oxidative stressors that CncC has evolved to protect flies against?	• How does Nrf2 prevent the degeneration of dopaminergic neurons in vivo?
• What are the reasons for the developmental lethality of flies homozygous for *cncC* and *keap1* mutant alleles?	• What are the mechanisms by which Nrf2 affects and is affected by the metabolism of sugars and lipids?

advantages of flies, new insights have already been gleaned on the functions of the Nrf2 system; albeit few in number, these advances are of substantial impact, as they pertain to the roles of Nrf2 in the regulation of longevity [88], the degeneration of dopaminergic neurons in Parkinson disease [104], and the resistance to the environmental pollutant methylmercury [105].

22.1.5.1 The Keap1/Nrf2 Pathway as a System Controlling Longevity One of the most interesting biological questions is whether and how the aging process is regulated and how longevity and youthfulness might be extended. According to the oxidative stress theory of aging, oxidative damage to biological macromolecules is a key driver of aging; conversely, delaying the accumulation of oxidation products in the cells and tissues of an organism could promote longevity [106]. Studies in model organisms have supported this hypothesis by showing that life span extension by various genetic or dietary manipulations correlates with increased oxidative stress resistance, as well as that the overexpression of antioxidant genes can not only augment oxidative stress tolerance but can also promote longevity [107–110]. As a master regulator of antioxidant and detoxification responses, Nrf2 was a plausible regulator of the aging process. Indeed, SKN-1 was shown to be required not only for normal life span in *C.elegans* [77] but also for the life span-extending function of caloric restriction through its function in a pair of specialized neuroendocrine cells in the worms' brain [111]. Having characterized the Nrf2 system in flies, we undertook a study to address whether activation of Nrf2 signaling could promote longevity under normal laboratory growth conditions [88].

For this test we employed flies heterozygous for either of two independent *keap1* alleles. These flies showed elevated mRNA levels of the CncC target gene *gstD1* (Fig. 22.5), suggesting that loss of one copy of the *keap1* gene can result in a gain of CncC function in vivo [88]. Male heterozygous *keap1* mutant flies showed increased resistance to Paraquat and also had a median life span significantly longer than that of their otherwise genetically identical siblings (Fig. 22.5) [88]. These findings demonstrated that partial loss of function of the Nrf2 pathway's negative regulator can have significant beneficial effects on the oxidative stress tolerance and longevity of male *Drosophila*. This was consistent with the oxidative stress hypothesis of aging and also provided the first evidence for a role of Keap1 in life span regulation. Interestingly, female *keap1* heterozygotes did not show significant differences in either Paraquat resistance or longevity (Fig. 22.5). Although we have not yet thoroughly investigated the underlying reasons for this sexual dimorphism, we suspect that it reflects hormonal or metabolic differences between the sexes. A subsequently published study in *C. elegans* showed that SKN-1 mediates the life span extension associated with reduced insulin/IGF-like signaling and that its transgenic overexpression at certain levels can also confer longevity [112]. Taken together, these studies in invertebrates revealed a new role for Nrf2 signaling as an antiaging and life span-extending factor that warrants detailed characterization in invertebrate and vertebrate models [further discussed in 8].

22.1.5.2 Protective Role of Nrf2 Against Parkinson Disease Oxidative stress in the nervous system is associated with neuronal cell death during the pathogenesis of multiple neurodegenerative disorders, including Parkinson disease (PD) [113, 114]. PD is an age-related disorder in which severe oxidative damage occurs in the substantia nigra, resulting in the degeneration and loss of dopaminergic (DA) neurons [115]; this manifests clinically as progressive functional impairment with ultimate mortality. Effective treatments for PD are lacking, and thus a better understanding of the mechanisms regulating DA neuron survival and death is urgently warranted. A series of recent studies have implicated the Nrf2 signaling pathway as an important factor for DA neuron survival during PD pathogenesis. Notably, compounds that are approved treatments for PD, such as bromocryptine and selegiline, were found to activate Nrf2 [116, 117]. Genetic and pharmacological studies also documented the importance of Nrf2 as a protective factor against PD in mouse models of the disease in which DA neuronal toxicity is elicited through administration of the prooxidant compounds 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA) [44, 118–120]. Furthermore, a recent human study documented that the few surviving DA neurons of postmortem brains from PD patients showed increased abundance and nuclear localization of Nrf2 [68]. Consistently, other studies have shown that the expression or function of p62 and DJ-1, which are both proteins modulating Nrf2, is compromised in neurodegeneration, likely leading to impaired antioxidant response. p62 is a cytoplasmic protein that mediates the formation of ubiquitinated aggregates that are removed by autophagy [121, 122], and it is localized to intracellular aggregates in various neurodegenerative diseases [123]. p62 binds directly to Keap1 and increases the protein stability of Nrf2 [124–127]; moreover, Nrf2 upregulates the transcription of *p62* [126, 128–130]. The *p62* promoter shows increased oxidation in PD leading to reduced *p62* gene transcription, which may account for Nrf2 dysfunction [131]. Regarding DJ-1, inherited loss-of-function mutations in its gene (*PARK7*) are associated with early-onset

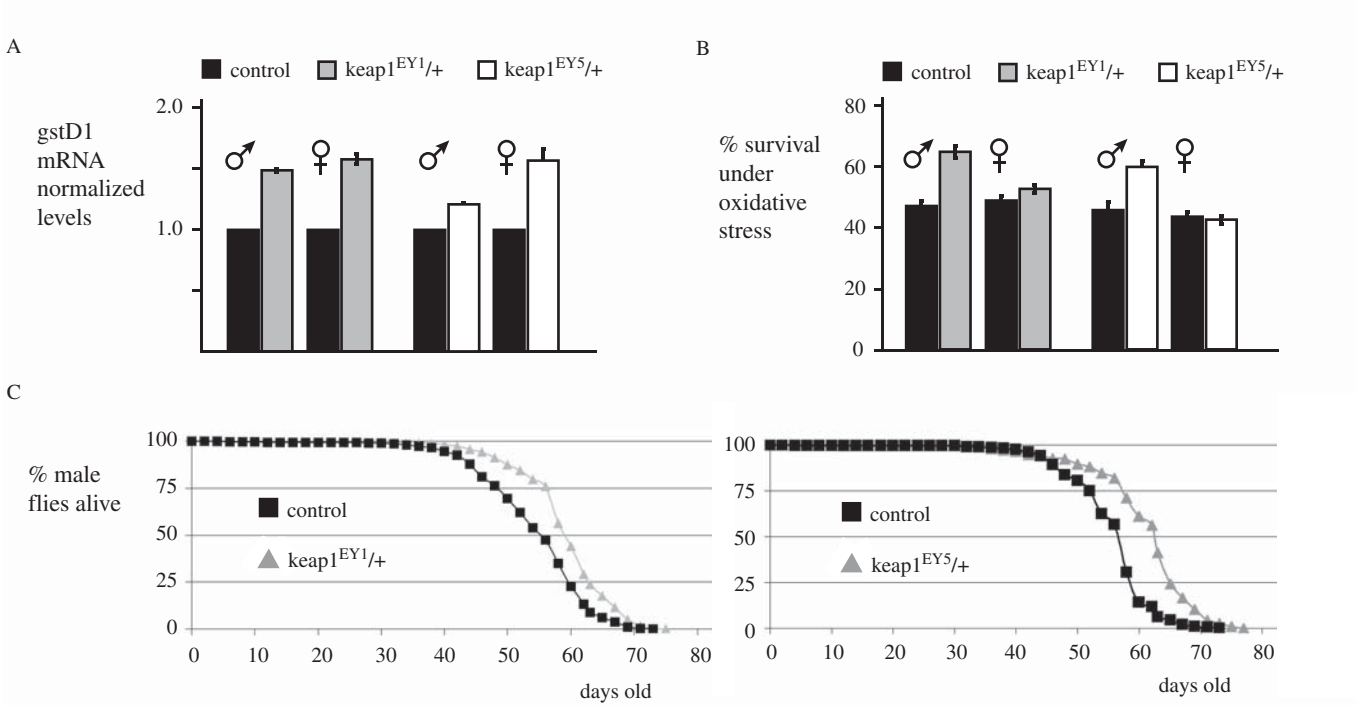

Fig. 22.5 Heterozygosity for *keap1* is associated with increased oxidative stress resistance and extended longevity in male *Drosophila*. (A) The $keap1^{EY1}$ and $keap1^{EY5}$ alleles were created by the reinsertions of mobilized transposable elements in the coding sequence of the *keap1* gene. Both alleles cause larval lethality and are presumed nulls. One-day-old male and female *keap1* heterozygous flies have significantly elevated *gstD1* mRNA expression levels (normalized to *actin* mRNA levels) as quantified by real-time RT-PCR. Control flies were siblings of the *keap1* heterozygotes and were genetically identical with them except that they were homozygous wild-type for *keap1*. Data are shown as means ± SE of three experiments performed in duplicate. (B) Male, but not female, heterozygous *keap1* flies show a significantly higher survival rate after exposure to Paraquat than their otherwise genetically identical wild-type sibling controls. Data are shown as means ± SE of four experiments performed in triplicate. (C) Under standard culture conditions, male heterozygous *keap1* flies live significantly longer than their otherwise genetically identical wild-type sibling controls. Data are shown as percentage of flies alive at each age; 500–700 flies of each genotype and sex were assayed. This figure is adapted from Sykiotis GP and Bohmann D, Keap1/Nrf2 signaling regulates oxidative stress tolerance and lifespan in *Drosophila*, *Dev Cell*, vol. 14, p. 76–85, Copyright 2008, with permission from Elsevier.

Parkinsonism [132, 133]. DJ-1 protects neurons from oxidative stress, but its functionality is lost upon oxidation [134]. Because DJ-1 stabilizes Nrf2 by preventing its association with Keap1, the oxidation of DJ-1 in PD could compromise Nrf2 signaling and precipitate the collapse of the antioxidant response system [135]. In summary, multiple independent lines of evidence highlight Nrf2 as an important factor that protects against PD.

In an effort to elucidate PD pathogenesis, several *Drosophila* models of the disease have been created by manipulating genes implicated in rare hereditary forms of early-onset parkinsonism. These include flies harboring loss-of-function mutations in the genes encoding parkin or transgenically overexpressing human α-synuclein in the DA neurons [136–138]. The resulting phenotypes are reminiscent of human PD and include premature loss of DA neurons, locomotor deficits, and increased sensitivity to oxidative stress [136–143]. As genetic models of PD, these flies facilitate the in vivo discovery of genes, pathways, and compounds that may be useful for the prevention and/or treatment of this debilitating disorder. Loss-of-function *parkin* mutations lead to the degeneration of a cluster of DA neurons termed protocerebral posterior lateral 1 (PPL1). This neuronal loss was exacerbated by a loss-of-function allele of the detoxification gene *gstS1*; conversely, overexpression of gstS1 specifically in DA neurons reduced *parkin*-associated DA neuronal loss [139]. Significant neuronal loss in the PPL1 cluster is also observed when human α-synuclein is expressed in DA neurons [138]. Loss-of-function mutations in *gstS1* or in *gclm*, which encodes the rate-limiting enzyme in the synthesis of the major antioxidant glutathione, further exacerbate the loss of DA neurons due to α-synuclein toxicity [138]. Conversely, overexpression of either of these enzymes

significantly protected DA neurons from α-synuclein-induced degeneration [138]. Importantly, the administration of the potent Nrf2 inducers allyl disulfide or sulphoraphane upregulated both *gstS1* and *gclm* and prevented the loss of DA neurons in both the parkin and α-synuclein fly models of PD [138].

More recently it was shown that decaffeinated coffee and nicotine-free tobacco, both of which can activate Nrf2 signaling, provide neuroprotection in the *parkin* and α-synuclein fly PD models [104]. RNAi-mediated knockdown of Cnc enhanced the toxicity of α-synuclein and suppressed the rescuing effect of decaffeinated coffee and nicotine-free tobacco [104]. Unfortunately, the transgene employed to knock down Cnc was non-isoform selective, that is, it was designed to target all three Cnc isoforms [104]. It therefore remains to be formally tested whether the Nrf2 homolog CncC is the isoform protecting DA neurons from α-synuclein toxicity. Moreover, it will be important to investigate whether the genetic activation of Nrf2 signaling is sufficient to ameliorate the toxicity of α-synuclein. A recent study in a *C. elegans* model of PD induced by exposure to the heavy metal manganese showed that SKN-1 activation is required to protect DA neurons from manganese-induced degeneration [144]. The species conservation of the protective role of Nrf2 against DA neuron degeneration supports the use of the genetically more tractable invertebrate model organisms for studies investigating Nrf2 as a preventive and/or therapeutic target in PD. In the near future, we anticipate that such studies will be expanded to other genetic models of human neurodegenerative diseases.

22.1.5.3 Nrf2 and Methylmercury Toxicity Methylmercury (MeHg) is an environmental pollutant with electrophilic chemical properties [145], to which humans can be exposed through diets rich in predatory fish like tuna and swordfish. MeHg easily crosses the blood-brain barrier and has potent neurotoxic properties [146]. Therefore, elucidating the mechanisms of resistance to MeHg is an issue of major public health importance. MeHg was found to interact with recombinant Keap1 in vitro, and Nrf2 overexpression could attenuate MeHg-induced cytotoxicity in cultured mammalian neuroblastoma cells [147]. Similarly, MeHg was shown to activate Nrf2 in cultured astrocytes and microglial cells, and inhibition of Nrf2 activation correlated with increased MeHg toxicity [148, 149]. In addition, primary hepatocytes from Nrf2-deficient mice accumulated more MeHg and were more susceptible to MeHg-induced cytotoxicity than control cells [147]. In contrast, primary hepatocytes from hepatocyte-specific conditional Keap1-deficient mice accumulated less MeHg and were more resistant to its toxic effect [147]. Thus the activation of Nrf2 reduces MeHg toxicity, by both increasing its detoxification in the liver and augmenting the resistance of its target tissues.

Whereas a number of cytotoxic mechanisms of MeHg have been characterized in differentiated cells, its mode of action in the developing nervous system in vivo is less clear. In primate and rodent models, MeHg exposure has been shown to cause aberrant cell migration and disorganized patterning of the brain's cortical layers [150–152]. However, these animal models are not sufficiently accessible genetically to facilitate elucidation of the molecular and cellular pathways targeted by MeHg. Notably, a recent study established the *Drosophila* embryo as a platform for elucidating MeHg-sensitive pathways in neural development [153]. When developing fly embryos were exposed to MeHg, a dose-dependent inhibition of embryonic development was observed, evident as failure of the embryos to hatch to the larval stage. In addition, specific defects in neural development were documented, including abnormalities in neuronal and glial cell patterning consistent with disrupted migration; and pronounced defects in neurite outgrowth were observed in both central and peripheral neurons. Importantly, the ectopic expression of CncC enhanced embryonic development and hatching in the presence of MeHg [153]. Thus the protective role of Nrf2 against MeHg is conserved in *Drosophila*, highlighting the fly embryo as a facile model system for investigating mechanisms of MeHg resistance. Moreover, this work suggests that CncC may protect flies from a battery of heavy metals and environmental pollutants that they encounter in their natural environment. Consistently, in our initial characterization of CncC/Keap1 signaling we had shown that arsenic, another heavy metal and environmental pollutant known to activate Nrf2 [129], induces ARE-mediated transcriptional responses in *Drosophila* [88].

C. elegans has also been recently developed as a model for investigating the mechanisms of MeHg toxicity and resistance [154–156]. It was found that low, chronic exposure of worms to MeHg confers embryonic defects, developmental delays, decreases in brood size and animal viability, and, interestingly, DA neuron degeneration [156]. MeHg exposure resulted in the induction of glutathione-*S*-transferases [155, 156], which was largely dependent on SKN-1 [156]. Moreover, SKN-1 was shown to be expressed in the DA neurons, and reducing SKN-1 gene expression increased MeHg-induced animal vulnerability and DA neuron degeneration [156]. Taken together, these recent worm and fly studies highlight the utility of the genetically tractable invertebrate models for studies on MeHg toxicity and resistance. More such studies can be anticipated in the near future, including genetic screens for which these

organisms are ideally suited. The protective role of SKN-1 against MeHg toxicity to DA neurons [156] also prompts testing whether CncC has a similar role in flies, which would be consistent with the recent findings in the fly PD models reviewed in the previous section.

22.2 CONCLUSION

Because Nrf2 has emerged as a critical transcription factor for the maintenance of tissue homeostasis; the protection from oxidative and electrophilic stressors; and the prevention of diverse pathologies, it holds substantial promise as a therapeutic target for human diseases. To date, most progress in the Nrf2 field has resulted from studies in mice and cultured mammalian cells. Flies have recently been established as an invertebrate genetic model organism in which Nrf2 signaling is functionally conserved [88]. Thus, together with worms, they have the potential to complement vertebrates in this exciting research area. Through their unique experimental strengths, flies could also facilitate breakthroughs of potentially major impact on human health issues, such as the extension of the healthy life span, the prevention of neuronal degeneration, and the treatment of metabolic disorders [157], which are also generally accompanied by elevated levels of oxidative stress.

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