Identification and Characterization of Proteins with Novel Functions in Nrf2 Signaling

by

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Biographical Sketch

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Publications:

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Abstract

Oxidative stress causes widespread damage to biomolecules, leads to different pathological conditions and contributes to aging. The Nrf2 transcription factor, a major mediator of oxidative stress responses, controls gene expression programs that protect multiple organs from oxidative damage, delay the onset of some age-associated diseases and promote longevity at least in some organisms. In an unstressed condition, Nrf2 interacts with its cytoplasmic inhibitor Keap1, which targets it for proteasomal degradation. Oxidative stress prevents Keap1-mediated degradation of Nrf2, resulting in its accumulation and nuclear translocation. In the nucleus, Nrf2 dimerizes with a small Maf protein, binds to 'Antioxidant Response Elements' and induces multiple antioxidant and detoxification genes. Complete understanding of the molecular mechanisms of Nrf2 regulation is important to assess its role in normal physiology and disease. The key components of Nrf2 signaling are conserved in *Drosophila* where CncC is the homolog of mammalian Nrf2. In order to study mechanisms of Nrf2 function in *Drosophila*, cell-based and *in vivo* transcriptional reporters for Nrf2 were developed. A cell-based dsRNA library screen was carried out to find novel regulators of Nrf2 signaling. Among others we identified Cdk12 and Fs(1)h. Cdk12, a RNA PolII-CTD kinase, was found to be required for CncC target gene expression in a cell-autonomous manner and to be important for oxidative stress resistance. In contrast, Fs(1)h, the sole member of the BET

protein family in *Drosophila*, was identified as an inhibitor of CncC. Bromodomain-containing BET proteins have complex functions in chromosome organization and the control of gene expression. Fs(1)h was found to physically interact with CncC in a manner that requires the function of its bromodomains and the acetylation of CncC. Treatment of cultured *Drosophila* cells or adult flies with the BET protein inhibitor JQ1 de-represses CncC transcriptional activity and induces protective gene expression programs. The mechanism by which Fs(1)h inhibits CncC function is distinct from that of Keap1. Consistent with this, combinations of drugs that can specifically target Keap1 and Fs(1)h cause a synergistic and specific activation of CncC dependent gene expression. This synergism might be exploitable for the design of combinatorial therapeutic approaches, targeting Nrf2 in various diseases.

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Professor Dirk Bohmann supervised the work described in the thesis. The thesis advisory committee members, Professors Dirk Bohmann, Vera Gorbunova, Douglas Portman and Michael Welte, provided scientific inputs and suggestions.

The experiments described in the thesis were performed by the author, except for the dsRNA library screen, which was carried out by Kerstin Spirohn in the laboratory of Professor Michael Boutros.

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List of Abbreviations

AMP	Adenosine monophosphate
BTB	Broad complex, tramtrack, bric-a-brac
cDNA	Complementary deoxy ribonucleic acid
Cdk	Cyclin-dependent kinase
CncC	CncC Cap n collar protein isoform C
DNA	Deoxy ribonucleic acid
ERK1/2	Extracellular signal regulated kinase 1/2 Ras Rat sarcoma
Flp	Flipase
Fs(1)h	Female sterile (1) homeotic
GclC	Glutamyl cystein ligase- catalytic subunit
GclM	Glutamyl cystein ligase- modulatory subunit
GFP	Green fluorescent protein
GPX2	Glutathione peroxidase 2
GstD1	Glutathione S- transferase D1
GstE9	Glutathione S- transferase E9
HO-1	Hemoxygenase 1
Hs	Heat shock
IGF-1	Insulin-like growth factor-1

Keap1	Kelch-like ECH-associated protein 1
NF-κB	Nuclear factor kappa beta
NQO1	NAD(P)H dehydrogenase [quinone] 1
Nrf2	Nuclear factor-erythroid 2-related factor-2
qRT-PCR	Quantitative real time polymerase chain reaction
RFP	Red fluorescent protein
RNA	Ribonucleic acid
RNAi	RNA interference
TOR	Target of rapamycin
TRE	TPA response element
UAS	Upstream activating sequence

Chapter 1

General Introduction

1.1. Nrf2 signaling in redox regulation, aging and metabolism

1.1.1. Redox homeostasis and oxidative stress

Oxidative stress is characterized by an increase in the intracellular levels of reactive oxygen species (ROS). ROS are diverse and highly reactive ions and small molecules that include the hydroxyl radical ($^{\bullet}OH$), the superoxide anion ($^{\bullet}O2^{-}$) and hydrogen peroxide (H_2O_2). These are byproducts of oxygen metabolism in mitochondria and peroxisomes and are also produced by membrane-associated NAD(P)H oxidase, cytochrome C oxidase and xanthine oxidase. The redox homeostasis is maintained by counteracting intracellular production of ROS by antioxidant systems in the cell that include enzymes such as catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) and non-enzymatic ROS scavengers such as glutathione, ascorbate and carotenoids (Trachootham et al, 2008). Maintenance of redox homeostasis is essential for many biological processes such as the 'oxidative burst' in phagocytes during the innate immune

response and signal transduction during growth factor mediated proliferation (Finkel & Holbrook, 2000). Excess of intracellular ROS, on the other hand, damages nuclear and mitochondrial DNA, and modifies proteins and lipids. These types of damage impair cellular functions and can lead to cellular senescence or apoptosis (Beckman & Ames, 1998). In addition to the endogenous sources there are external sources of ROS. Different environmental factors such as ultraviolet rays, ionizing radiations, hyperthermia, toxins and chemicals generate high levels of ROS and often lead to oxidative stress. Oxidative stress causes widespread damage to different tissues and is implicated in a variety of diseases that include cancer, cardiovascular diseases, diabetes and neurodegenerative diseases.

1.1.2. Role of oxidative stress in aging

Aging is a multi-layered and complex phenomenon caused by multiple and often inter-related factors. The "free radical theory" of aging, first put forward by Harman, proposed that the cumulative damage to cellular structures caused by the intracellular oxygen radicals drives the process of aging (Harman, 1956). Subsequent studies in different organisms established oxidative stress as one of the principal contributors to the aging process. Mutation in *age-1* and *daf-2* gene, which are involved in insulin-like signaling pathway in *C. elegans*, extend the lifespan of worms and concomitantly increase stress tolerance at least in part by elevating superoxide dismutase and catalase activity (Johnson, 1990; Kenyon et al, 1993). Loss-of-function mutations of *daf-16*, a forkhead

transcription factor that is negatively regulated by *age-1* and *daf-2*, abrogate this longevity phenotype and decrease stress resistance (Murakami & Johnson, 1996; Ogg et al, 1997). The relation between oxidative stress and aging was corroborated by similar findings in *Drosophila* where mutations in *chico*, insulin receptor substrate, and *InR*, insulin-like receptor, resulted in extended lifespan and heightened SOD activity (Aigaki et al, 2002; Clancy et al, 2001; Tatar et al, 2001). *Drosophila FOXO* (*dFOXO*), which is a homolog of *daf-16* and is suppressed by insulin signaling, also extends lifespan and confers stress resistance when overexpressed (Hwangbo et al, 2004). Furthermore, oxidative stress has been shown to be a contributor to many age-related cardiovascular, respiratory and neurological diseases in mammals (Ito & Barnes, 2009; Pratico, 2008; Ungvari et al, 2008).

1.1.3. Cellular signaling in oxidative stress response

Oxidative stress activates signaling pathways such as NF-κB, JNK, ERK, p38 MAPK, PI(3)K/Akt, p53, HSF-1, FOXO and Nrf2 (Akerfelt et al, 2010; Bowie & O'Neill, 2000; Budanov, 2014; Kim & Choi, 2010; Niture et al, 2014; Wang et al, 2014). These signaling pathways are principal mediators of the cellular oxidative stress response. Cells, depending on their adaptability and their ability to resist stress, respond differently to oxidative stress. They may trigger pro-survival mechanisms that include increased production of antioxidants accompanied by repair and replacement of the damaged macromolecules. Alternatively, they may activate senescence or undergo apoptosis to

remove malfunctioning cells and thereby preserve tissue integrity. The above-mentioned pathways often interact among themselves in a cooperative or antagonistic fashion to bring about a coordinated response to oxidative stress (Finkel & Holbrook, 2000). Stress responsive pathways, serving as central mediators of cell defense against oxidative stress, also affect longevity of the organism.

1.1.4. Nrf2 signaling and its functions

Nrf2 is a master regulator of the oxidative stress response and has been shown to be involved in the regulation of lifespan. Nrf2 is a bZIP transcription factor that is tethered to its cytoplasmic inhibitor Keap1 in unstressed conditions (Katoh et al, 2005; Motohashi & Yamamoto, 2004). Keap1 limits Nrf2 activity by sequestering it in the cytoplasm, targeting it for degradation by the proteasome and thereby preventing its binding to the Antioxidant Response Element (AREs) in the regulatory regions of its target genes. In the absence of nuclear Nrf2 the AREs are occupied by homodimers of a small Maf protein (Blank, 2008; Motohashi et al, 2002). Oxidative stress or electrophilic xenobiotics trigger cause sulfhydryl modification of Keap1 that prevents Nrf2 degradation and leads to an increase of its intra-nuclear concentration. Inside the nucleus, Nrf2 forms heterodimers with small Maf and binds to ARE elements to up-regulate a battery of anti-oxidant enzymes and detoxifying proteins such as thioredoxins and glutathione-synthesizing enzymes, glutathione S-transferases, molecular chaperones and proteasomal subunits (Fig. 1.1) (Itoh et al, 1997; Marini et al, 1997; Motohashi et al, 2004). Nrf2 also drives

the basal level transcription of its target genes.

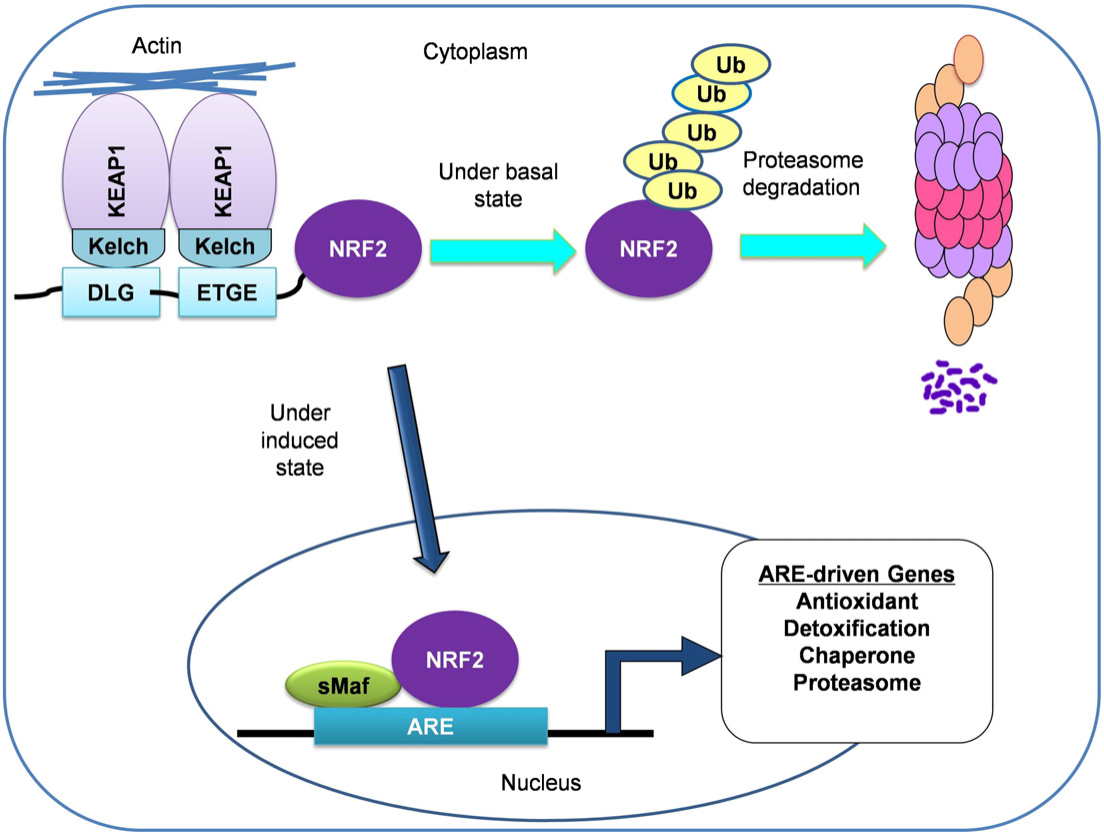


Figure 1.1. Nrf2 signaling pathway. Under unstressed basal condition, Nrf2 binds to Keap1 through the DLG and ETGE motif in the Neh2 domain. This leads to ubiquitination and proteasomal degradation of Nrf2. In the induced state, Nrf2 is protected from Keap1 mediated proteasomal degradation. This results in increased nuclear localization of Nrf2. In the nucleus, Nrf2 dimerizes with small Maf proteins through Neh1 domain, binds to antioxidant response elements (AREs) at the promoter region of the target genes and induce their transcription. (Adapted from (Namani et al, 2014)).

Nrf2-induced antioxidant and detoxification genes protect the organism against oxidative stress. Absence of Nrf2 in mice (*nrf2*$^{-/-}$) renders them highly susceptible to diverse oxidative and electrophilic stresses (Kensler et al, 2007). A wide array of studies has established that Nrf2 signaling protects against multiple diseases such as cancer, neurodegenerative diseases, pulmonary diseases and inflammation where oxidative stress plays a major role (Kensler et al, 2007; Motohashi & Yamamoto, 2004; Sykiotis & Bohmann, 2010). It is interesting to note that many of these are age-related diseases and therefore it can be concluded that Nrf2 protects different organs from age-associated dysfunctions. In addition to the oxidative stress response, Nrf2 has also been implicated in the regulation of lipid and glucose metabolism, and in stem cell maintenance (Hochmuth et al, 2011; Kitteringham et al, 2010; Mitsuishi et al, 2012; Shin et al, 2009).

1.1.5. Nrf2 protein structure and the conservation of Nrf2 signaling in *Drosophila*

Seven functional Neh [Nrf2–ECH (erythroid cell-derived protein with CNC homology) homology] domains, namely Neh1– Neh7 (Jaramillo & Zhang, 2013) have been identified in the Nrf2 protein. The Neh1 domain contains a bZIP DNA-binding and dimerization motif that allows Nrf2 to bind antioxidant response elements (AREs) in DNA and dimerize with other transcription factors such as Maf (Moi et al, 1994). The Neh2 domain is located near the N-terminus of Nrf2 and contains DLG and ETGE motifs that interact with Keap1. The Neh2 domain also contains seven lysine residues that are

sites of ubiquitination (Zhang et al, 2004). Keap1 acts as an adaptor for a Cullin 3-dependent E3 ligase that promotes ubiquitination of Nrf2 and directs it for proteasomal degradation (Cullinan et al, 2004; Furukawa & Xiong, 2005; Kobayashi et al, 2004; Zhang et al, 2004). The Neh3 domain binds to the Chromodomain-helicase-DNA-binding protein 6 (CHD6) that acts as an Nrf2 transcriptional co-activator (Nioi et al, 2005). The Neh4 and Neh5 domains interact with transcriptional co-activator CBP that acetylates Nrf2 and induces transactivation of Nrf2 target genes (Katoh et al, 2001; Zhu & Fahl, 2001). The Neh6 domain interacts with the F-box protein family member β-TrCP that promotes Nrf2 degradation in a Cullin 1-dependent fashion (Chowdhry et al, 2013; Rada et al, 2011). The Neh7 domain interacts with the retinoic X receptor that represses Nrf2 target gene expression (Fig. 1.2.) (Wang et al, 2013).

The Nrf2/Keap1 signaling module is conserved in *Drosophila* where CncC, expressed from the *Cap'n'collar* locus, is the functional and structural homologue of Nrf2. The mammalian Keap1 and four small Maf proteins also have *Drosophila* counterparts: dKeap1 and a single Maf-S. Like mammalian Nrf2, *Drosophila* CncC is activated by oxidative stress and electrophilic drugs like oltipraz and its activation leads to induction of a battery of antioxidant and detoxification genes. Like mammalian Nrf2, CncC also confers protection against oxidative stress. In addition, dKeap1 heterozygous mutation that leads to CncC activation extends lifespan in *Drosophila* (Sykiotis & Bohmann, 2008).

Unlike *Drosophila cnc* loss-of-function mutants that die during development, Nrf2 deletion in mammals is not lethal probably because of the compensatory functions of the related Nrf1 and Nrf3 proteins (Blackwell et al, 1994; Harding et al, 1995; Mohler et al, 1995). Importantly, Nrf1 is also reported to activate antioxidant genes and is activated by oxidative stress (Chen et al, 2003; Ohtsuji et al, 2008; Zhang et al, 2014b). On the other hand, it also differs from Nrf2 in some aspects of its function and regulation. For example, induction of proteasome subunit genes by TorC1 is dependent on Nrf1 but not Nrf2 (Zhang et al, 2014a; Zhang et al, 2014b). Moreover, unlike Nrf2 deficiency, Nrf1 deficiency in mice leads to developmental lethality (Chan et al, 1998; Chen et al, 2003). Even the mechanism of Nrf1 activation is different from that of Nrf2. Nrf1 attaches to the ER membrane via its NH2-terminally localized NHB1 domain. Deglycolsylation of Nrf1, as seen in response to ER stress, changes the conformation of transcription activation domain of Nrf1 and that ultimately results in target gene activation (Zhang et al, 2014b). These findings raise the question of whether CncC-related proteins in *Drosophila* carry out such similar yet non-redundant antioxidant and proteostatic functions.

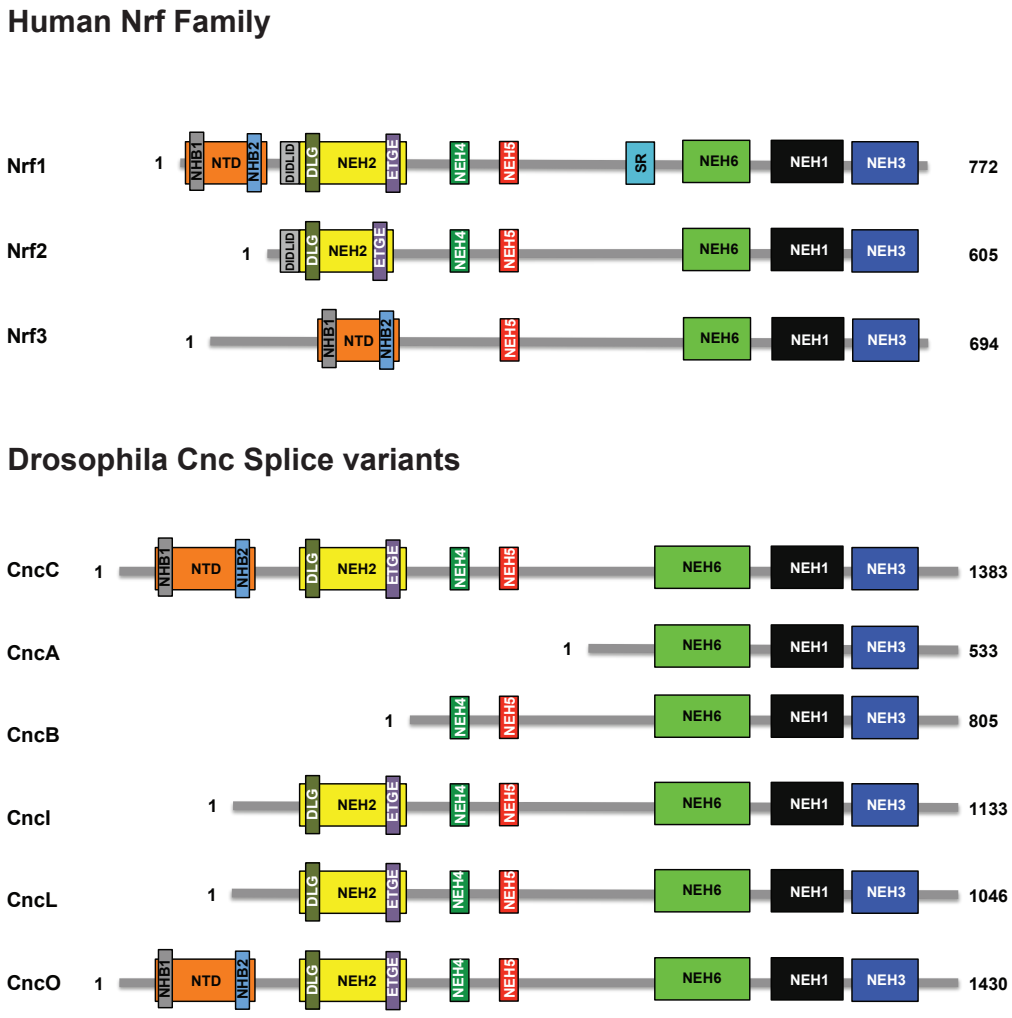


Figure 1.2. Domain organization of cap'n'collar (CNC) transcription factors found in humans and *Drosophila*. The Neh1 domain, which contains CNC motif and binds to DNA, as well as Neh3 and Neh6 domains, are present in all CNC proteins. The Neh2 domain, which binds to Keap1, is absent from Nrf3 in humans and in CncA and CncB in *Drosophila*. The N-terminal domain (NTD), which contains NHB1/2 subdomains that are believed to be important for endoplasmin reticulum localization, is present in Nrf1, Nrf3, CncC, and CncO. (Adapted from (Pitoniak & Bohmann, 2015)).

In addition to CncC, *Cap'n'collar* locus also produces other splice-variants such as CncA, CncB, CncD, CncI, etc. The bZIP DNA binding and dimerization region of Cnc proteins shows homology to the corresponding Neh1 and Neh3 domains in the mammalian transcription factors NF-E2 and Nrf1, 2 and 3 (Harding et al, 1995; Mohler et al, 1995). *Drosophila* Cnc products also contain peptide sequences that resemble some of other domains that are conserved among mammalian cap'n'collar proteins such as the Neh3, 4, 5 and 6 domains. The Keap1 interacting Neh2 motif is present in longer splice variants such as CncC and CncI but not in shorter ones namely CncA and CncB. Interestingly, some of the Cnc splice-variants contain peptide sequences with homology to the Nrf1-specific NHB1 domain (Grimberg et al, 2011). It is therefore possible that the *Drosophila cnc* locus combines the functions of the mammalian Nrf1 and Nrf2 genes. If experimentally validated, this would make *Drosophila* an ideal system to compare and contrast the functions and regulation of Nrf1 and Nrf2 (Pitoniak & Bohmann, 2015).

1.2. Regulation of Nrf2 signaling

1.2.1. Regulation of Nrf2 by oxidative stress and Keap1

Oxidative stress activates Nrf2 signaling by modifying its cytoplasmic inhibitory protein Keap1. Keap1 protein contains five different domains, namely NTR, BTB, IVR, DGR and CTR. The DGR domain contains Kelch motifs that interact with Nrf2 (Keum and

Choi, 2014). The BTB domain forms a homodimer for binding to the Cullin-3 E3 ubiquitin ligase complex and this interaction leads to ubiquitination and proteolytic degradation of Nrf2 protein (Furukawa & Xiong, 2005; Kobayashi et al, 2004). Keap1 contains many cysteine residues several of which are conserved across different species. The IVR domain contains four highly reactive cysteine residues and transient transfection assays revealed that two cysteine residues out of these four are important for Keap1-dependent Nrf2 repression (Wakabayashi et al, 2004; Zhang & Hannink, 2003). Oxidative stress causes sulfhydryl modification of cysteine residues in Keap1 causing a conformational change in Keap1, which ultimately leads to Nrf2 activation (Dinkova-Kostova et al, 2002; Motohashi & Yamamoto, 2004).

The exact mechanism that links cysteine modifications in Keap1 to Nrf2 activation is not well understood. Two principal molecular models that explain Nrf2 activation are: (1) The 'Hinge and latch' model, in which sulfhydryl modifications in cysteine residues of the IVR domain of Keap1 disrupt the interaction between the DGR domain of Keap1 and the DLG domain of Nrf2 but not its ETGE domain. This causes a misalignment of the lysine residues of Nrf2 so that they can no longer be ubiquitinated; and (2) The 'Conformational cycling' model in which the Keap1/Nrf2 complex exists in two different conformations: an 'open' conformation where only the high affinity "ETGE" motif, but not the low affinity "DLG" motif in Nrf2 protein binds to Keap1 dimers and a 'closed' conformation where both the "DLG" and "ETGE" motifs bind to Keap1 dimers. Thiol

modification of Keap1 changes the conformation of Nrf2-Keap1 complex from a 'open' to a 'close' one, and thereby prevents Nrf2 degradation. In both models, cysteine modification of Nrf2-bound Keap1 prevents Nrf2 degradation. This allows the newly synthesized Nrf2 proteins to bypass Keap1 and translocate into the nucleus where they bind to AREs in DNA and induce expression of Nrf2 target genes (Kansanen et al, 2013; Keum & Choi, 2014).

1.2.2. Additional environmental and physiological regulators of Nrf2 signaling

In addition to oxidative stress and Keap1, other environmental and physiological signals such as calorie restriction, endoplasmic reticulum stress and insulin signaling also modify the activity of Nrf2 and its homologs in other organisms. Different molecular modifications of Nrf2 such as phosphorylation, acetylation as well as other proteins that interact with Nrf2 or Keap1, bring about changes in the Nrf2 activity. Nrf2 therefore acts as an integrator of multiple environmental, physiological and molecular signals.

1.2.2.1. Calorie restriction and Nrf2 signaling

Calorie restriction (CR) is the best established method for extending lifespan in different species. CR also improves other health parameters. For example, it enhances insulin sensitivity, lowers cancer risk, lowers blood pressure and improves neuronal function

(Fontana et al, 2010). Although there is no report indicating the importance of Nrf2 signaling in CR mediated lifespan extension in mammals, CR has been reported to activate antioxidant genes that are Nrf2 targets in mice and this induction was impaired in *nrf2*$^{-/-}$ mice. In addition, it was observed that CR mediated protection against cancer caused by carcinogens are also dependent on Nrf2 activity (Pearson et al, 2008; Sykiotis et al, 2011).

The role of Nrf2-like signaling in the longevity effects of CR in *C. elegans*, on the other hand, is well documented. A study in *C. elegans* reported that the activity of Skn-1, the Nrf2 homolog in worms, is required for lifespan extension in response to CR (Bishop & Guarente, 2007). Interestingly, Skn-1 activity in ASI neurons was important for lifespan extension. This implied that cell non-autonomous effect of Nrf2 signaling is involved for CR mediated lifespan extension in worms.

1.2.2.2. IIS/Akt signaling and Nrf2

Insulin and its downstream signaling mediated through PI3K/Akt are important anabolic signals and control growth. Experiments in multiple organisms have shown that loss-of-function mutations in the insulin signaling pathway can extend lifespan (Bluher et al, 2003; Clancy et al, 2001; Kenyon, 2005). Daf-16/Foxo, which is negatively regulated by

IIS/Akt signaling, has been identified as a key mediator of longevity in response to loss of insulin signaling (Junger et al, 2003; Puig & Tjian, 2005; Salih & Brunet, 2008). However, a study by Tullet et al also implicated Skn-1 as a downstream target of IIS/Akt signaling (Tullet et al, 2008). These authors have shown that, like Daf-16, the worm homolog of Foxo, Skn-1 is also phosphorylated and inhibited by Akt. It has also been observed that Daf-16 and Skn-1 function independently of each other as downstream effectors of Insulin signaling.

The mammalian Nrf2 signaling pathway, however, has been reported to be activated by PI3K/Akt signaling (Li et al, 2006; Wang et al, 2008a). Rizvi et al have recently observed that inhibition of PI3K/Akt signaling lead to reduced nuclear retention of Nrf2 and increased Nrf2 ubiquitination. PI3K/Akt inhibition, thereby, suppressed Nrf2 target genes and lead to an increase in oxidative cytotoxicity (Rizvi et al, 2014). Oxidative stress, on the other hand, leads to phosphorylation of Akt, which in turn phosphorylates and inhibits Gsk3β. Gsk3β represses Nrf2 signaling by promoting degradation of Nrf2 protein and by activating Fyn kinase, which phosphorylates Nrf2 and increases its nuclear export. Gsk3β inhibition by Akt, therefore, leads to Nrf2 activation. However, it is unclear why PI3K/Akt signaling has opposite effects on mammalian Nrf2 and Skn-1in *C.elegans*. It would be interesting to explore the physiological and evolutionary significance of these differential effects of PI3K/Akt signaling on Nrf2 and Skn-1.

1.2.2.3. ER stress and Nrf2 signaling

An excess of unfolded or misfolded polypeptides that overwhelms the chaperone system causes ER stress. The Unfolded protein response (UPR) facilitates refolding or clearing of these defective proteins that are potentially toxic to the cells. In addition to a variety of other pathways, UPR also activates Nrf2 signaling. UPR activates PERK, which in turn, activates Nrf2 by destabilizing Nrf2-Keap1 interaction (Cullinan et al, 2003). Moreover, JNK, activated by IRE1, promotes Nrf2-Keap1 dissociation and thereby activate Nrf2 signaling (Digaleh et al, 2013; Keum et al, 2003; Urano et al, 2000). In addition to Nrf2, UPR is also likely to activate Nrf1. As discussed in the section 1.1.5, Nrf1 is activated by deglycosylation. The activation of Nrf1 and Nrf2 signaling by ER stress ultimately leads to induction of multiple detoxification, chaperone and proteolytic genes that help the cell mount a potent ER stress response (Digaleh et al, 2013).

UPR is also reported to activate Skn-1, the Nrf2 homolog, in *C. elegans*. Glover-Cutter et al. found that ER stress in worms can activate Skn-1 target genes and Skn-1 is essential for resistance to ER stress. They also reported that Skn-1 binds to common downstream targets with XBP-1 and ATF6 to orchestrate the transcriptional ER stress response (Glover-Cutter et al, 2013). These studies indicate that activation of Nrf2-like signaling in response to ER stress is conserved across different species.

1.2.3. Additional molecular and chemical regulators of Nrf2 signaling

1.2.3.1. Regulation of Nrf2 signaling by kinases

Different kinases have been reported to regulate of Nrf2 signaling (Bryan et al, 2013) (Bryan et al 2013). For example, it was shown that PKC-δ mediated phosphorylation of Ser40 in mammalian Nrf2 promotes its stabilization and nuclear translocation (Bloom & Jaiswal, 2003; Huang et al, 2002; Niture et al, 2009). On the other hand, GSK-3β and Akt signaling promote degradation of Nrf2 in a Keap1-independent manner. F-box protein β -TrCP and Cullin 1 mediate this mechanism of Nrf2 regulation (Chowdhry et al, 2013). The Src-family tyrosine kinase Fyn can phosphorylate Tyr568 of Nrf2 and that leads to nuclear export of Nrf2 and its degradation (Jain & Jaiswal, 2006). Interestingly, GSK-3β can also phosphorylate Fyn and increase its nuclear accumulation and thereby, inhibit Nrf2 signaling (Jain & Jaiswal, 2007).

It has been reported that p38 phosphorylates Nrf2 and promotes its interaction with Keap1. This prevents nuclear translocation of Nrf2, resulting in suppression of target gene expression (Keum et al, 2006). In addition, PERK, when activated by ER stress has also been reported to phosphorylate and activate Nrf2 (Cullinan et al, 2003). However, the substrate sites of phosphorylation for some kinases are not known and it has been suggested that the coactivators of Nrf2 such as CBP instead of Nrf2 itself, might be the

targets of some of the protein kinases (Shen et al, 2004).

1.2.3.2. Regulation Nrf2 signaling by acetylation

p300/CBP, transcriptional co-activators that acetylate histones to facilitate chromatin decondensation and recruitment of the RNA polymerase machinery (Ogryzko et al, 1996; Roth et al, 2001), have been reported to be associated with Nrf2 protein (Katoh et al, 2001; Sun et al, 2009). p300/CBP binds to Nrf2 at its Neh4 and Neh5 domains in response to oxidative stress and acetylates a number of lysine residues within the Neh1 DNA binding region of Nrf2. Mutational analysis of these lysine residues revealed that acetylation of multiple residues is required for full Nrf2 activation. The acetylation of Nrf2 by CBP enhances its binding to the ARE sites in the DNA and enhances its transactivation potential but does not affect its stability (Sun et al, 2009). Recent studies have shown that acetylation of Lysine residues in other domains of Nrf2 such as Neh2 and Neh3 also modulates Nrf2 activity (Kawai et al, 2011).

1.2.3.3. Regulation Nrf2 signaling by nuclear receptors

Nrf2 signaling is regulated by different nuclear receptors and retinoid receptor is one of them. There are two distinct classes of receptors for retinoids, namely retinoic acid receptor (RAR) and retinoid X receptor (RXR) (Namani et al, 2014). It was reported that

all-trans retinoic acid (ATRA) can inhibit ARE-driven transcription and that it does so without affecting the stability and the nuclear accumulation of Nrf2 (Wang et al, 2007). It was also observed that the expression of Nrf2 target genes was increased in the small intestine of mice that were fed a vitamin A-deficient diet. This induction was repressed by ATRA in an Nrf2-dependent manner. It was also established that ATRA works principally through the RARα receptor that forms a protein complex with Nrf2 and affects its binding to DNA and thereby, suppress Nrf2 target gene expression. In addition to RARα, RXRα has also been shown to inhibit Nrf2. A recent study by Wang et al showed that RXRα inhibits both basal and inducible expression of Nrf2 target genes. Moreover, GST pull-down experiments revealed that the DNA binding domain (DBD) of RXRα physically interacts with Nrf2 at its Neh7 domain to prevent transactivation of Nrf2 target genes (Wang et al, 2013). Interestingly, RXRα rendered A549 cells, where Nrf2 is constitutively active due to mutation in Keap1 (Singh et al, 2006), more sensitive to anticancer drug like doxorubicin.

Estradiol (E2) and other estrogens are reported to suppress Nrf2 target gene expression independently of Keap1-mediated degradation (Ansell et al, 2004; Ansell et al, 2005). Estrogen receptor ERα, in response to ligands such as E2, physically interacts with Nrf2 and prevents activation of the Nrf2 target genes. Studies by Yao et al showed that ERα binds to the promoter region of the Nrf2 target gene NQO1 and inhibits its transcription in breast cancer cells (Yao et al, 2010). This inhibition is reversed by the anti-estrogen

drug shikonin. In addition to retinoid and estrogen receptors, glucocorticoid receptor (GR) is also implicated in the regulation of Nrf2 signaling. Ki et al showed that the synthetic glucocorticoid dexamethasone suppresses the induction of the Nrf2 target gene GSTA2 by oltipraz or tBHQ (Ki et al, 2005). They found that SMRT, a co-repressor of the steroid–GR complex, binds with the Neh4/5 domain of NRF2 and represses the expression of NRF2 target gene. Moreover, 11β-HSD1, that activates GR through glucocorticoids, also suppresses the Nrf2-dependent antioxidant response and increases sensitivity to H_2O_2 (Kratschmar et al, 2012).

1.2.3.4. Chemical regulators of Nrf2 signaling

Nrf2 signaling is activated by different types of oxidative stressors such as paraquat which interferes with the mitochondrial electron transport chain (Castello et al, 2007) or hydrogen peroxide (a reactive oxygen species). In addition to these compounds, a variety of small molecules have been identified as inducers of Nrf2 signaling. These molecules can be classified into 11 broad classes: (1) Michael acceptors like curcumin, bardoxolone methyl and diethyl maleate (2) oxidizable diphenols and quinones such as tBHQ and resveratrol, (3) isothiocyanates and sulfoxythiocarbamates like sulforaphane, (4) dithiolethiones and diallyl sulfides such as oltipraz, (5) vicinal dimercaptans like (R)-lipoic acid, (6) trivalent arsenicals such as sodium arsenite, (7) selenium-based compounds like Ebselen, (8) polyenes such as lycopene, (9) hydroperoxides like tert-butyl hydroperoxide, (10) heavy metals and metal complexes such as cadmium chloride,

and (11) others inducers like dimethyl fumarate (DMF) (Magesh et al, 2012). A number of small molecules inducers such as sulforaphane, bardoxolone methyl, oltipraz, DMF and Ebselen are currently undergoing clinical trials for the treatment of a variety of diseases and conditions such as breast and prostate cancer, cystic fibrosis, asthma, COPD, chronic kidney disease in type 2 diabetes, multiple sclerosis and non- alcoholic fatty liver disease (English & Aloi, 2015; Magesh et al, 2012). However, most of the currently known ARE inducers affect Keap1-Nrf2 interaction and most likely form covalent adducts with the sulfhydryl groups of cysteines in Keap1. They, therefore, carry a risk of "off-target" toxic effects due to their potential to react with other cysteine-containing proteins (Garber, 2012; Zhang & Munday, 2008). In addition to the Nrf2 inducers, all-trans-retinoic acid (ATRA) has been identified as an inhibitor of Nrf2 signaling that prevents Nrf2 binding to ARE (Wang et al, 2007).

1.3. Dysregulation of Nrf2 signaling in cancer and aging

1.3.1. Dysregulation of Nrf2 signaling in cancer

Nrf2 signaling has dual role in cancer. Due to its cytoprotective activities it can suppress chemical carcinogenesis in model organisms. However, the same cytoprotective properties of Nrf2 give cancer cells protection against endogenous oxidative stress and oxidative stress caused by exogenous sources such as chemotherapeutic drugs and

radiation therapy. In addition, Nrf2 also promotes cell proliferation by channelizing glucose and glutamate towards purine biosynthesis and by modulating the pentose phosphate pathway in cancer cells (Mitsuishi et al, 2012). Nrf2 signaling, therefore, despite preventing cancer in normal and premalignant tissues, provides a growth and survival advantage to malignant tissues. It is, therefore, not surprising that high Nrf2 activity has been identified in various types of cancer (Kansanen et al, 2013; Sporn & Liby, 2012).

There are several ways in which constitutive activation of Nrf2 is achieved in different types of cancer: (1) Mutations in Keap1 or in Nrf2 that affect their interaction and causes activation of Nrf2 signaling (Hayes & McMahon, 2009). For example, somatic mutations in Keap1 that resulted in constitutive activation of Nrf2 are identified in lung, biliary tract and gall bladder cancers (Ohta et al, 2008; Shibata et al, 2008; Singh et al, 2006). Similarly, multiple mutations in the DLG and ETGE motifs of Nrf2 have been identified in lung cancer, and head and neck cancer (Shibata et al, 2008); (2) Reduction in Keap1 expression due to epigenetic silencing. Loss of Keap1 mRNA expression was reported in several lung tumors and it was caused by hypermethylation of CpG sites in the *keap1* promoter (Wang et al, 2008b); (3) Increase in disruptor proteins such as p62 that destabilize Nrf2-Keap1 interaction. Since p62 interacts with Keap1 at its Nrf2-binding site, increased p62 levels, as seen in conditions of autophagy deficiency, would affect Nrf2–Keap1 interaction and thereby, would activate Nrf2 target genes. Inami et al

reported that liver-specific inhibition of autophagy lead to formation of hepatocellular carcinoma where enhanced p62 protein levels were accompanied by increased Nrf2 signaling (Inami et al, 2011); (4) Transcriptional induction of Nrf2. Overexpression of oncogenic K-Ras, B-Raf and c-Myc reduces intracellular ROS level by transcriptional activation of Nrf2. Genetic inhibition of Nrf2 prevents K-Ras induced cell proliferation and tumorigenesis (DeNicola et al, 2011); and (5) Post-translational modification of Keap1. Loss of fumarate hydratase activity in familial papillary renal carcinoma leads to succinylation of cysteine residues of Keap1. This prevents Nrf2-Keap1 interaction and leads to Nrf2 pathway activation (Adam et al, 2011; Ooi et al, 2011). Pharmacological inhibition of Nrf2 signaling, therefore, might be an effective way to treat these types of cancer where Nrf2 is constitutively active.

1.3.2. Decline in Nrf2 signaling with age

Nrf2 signaling protects organisms against different age-related diseases. However, multiple studies have shown that Nrf2 signaling declines with age and that might contribute to progressive age-related deterioration of various physiological processes. A decrease in the Nrf2 level in the rat aorta makes the Nrf2 target gene less responsive to H_2O_2 and glucose induction and more prone to apoptosis in response to oxidative stress (Ungvari et al, 2011). Similarly, a decrease in Nrf2 and a concomitant reduction in its ARE binding led to reduced gluthathione levels in the livers of old rats. The Nrf2 activating chemo-preventive agent (R)-α-lipoic acid restored the Nrf2 activity and the

glutathione level in the hepatic tissue (Suh et al, 2004). An age-associated reduction of Nrf2 activity was observed in neuronal tissue as well. A loss of PI3K/Akt activity and the resultant activation of Gsk3β led to the inhibition of Nrf2 signaling in the neuroprogenitor cells in the subventricular zone of the brain in old mice. Nrf2 induction in response to MPTP, a neurotoxin that destroys dopaminergic neurons and elicits permanent Parkinson's disease-like condition, was also impaired in old age (L'Episcopo et al, 2013). This decline is Nrf2 signaling in these neuronal cells causes oxidative stress that is a hallmark of Parkinson's disease (PD). Multiple studies have shown that pharmacological activation of Nrf2 protects against PD (Burton et al, 2006; Hwang & Jeong, 2008; Yang et al, 2009). Similarly, overexpression of Nrf2 in astrocytes protects dopaminergic neurons from neurotoxins such as MPTP or 6-hydroxydopamine (Chen et al, 2009; Jakel et al, 2007). Interestingly, pharmacological or genetic activation of CncC signaling in dopaminergic neurons also prevents neuron loss in *Drosophila* PD model (Barone et al, 2011; Trinh et al, 2008).

PI3K/Akt signaling might also be involved in the maintenance of Nrf2 activity in aged skeletal muscle. Safdar et al. observed that Nrf2 activity in active old people is higher in comparison to that in sedentary old subjects. This difference in Nrf2 activity is accompanied by an increase in Akt phosphorylation and a decrease in GSK3β phosphorylation (Safdar et al, 2010). Exercise also exerts beneficial effects on the Nrf2 activity in cardiac muscle. Gounder et al reported that moderate endurance training in old

mice could restore Nrf2 activity in myocardial tissue (Gounder et al, 2012).

Age-related decline in Nrf2-like signaling in invertebrate organisms has also been observed. It has been reported that Skn-1 induction in *C. elegans* declines with age. This also contributes to the lack of juglone-mediated hormetic enhancement in oxidative stress defense in old worms (Przybysz et al, 2009). Similar age-related reduction in the inducibility of CncC signaling was observed in *Drosophila*. This resulted in higher oxidative stress sensitivity, cardiac arrhythmia and impaired motor function. Overexpression of the CncC binding partner Maf-S restored the CncC inducibility in aged flies and thereby improved oxidative stress resistance, cardiac and motor functions (Rahman et al, 2013). All these studies establish that age-related decline in Nrf2 function is present in multiple tissues in different species and this decline contributes to many diseases and frailties of old age. Pharmacological, environmental and genetic restoration of Nrf2 activity may improve oxidative stress resistance and various other physiological functions in older organisms.

1.4. Aim of the thesis

In addition to the established role of Nrf2 signaling in the oxidative stress response, recent studies have implicated Nrf2 and its homologs in the regulation of multiple metabolic processes, in stem cell maintenance and in longevity. Consistent with its

expanded scope to influence on cellular physiology, it has been found that different environmental and physiological signals other than oxidative stress such as ER stress, IIS/Akt signaling and calorie restriction also regulate Nrf2 signaling. A better understanding of the molecular mechanisms of Nrf2 regulation would, therefore, be important to explain how it responds to different signals and how different molecular players influence the functions of Nrf2. In this study we wanted to identify novel molecular regulators of Nrf2 signaling in *Drosophila* and characterize how they regulate Nrf2. In order to do large-scale screens to identify Nrf2 regulators and to study their effect in the physiological context, we wanted to generate cell-based and *in vivo* reporters of Nrf2 signaling. We intended to carry out a large-scale dsRNA library screen to identify novel regulators of Nrf2 signaling. Finally, we wanted to characterize one or more of the regulators identified in the screen to advance our understanding of the molecular mechanisms of regulation of Nrf2 signaling. This study is important to find probable links between different signals and Nrf2 pathway, and also to identify potential targets for therapeutic modulation of Nrf2 signaling.

Chapter 2

Generation of Nrf2 reporters for cell-based studies and studies in *Drosophila*

2.1. Introduction

Signal transduction mechanisms that modulate transcription profiles in response to extracellular inputs control all major biological processes, including development, growth and homeostasis. Our knowledge of signal-dependent transcription control has been derived from studies *in vitro* and in cultured cells, and from genetic experiments in model organisms. Such research has yielded detailed molecular descriptions of many signaling and gene regulation processes. Questions that are, however, much less well understood include: How are spatial and temporal patterns of gene expression regulated in the two and three dimensions of epithelia or tissues? How do multiple signaling systems and transcription factors interact and form complex gene control networks in the intact organism? Addressing such questions requires experimental tools with which the activity of signal-dependent transcription factors can be monitored in live tissues with good temporal and spatial resolution. To this end, reporter genes that can register the activity of transcription factors or other signaling proteins have been designed. A widespread type of reporter relies on fusion genes in which synthetic DNA elements, that represent defined

binding sites for a transcription factor of interest, are inserted upstream of sequences encoding protein products such as fluorescent proteins and enzymes, which are easy to visualize or quantify, are not normally present in the test organism, and for which sensitive assays are available such as chloramphenicol acetyl transferase or luciferase. Such reporter genes have been used in tissue culture or in transgenic animals (Jiang et al, 2008). Transcriptional *Drosophila* GFP or RFP reporters have been employed in the analysis of specific signaling pathways and regulatory processes in the context of an intact organism (Barolo et al, 2000).

One technical limitation of most transgenic *Drosophila* reporter lines published so far is that they have been generated by random P-element integration into the genome of a recipient strain. Consequently, the insertion sites and their genomic environment are undefined and these reporters can be subject to variable and unpredictable position effects. The influence of nearby gene regulatory elements or the overall epigenetic state at the integration site can substantially affect the expression characteristics and response patterns of transgenes (Daniels et al, 1986; Manseau et al, 1997). Such inherent variability due to different insertion sites complicates comparisons between reporters. It is therefore difficult to directly relate the activity and responsiveness of a set of reporter mutants or to generate negative control reporters that differ from experimental reporters only by disruption of a DNA binding element.

The development of targeted methods for site specific transgene integration into the *Drosophila* germline can alleviate problems arising from random position effects. The feasibility of such strategies has recently been documented (Boy et al, 2010; Rogers & Williams, 2011). We therefore used a targeted reporter gene insertion strategy that relies on the ΦC31 method (Groth et al, 2004). The modular system of reporters uses eGFP, or DsRed.T4 constructs that are integrated in defined genomic locations. Corresponding firefly luciferase reporter constructs for cell culture experiments were generated as well, and can complement *in vivo* assays by providing platforms for rapid quantitative analyses and high throughput screens. This standardized system permits comparative reporter analysis across different signaling pathways using *in vivo* and *in vitro* platforms.

2.2. Results and Discussion

The continued integrity of cells and organisms depends on their ability to protect themselves against oxidative stress and fluctuations in redox state. Multiple signaling systems have evolved that can sense oxidative and genotoxic stresses and orchestrate rapid and effective responses in order to deflect or repair potentially harmful damage to cells, tissues and organisms. Conserved stress-inducible signaling systems include: the JNK, p38, p53, NF-kB and Nrf2 pathways (reviewed in (Finkel & Holbrook, 2000)). The molecular constituents that relay stress signals and their epistatic relationship are, in most cases, well described. However, the interplay among these pathways, signal response profiles and biological functions in the organism are not comprehensively understood.

To begin a systematic comparative analysis of stress signaling pathways in specific organ systems and developmental processes, we generated a modular reporter system for monitoring the activities of two major stress responsive signaling pathways, the JNK and Nrf2 cascades, in *Drosophila*.

JNK (c-Jun NH_2-terminal Kinase) is the eponymous enzyme of a MAP kinase pathway that has been implicated in stress responses, embryonic development, wound healing, apoptosis and cancer (Davis, 2000; Kockel et al, 2001; Sykiotis & Bohmann, 2007; Weston & Davis, 2007). JNK activity can be induced by multiple signals, including oxidative stress, UV radiation, inflammatory cytokines and epithelial wounding. The prototype substrate for JNK is the AP-1 transcription factor c-Jun, which upon binding to JNK and phosphorylation at several N-terminally located amino acid residues acquires the ability to activate its target genes as a heterodimer with Fos or ATF2 proteins (Hess et al, 2004; Lopez-Bergami et al, 2010). Binding sites for AP-1 transcription factor dimers are referred to as TRE (for TPA or tetradecanoylphorbol acetate response element). Such TREs confer transcription activation in response to JNK signaling to *cis*-linked genes. The JNK signaling system in the fruitfly is structurally and functionally similar to that of mammals. The *Drosophila* homolog of JNK is encoded by the gene *basket* (*bsk*) (Riesgo-Escovar et al, 1996). Basket is activated by the JNKK Hemipterous (Hep) which in turn responds to one of several JNKKKs (Glise et al, 1995; Sykiotis & Bohmann, 2007). *Drosophila* Jun and Fos homologs are regulated by JNK phosphorylation (Ciapponi et al,

2001; Peverali et al, 1996) and have the same DNA binding specificity as their mammalian counterparts.

Nrf2 is, like c-Jun, a leucine zipper transcription factor. The Nrf2 activating signaling pathway is turned on by diverse signals and stressors, some, but not all of which, also activate JNK (Baird & Dinkova-Kostova, 2011; Osburn & Kensler, 2008; Sykiotis & Bohmann, 2010). Several compounds, including sulforaphane, oltipraz and lipoic acid, activate Nrf2 without causing evident cell stress. These drugs have therapeutic potential, for example in cancer chemoprevention (Hur & Gray, 2011). Upon activation, Nrf2 dimerizes with the small leucine zipper protein Maf-S (Blank, 2008) and binds to its cognate promoter elements called AREs (for antioxidant response element). Genes thus regulated encode, among other proteins, redox regulators and phase II detoxification enzymes. Defects in the Nrf2 pathway cause stress sensitivity and Nrf2-deficient mouse mutants are predisposed to hyper-inflammatory diseases, cancer, neurodegeneration and metabolic dysfunction (Osburn & Kensler, 2008; Sykiotis & Bohmann, 2010).

The Nrf2 signaling system is functionally and structurally conserved in *Drosophila* (Sykiotis & Bohmann, 2008; Sykiotis & Bohmann, 2010). The fly homolog of Nrf2 is the Cap'n'collar splice form C (CncC) protein. CncC is regulated by interaction with an Nrf2-specific E3 enzyme, the *Drosophila* Keap1, and controls the expression of stress

defense, detoxification and antioxidant genes reminiscent of the mammalian transcription factor. CncC function has been shown to confer resistance against acute oxidative stress, promote longevity, and regulate somatic stem cell maintenance in the *Drosophila* intestine (Hochmuth et al, 2011; Sykiotis & Bohmann, 2008).

The transcriptional responses to JNK and Nrf2 signaling, are limited by negative feedback mechanisms mediated by the induction of specific inhibitors. The *Drosophila* JNK pathway, for example, stimulates the expression of the JNK phosphatase Puckered, which will eventually decrease the activity of the pathway by abrogating JNK kinase activity (Martin-Blanco et al, 1998). Nrf2 is subject to a similar feedback control involving the transcriptional upregulation of Keap1 (Fig. 2.1A) (Katoh et al, 2005; Motohashi & Yamamoto, 2004).

The JNK and Nrf2 signaling pathways share several functional and molecular characteristics. The spectrum of extracellular signals and stressors that can activate JNK and Nrf2, respectively, is overlapping but not identical. Similarly, JNK activated transcription factors and Nrf2 have common, but also independent target genes. Whether there is significant cross-talk between JNK and Nrf2, and whether they function in parallel or synergistically in different cell types or organs has not systematically been investigated.

2.2.1. Generation of *in vivo* and cell-based reporters

To elucidate the function and relationship of Nrf2 and JNK signaling, we designed a system of reporter genes for monitoring the functions of Nrf2 and JNK in intact organisms, tissues and cell culture. For the generation of *in vivo* reporter genes we used the previously described pelican plasmids as a starting point (Barolo et al, 2000). The key components of the new system are described below:

Synthetic transcription factor binding sites

Based on previously determined sequence preferences (Kerppola & Curran, 1994; Veraksa et al, 2000), we designed DNA elements that would specifically bind either Nrf2/MafS dimers or Jun/Fos dimers (Fig. 2.1B). These oligonucleotides should therefore function as Nrf2-inducible ARE, or AP-1 responsive TRE elements, respectively. As a negative control, we generated a mutated version of the oligonucleotides, named mRE, which should not bind either Nrf2 or AP-1 as predicted based on the specificity of the respective mammalian homologs (Kerppola & Curran, 1994).

Reporter genes

Four head-to-tail concatenated copies of the ARE, TRE or mRE elements were placed upstream of a basal promoter to control the expression of either a green or red fluorescent

protein, eGFP or DsRed.T4, respectively (Fig. 2.1C). These two proteins have distinct fluorescence spectra, which make it possible to assess their levels and distribution separately in the same sample. eGFP or DsRed.T4 have similar maturation time, facilitating the comparative analysis of Nrf2 and JNK signal response kinetics (Barolo et al, 2000). We will refer to these reporter constructs with names that indicate the nature of the promoter element and the reporter gene, as in ARE-red, TRE-green, mRE-green, etc.

Site-directed transgenesis with ΦC31

Conventional P-element-mediated transformation results in transgene insertion into random genomic loci. Consequently, the integrated gene can be exposed to variable position effects, which may seriously confound the interpretation of reporter experiments, especially when multiple different constructs are to be compared. In order to circumvent this problem, transgenic *Drosophila* lines carrying the ARE, TRE or mRE reporter genes in predetermined locations were generated using the ΦC31 recombinase mediated site directed transgenesis technique. This method utilizes the sequence-specific DNA recombinase of bacteriophage ΦC31 to target the transgenic construct carrying attB recombination sequence to a defined genomic position bearing a preplaced attP recipient site (Groth et al, 2004). Two different docking sites, attP40 and attP16, on the left and the right arm, respectively, of the second chromosome were targeted for integration of the different reporter constructs (Fig. 2.1C). These genomic locations were chosen because previous studies had shown low basal and high induced expression of transgenes

integrated at these sites (Markstein et al, 2008). As an additional safeguard against spurious or artifactual influences on reporter gene activity the response element-reporter cassette was flanked by gypsy insulators (Markstein et al, 2008). Several strains carrying different combinations of ARE, TRE, and mRE driving DsRed.T4 or eGFP reporters in either the 2L or 2R integration site were generated (see Table 2.1 for nomenclature).

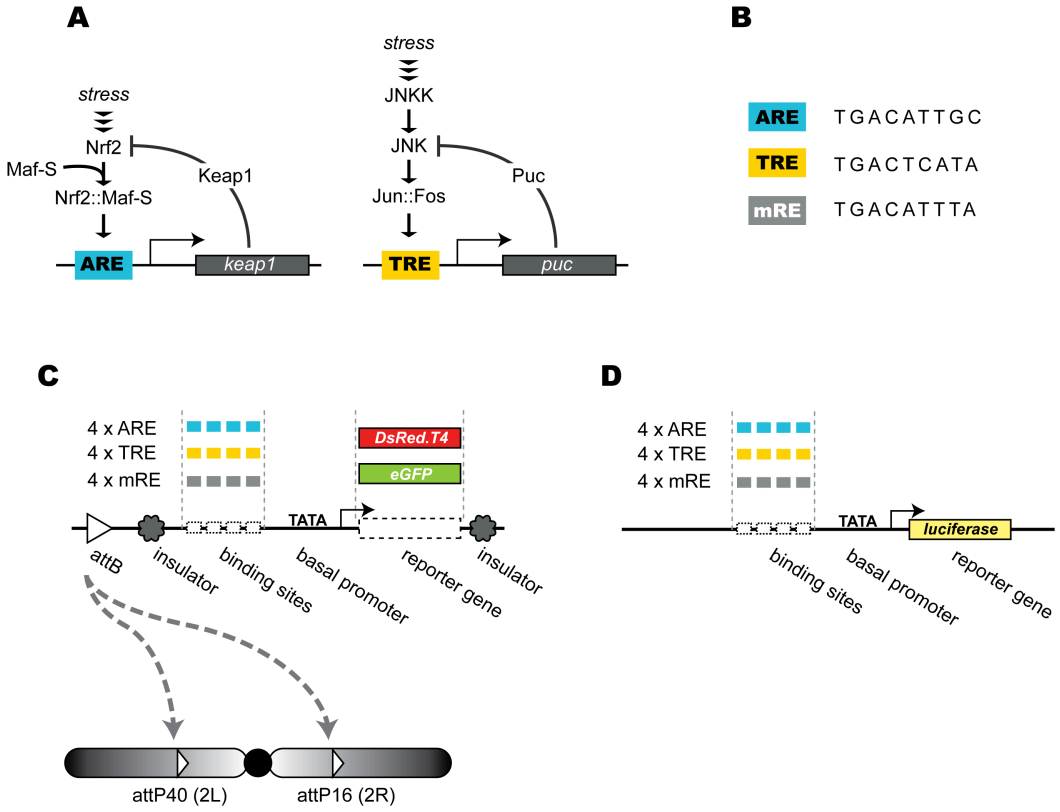


Figure 2.1.Design of ARE and TRE reporter genes.

(A) Simplified cartoons of stress signaling by the Nrf2 and JNK signaling pathways. Upon exposure to stress or cancer chemopreventive drugs, Nrf2 is relieved from repression by its cognate inhibitor Keap1. Nrf2 then dimerizes with Maf-S and binds to AREs of its target genes to activate their transcription. Activation of the *keap1* gene itself by Nrf2 engages a negative feedback loop that limits Nrf2 responses. The JNK cascade with the stress inducible JNKK and JNK activates the Jun::Fos dimer by phosphorylation. This leads to the activation of genes via Fos::Jun binding sites or TREs. Activation of the gene encoding the JNK phosphatase, Puckered causes negative feedback regulation.

(B) Sequence of the artificial TRE and ARE elements used for the construction of the Nrf2 and JNK reporters. The sequences correspond to optimal CncC/Maf and Jun/Fos binding sites as reported by Kerppola and Curran (Kerppola & Curran, 1994). A mutant version of the response elements (mRE) that does not bind either Jun/Fos or Nrf2/Maf dimers was generated as a control construct.

(C) Modular design of *in vivo* reporter constructs. Each transgenic cassette carries an attB site for ΦC31 recombinase-catalyzed genomic integration, a minimal heat shock protein 70 (Hsp70) promoter (TATA) and eGFP or DsRed.T4 reporter gene driven by four head-to-tail concatenated copies of the ARE, TRE or mRE elements. The promoter-reporter cassettes are flanked by gypsy

insulators to prevent 'position effects' of surrounding chromatin. Transgenic flies carry reporter transgenes at predetermined locations, attP 16 on the right arm of chromosome 2 and attP 40 on the left arm of the same chromosome.

(D) Design of reporters for cell culture experiments. In a similar design as shown in C, the firefly luciferase reporter gene was placed under a minimal heat shock protein 70 (Hsp70) promoter (TATA) and four head-to-tail concatenated copies of the ARE, TRE or mRE elements.

Table 2.1. Transgenic reporter fly line

Name	Promoter	Reporter	Location
ARE-green-2L	4×ARE	eGFP	attP40
TRE-red-2R	4×TRE	DsRed.T4	attP16
ARE-green-2R	4×ARE	eGFP	attP16
TRE-red-2L	4×TRE	DsRed.T4	attP40
ARE-red-2L	4×ARE	DsRed.T4	attP40
TRE-green-2R	4×TRE	eGFP	attP16
mRE- red-2L	4×mRE	DsRed.T4	attP40

Reporters for cell culture experiments

As a complement to the *in vivo* reporters in transgenic flies, we generated matching cell-based reporters, namely ARE-fluc, TRE-fluc and mRE-fluc that carry the firefly luciferase gene under the control of the same synthetic Nrf2 and AP-1 binding sites (Fig.

2.1D). These *in vitro* reporters can be used to carry out, among other experiments, large-scale screens for genetic regulators and chemical modulators of CncC and JNK pathways (Chatterjee & Bohmann, 2012).

2.2.2. Functional Characterization

In vivo reporters

The readout of fluorescent ARE or TRE reporters can be monitored in intact flies using a UV equipped stereo microscope. Fig. 2.2 shows that in standard non-stressed conditions both ARE and TRE-dependent reporters have measureable basal levels of activity. Interestingly, the tissue distribution of the ARE and TRE dependent signals differs, with the ARE reporter showing prominent activity in the abdomen and the antennae, whereas TRE activity was strongest in the thorax. This evident tissue specificity is independent of the genomic integration site. A qualitative analysis shows that the activity pattern is maintained regardless of whether the reporters reside in the attP16 or attP40 location (Fig. 2.7). The ARE and TRE reporters will be instrumental to dissect tissue and organ-specific functions of JNK and Nrf2 signaling.

In order to test the inducibility and specificity of the ARE and TRE reporters in their respective transgenic fly lines, we assessed the effect of activating the Nrf2 and JNK pathways genetically. To this end, the specific inhibitors of JNK or Nrf2 signaling, Puc and dKeap1, respectively, were removed by expression of corresponding RNAis under the control of the ubiquitously active armadillo Gal4 driver. The resulting changes in reporter gene activity were assessed at the whole animal level by observation under a stereo microscope (Fig. 2.2). Knockdown of dKeap1 specifically activated the ARE-green reporter, but not the TRE-red reporter. Conversely, removal of Puc activated TRE-red reporter in flies (Fig. 2.2), but left ARE-green unaffected.

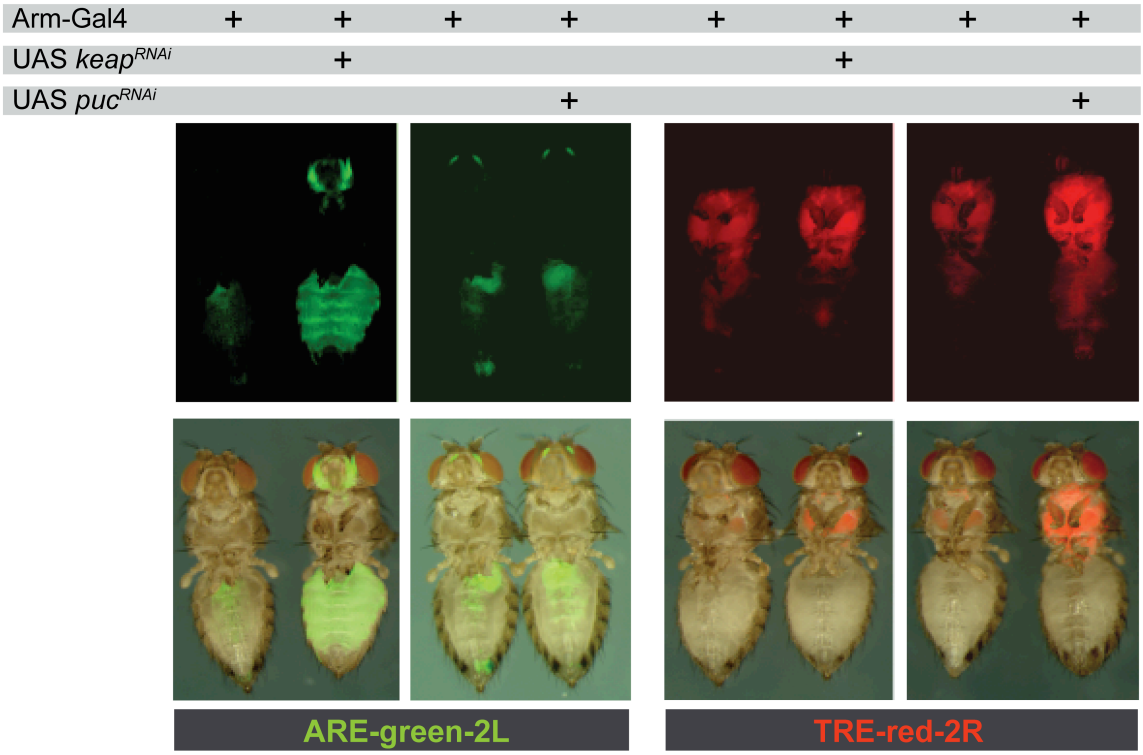


Figure 2.2. Transgenic ARE reporters and TRE reporters are specifically responsive to Nrf2 and JNK signaling respectively. Loss of dKeap1 function specifically activates Nrf2 activity *in vivo* (Sykiotis & Bohmann, 2008). Consequently, expressing a Keap1-specific RNAi under the control of the ubiquitously active arm-Gal4 driver activated the ARE-green reporter, but not the TRE-red reporter. Conversely, arm-Gal4 driven expression of RNAi targeting Puc, the negative regulator of JNK signaling, stimulated TRE-red reporter activity but did not affect the ARE-green reporter. The upper panels show red and green fluorescence in adult 5-day-old female flies. These fluorescence images were superimposed onto the bright-field images of the same flies, as shown in the lower panels. 15 to 20 flies were analyzed for each genotype and all showed similar results. The images shown show representative, randomly selected specimens.

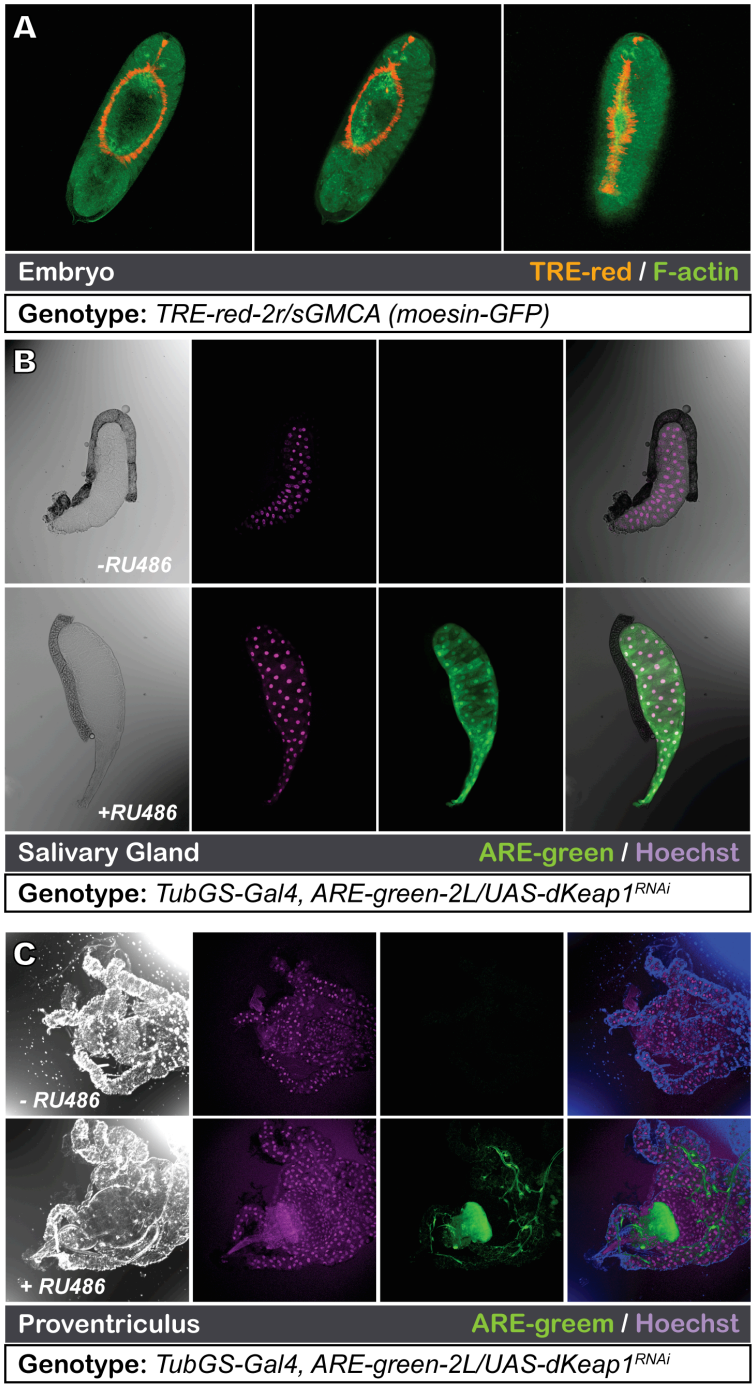


Figure 2.3. ARE and TRE reporters faithfully reproduce known patterns of JNK and Nrf2 signaling.

(A) The TRE-red reporter marks the leading edge cells of stage 13 to 15 embryos where JNK is known to be active during dorsal closure. In addition to the TRE-red-2R reporter, the embryos

expressed a moesin GFP fusion protein (sGMCA (Hutson et al, 2003)) to visualize the actin cytoskeleton (green). Three embryos at progressively later stages of dorsal closure are shown.

(B) ARE-green reporter activity was detected in different larval tissues in which Nrf2 function has previously been reported (Sykiotis & Bohmann, 2008). In 3rd instar larval salivary glands Nrf2 reporter activity was undetectable at basal levels (top panel, genotype TubGSGal4, ARE-green-2L/UAS-dKeap1^{RNAi}, no RU 486 treatment), but was prominent after knockdown of Keap1 expression (same genotype as above, but keap1^{RNAi} expression was induced by RU486). The nuclei are stained with Hoechst (purple). The left panels show a micrograph of the salivary glands.

(C) Cells in the proventriculus of the digestive tract show prominent Nrf2 activity after Keap1 knockdown. Conditions and genotypes are as described in panel B.

Confirming the fidelity of the *in vivo* reporter lines, we found that they mark tissues in which the JNK or Nrf2 signaling is known to be active (Fig. 2.3). For example, the TRE reporter strongly labels cells in the leading edge of the embryonic epidermis during dorsal closure, a morphogenetic process that requires developmentally regulated JNK mediated gene activation in this location (Glise & Noselli, 1997; Homsy et al, 2006; Kockel et al, 1997; Noselli, 1998). High inducible Nrf2 target gene and CncC mRNA expression, has been described in the proventriulus, midgut and salivary glands of *Drosophila* larvae (Sykiotis & Bohmann, 2008). This activity pattern can be recapitulated in larvae expressing *keap1^{RNAi}* (used in Sykiotis and Bohmann, 2008 (Sykiotis & Bohmann, 2008)), as visualized with the ARE-green reporter (Fig. 2.3). These results show that the ARE and TRE reporters can be used to follow the *in vivo* CncC and JNK activity of *Drosophila* at various stages of development and during different physiological processes.

In vitro reporters

Complementing the *in vivo* experiments described above, cell culture assays were designed to test the specificity of the ARE and TRE luciferase reporters in S2 cells. The JNK pathway can specifically be activated in this system by transient expression of a constitutively active version of JNKK (Hep^{act}, in which regulatory phosphorylation sites, the serine and threonine residues at positions 326 and 330, were replaced with aspartic acid), a kinase that selectively phosphorylates JNK and causes its robust activation (Jasper et al, 2001; Weber et al, 2000). Under these conditions, AP-1 activity, as measured by the TRE-fluc reporter and luciferase assays, increases strongly. In contrast, the ARE-fluc reporters did not respond in parallel experiments, documenting the specificity of the assay system. Conversely, when the *Drosophila* Nrf2 pathway was genetically activated, by overexpression of CncC, enhanced ARE but not TRE activity was observed (Fig. 2.4A)

In loss-of-function experiments, the activities of ARE-fluc and TRE-fluc reporters were specifically downregulated by dsRNA-mediated knock-down of CncC and Bsk (Fig. 2.4B). These results show that the ARE-luc reporter is specific to CncC signaling, whereas the TRE-fluc is specific to JNK activity in S2 cells. Taken together, the cell-based and *in vivo* studies showed that the genetic modulation of one pathway does not affect the activity of the reporter for the other pathway. Therefore, the ARE and TRE

reporters can be reliably used to monitor CncC and JNK activity both *in vivo* and in cell-based experiments.

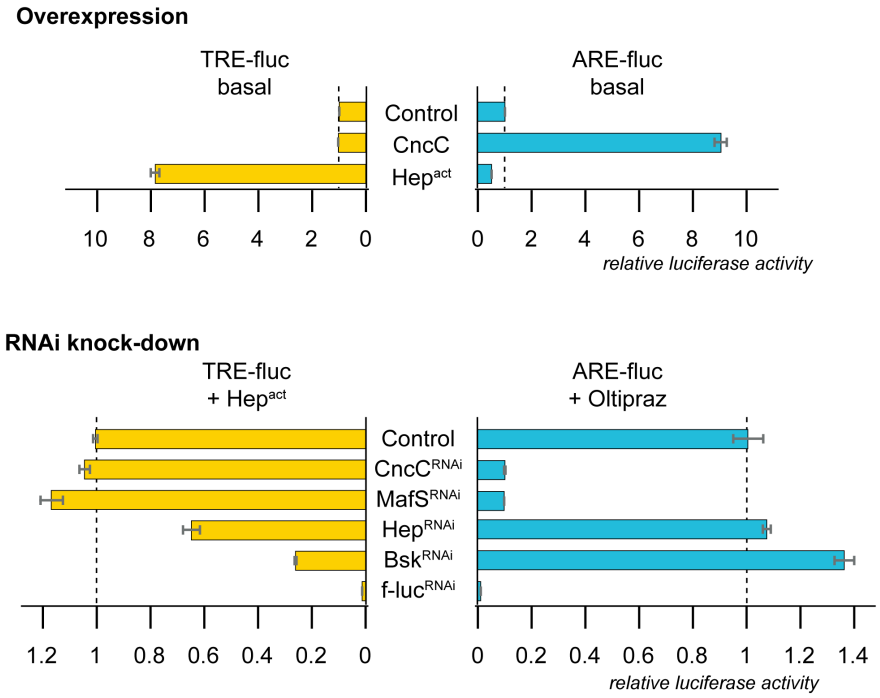


Figure 2.4. Signal specificity of cell-based reporters

(A) Artificial stimulation of JNK and Nrf2 signaling in S2 cells specifically activates the TRE-fluc or ARE-fluc reporter, respectively. Expression of a constitutively active mutant of *Drosophila* JNKK (Hep^{act}) activated a co-transfected TRE-fluc reporter, but not an ARE-fluc reporter. Conversely, overexpression of CncC increased the activity of the ARE and not the TRE reporter. Firefly luciferase activity was normalized using a cotransfected renilla luciferase construct (Act5C rluc) as a reference. The activity under control conditions (reporters co-transfected with pAct-Gal4) was set to 1.

(B) Signal-dependent activation of TRE and ARE reporters specifically requires JNK or Nrf2 pathway components. S2 cells were transfected with TRE-fluc reporters and JNK activity was stimulated by co-expression of constitutively active *Drosophila* JNKK (Hep^{act}) as indicated. Cells transfected with the ARE-fluc reporter were treated with the specific Nrf2 activator Oltipraz. dsRNA mediated knockdown of CncC, or MafS compromised only Oltipraz-induced ARE-fluc activity. Conversely, dsRNA against Hep (JNKK) and Bsk (JNK) only reduced TRE activity. Firefly luciferase activity was normalized to renilla luciferase activity driven by Act5C promoter. The activity of the activated reporters under control conditions was set to 1.

Error bars indicate standard deviation of 3 biological replicates.

2.2.3. Comparative analysis of stress JNK and Nrf2 responses in adult flies and cell culture

Due to the defined genomic integration sites of the *in vivo* reporters and the complementary spectral characteristics of eGFP and dsRedT4, it is possible to generate fly stocks carrying two different reporters that can be tracked separately. For example, we constructed a recombinant *Drosophila* line that carries 2 copies of ARE-green and 2 copies of TRE-red reporters at the attP40 and attP16 sites respectively, on the second chromosome. In these flies, JNK signaling can be tracked as red fluorescence whereas Nrf2 activation elicits a green signal.

Using these double reporter flies, JNK and Nrf2 responses to different insults, as well as the temporal and spatial characteristics of their induction, can readily be compared in the intact organism. Fig. 2.5 shows an example for such an analysis. Both JNK and Nrf2 activity were markedly elevated upon exposure of the double reporter flies to the oxidative stressor paraquat, a free radical generator. Similar effects were observed in response to treatment with hydrogen peroxide (an intracellular reactive oxygen species), sodium arsenite (a heavy metal), or DEM (diethyl maleate, a glutathione-depleting agent) (data not shown). In contrast, exposure to Oltipraz, a cancer chemopreventive drug known to mediate its salutary effects by boosting Nrf2 activity (Kensler et al, 1999; Ramos-Gomez et al, 2001), raised the ARE-GFP signal, but had no effect on the activity of the TRE reporter.

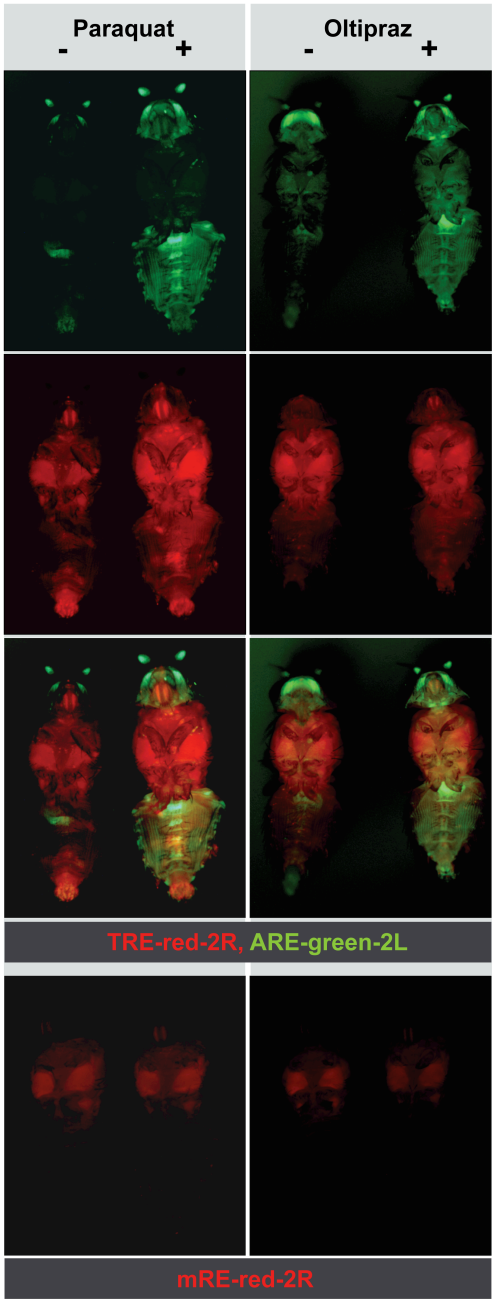


Figure 2.5. Simultaneous measurement of Nrf2 and JNK responses *in vivo*. Exposure of flies that carry two copies each of both the TRE-red-2R and the ARE-green-2L reporters to the oxidative stressor paraquat increased both ARE and TRE activities, however with different spatial specificity. Oral application of the cancer chemopreventive drug oltipraz, on the other hand, only induced ARE and not TRE activity. The upper and the middle panels show green and red fluorescence separately in the same flies. These green and red fluorescence images are merged in the lower panel. The control food for oltipraz treatment was supplemented with 1% DMSO, the solvent used for oltipraz. The control reporter stock mRE-red-2L did not respond to either paraquat or oltipraz.

To test whether signal specific pathway activation, as documented *in vivo*, can also be observed in S2 cultures, experiments using the ARE, TRE and mRE-fluc reporters were conducted. We found that Nrf2 activity, as measured by a transiently transfected ARE-fluc reporter, was selectively induced by treatment of S2 cells with Oltpraz or Sulforaphane (Fig. 2.6 and data not shown). TRE-fluc does not respond to these drugs. In contrast, general stressors DEM and arsenite stimulate both the ARE and the TRE reporter (Fig 2.6). The mRE reporter is not affected by any of these drugs. These experiments show the utility of the reporter system for studying CncC and JNK

responses in cell culture, allowing further molecular and biochemical characterization of different stressful or pharmacological activators and inhibitors.

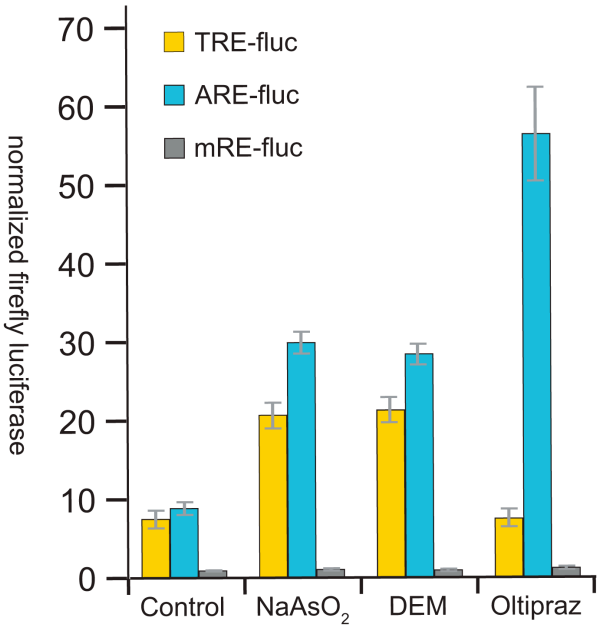


Figure 2.6. Stimulation of cell-based reporters by sulforaphane, oltipraz and stressors. S2 cells transiently transfected with TRE-fluc, ARE-fluc or mRE-fluc plasmids, as indicated, were exposed to sodium arsenite ($NaAsO_2$), Diethyl Maleate (DEM) or oltipraz. The firefly luciferase activity was measured 24 hrs after drug treatment and normalized to renilla luciferase activity expressed from a co-transfected Act5C renilla luciferase construct. Note that both TRE and ARE reporters respond to the stressors, Arsenite and DEM, but only ARE-fluc responds to Oltipraz. The mRE reporter responds neither to oltipraz nor to the stressors. The activity of mRE-fluc reporter under control conditions was set to 1. Error bars indicate standard deviation of 3 biological replicates.

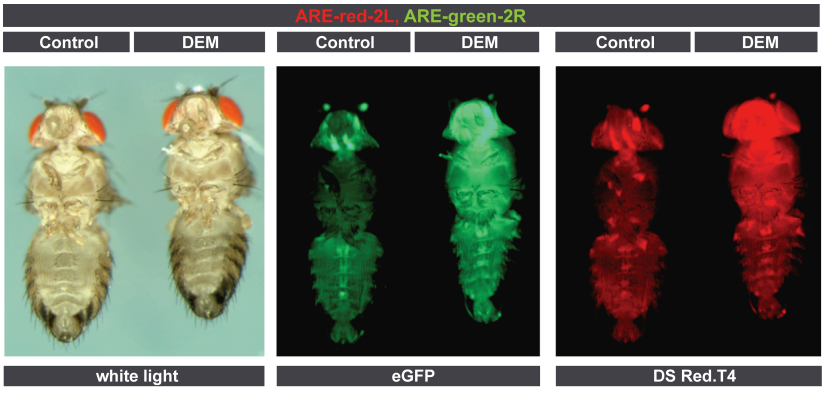


Figure. 2.7. Insertion site or type of reporter gene do not affect *in vivo* ARE activity. ARE-red-2L, ARE-green-2R double Nrf2 reporter flies were exposed to DEM stress. Both basal level and DEM-induced activities of ARE reporters at two different sites, attP40 (2L) and attP16 (2R), containing different reporter genes (eGFP and DsRedT4) were similar.

Chapter 3:

dsRNA library screen for the identification of novel regulators of Nrf2 signaling

3.1. dsRNA library screen

3.1.1. Introduction

A vast number of studies have established Nrf2 as a master regulator of oxidative stress responses (Kensler et al, 2007; Motohashi & Yamamoto, 2004; Sykiotis & Bohmann, 2010). However, over the last few years Nrf2 and the related Nrf1 transcription factor have been implicated in a range of biological processes in addition to stress responses. Examples include the regulation of energy metabolism (Hayes & Dinkova-Kostova, 2014; Sykiotis et al, 2011), stem cell maintenance (Hochmuth et al, 2011) and aging (Sykiotis & Bohmann, 2008; Tullet et al, 2008). These functions are probably regulated by signals other than toxic insults and presumably require a different transcriptional response, in terms of kinetics and target gene profile. Consistent with this expanded range of Nrf2 functions, it has emerged that the mechanisms that regulate the activity of the transcription factor are also more complex than previously recognized. Several mechanism and cofactors in addition to those associated with the canonical Keap1-

dependent pathway have been discovered to regulate Nrf2 in such situations. As discussed in the section 1.2.3.1, multiple kinases have been reported to regulate mammalian Nrf2 signaling. However, the mechanisms of the regulatory effects of several of those kinases on Nrf2, and how they connect Nrf2 signaling pathway to other physiological and environmental signals, are not clear. In order to further explore the complexities of Nrf2 function and regulation we conducted a cell-based kinome-wide dsRNA library screen.

A cell-based dsRNA library screen can be very effective to identify the regulators of Nrf2 pathway. Such a screen is fast and can identify essential genes that would not be identified in classical mutant screens because loss-of-function mutations would often be lethal or cause pleiotropic phenotypes. *Drosophila* cells are particularly well suited for this screen because (1) the Nrf2 pathway is conserved between *Drosophila* and mammals, (2) *Drosophila* has less genetic redundancy compared to the mammalian genome, (3) the cells can take up dsRNAs directly from the media and (4) there is no interferon-mediated shutdown of translation when *Drosophila* cells are challenged with dsRNAs (Boutros et al, 2004; Wang & Carmichael, 2004).

3.1.2. Results and Discussion

3.1.2.1. Identification of putative regulators of CncC signaling

In order to identify novel regulators of CncC signaling, a cell-based, kinome-wide dsRNA library screen was conducted in collaboration with the laboratory of Dr. Michael Boutros. The ARE-fluc and actin-renilla-luciferase (RL-Act) plasmid constructs were used in a 384-well robotic platform as reporter for CncC transcriptional activity and internal control, respectively (Chatterjee & Bohmann, 2012). 420 genes that encode presumptive kinases were tested in the screen to identify kinases that affect ARE-fluc reporter gene activity in transiently transfected *Drosophila* Schneider 2 (S2) cells under basal conditions (Fig 3.1). In order to exclude the possibility of off-target effects, each of the kinases was knocked down with three independent dsRNAs (200-700 bp) that targeted different parts of the gene. The Z-score for each dsRNA was calculated using the formula, z score = (individual value – mean) / standard deviation.

Genes that, when knocked down, caused a significant decrease in ARE-fluc reporter activity for multiple dsRNAs (Z-scores below -1.65), were classified as putative positive regulators of Nrf2 signaling. On the other hand, conditions, where knock down caused a consistent increase in luciferase activity (Z-scores above +1.65), identified potential negative regulators. In control experiments, which were conducted to validate the assay

system, knock down of CncC and MafS caused a significant decrease of ARE-fluc activity. Keap1 knock down, on the other hand, increased ARE-fluc signal.

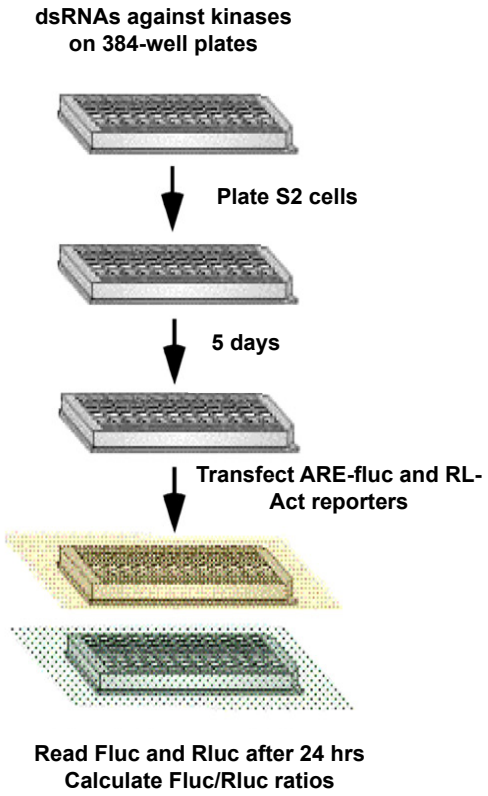


Figure 3.1. Schematic representation of the dsRNA library screen. S2 cells were incubated with dsRNAs against *Drosophila* kinases in a 384-well plate for 5 days, followed by transfection with ARE-fluc and RL-Act plasmids. The firefly and Renilla luciferase activities were measured after 1 day and the individual value for each well was calculated by dividing the firefly luciferase activity with the Renilla luciferase activity. Z-score for each well was calculated by the formula: Z score = (individual value – mean) / standard deviation. A positive Z-score indicated increase in individual value and hence, a knock down of a negative regulator. Conversely, a negative Z-score signaled reduction in individual value and therefore, a knock down of a positive regulator. (Adapted from Stielow et al, 2008, (Stielow et al, 2008)).

Six putative positive regulators and two putative negative regulators of CncC signaling were identified in this screen (Table 3.1). We found *casein kinase 2*, which had previously been reported to activate mammalian Nrf2, as a putative positive regulator in this screen (Apopa et al, 2008). However, it is important to validate the role of these candidates in the regulation of CncC signaling through secondary and other functional

assays. The validation of Cdk12, which was identified as a putative positive regulator in the screen, is discussed here.

Table 3.1. Putative regulators of CncC signaling identified in the dsRNA library screen.

	Gene	dsRNA 1	dsRNA 2	dsRNA 3
Controls	**CncC**	**-4.52**		
	MafS	**-3.61**		
	Keap1	**0.7**		
Inducers	**Cdk12**	**-25.1**	**-28.27**	**-39.64**
	Smg1	**-1.47**	**-3.9**	**-6.16**
	Fray	**-3.4**	**-3.82**	**-0.19**
	Madm	**-1.92**	**-2.86**	**-3.29**
	Psi	**-0.93**	**-2.84**	**-3.02**
	CkII-alpha	**-1.93**	**-1.94**	**-1.95**
Suppressors	**Fs(1)h**	**1.96**	**1.9**	**1.6**
	Nipped-A	**1.27**	**1.81**	**1.81**

The Z-scores calculated for the effects of knocking down putative regulators identified in the dsRNA library screen on ARE-fluc readouts are tabulated here. Six putative inducers represented by negative Z-scores and two putative suppressors represented by positive Z-scores were identified in the screen. dsRNAs against CncC, MafS and Keap1 were used as controls.

3.2. Validation of Cdk12 as a required factor in Nrf2 dependent gene activation

3.2.1. Introduction

Cdk12, a RNA pol II CTD kinase, phosphorylates serine residues in the COOH-terminal domain (CTD) of the largest subunit of RNA polymerase II during transcription. The CTD in *Drosophila* contains of 42 seven amino acid (YSPTSPS) repeats (Zehring et al, 1988) and phosphorylation of serine residues in these repeats is necessary for the initiation and elongation of transcription, and for some steps of RNA processing such as 5′ capping and splicing (Phatnani & Greenleaf, 2006). Recent studies have found that CDK12, in a complex with a Cyclin K, can specifically phosphorylate the second serine (Ser2) residue in the CTD repeat sequence (Bartkowiak et al, 2010). In addition to Cdk12, Cdk9, in a complex with Cyclin T, can also phosphorylate Ser2 in the CTD heptad repeats in *Drosophila* (Bowman & Kelly, 2014). Interestingly, Bartkowiak et al found that CDK12 is enriched toward the 3′ end of genes whereas Cdk9 is enriched toward the 5′ end. This observation along with the finding that inhibition of Cdk9 activity often decreases all Ser2 phosphorylation led Bartkowiak and Greenleaf to propose a model where Cdk9 causes Ser2 phosphorylation of RNA Polymerase II CTD at the 5′ end of the gene and, this is upstream of and required for Cdk12 mediated Ser2 phosphorylation at the 3′ end of the gene (Bartkowiak et al, 2010; Bartkowiak et al, 2011). A recent study has found that CDK12 is important for the transcription of only a small subset of human genes. This group was enriched in long genes with high numbers

of exons, such as DNA damage response genes (Blazek et al, 2011). Cdk12/Cyclin K might also be important for cellular differentiation. Dai et al reported that pluripotent embryonic stem cells (ESCs) have high levels of Cyclin K, which decrease during differentiation. In addition, artificial reduction of Cdk12 levels in ESCs resulted in spurious differentiation (Dai et al, 2012).

3.2.2. Results and Discussion

3.2.2.1. Cdk12 is required for Nrf2 activity

In order to study whether CDK12 is important for the increase in ARE reporter activity in response to Nrf2 induction, we exposed S2 cells treated with Cdk12 dsRNA to selective Nrf2 activator oltipraz. It was observed that knock down of Cdk12 with two different dsRNAs consistently affected both the basal and oltipraz-induced ARE-luciferase activity (Fig. 3.2A). In addition, knock down of Cyclin K, the regulatory subunit of Cdk12, also reduced Nrf2 reporter activity (Fig 3.2B). These results indicate that Cdk12 is required for both the basal and oltipraz-induced Nrf2 activity in *Drosophila* S2 cells and it requires its binding partner Cyclin K for this function.

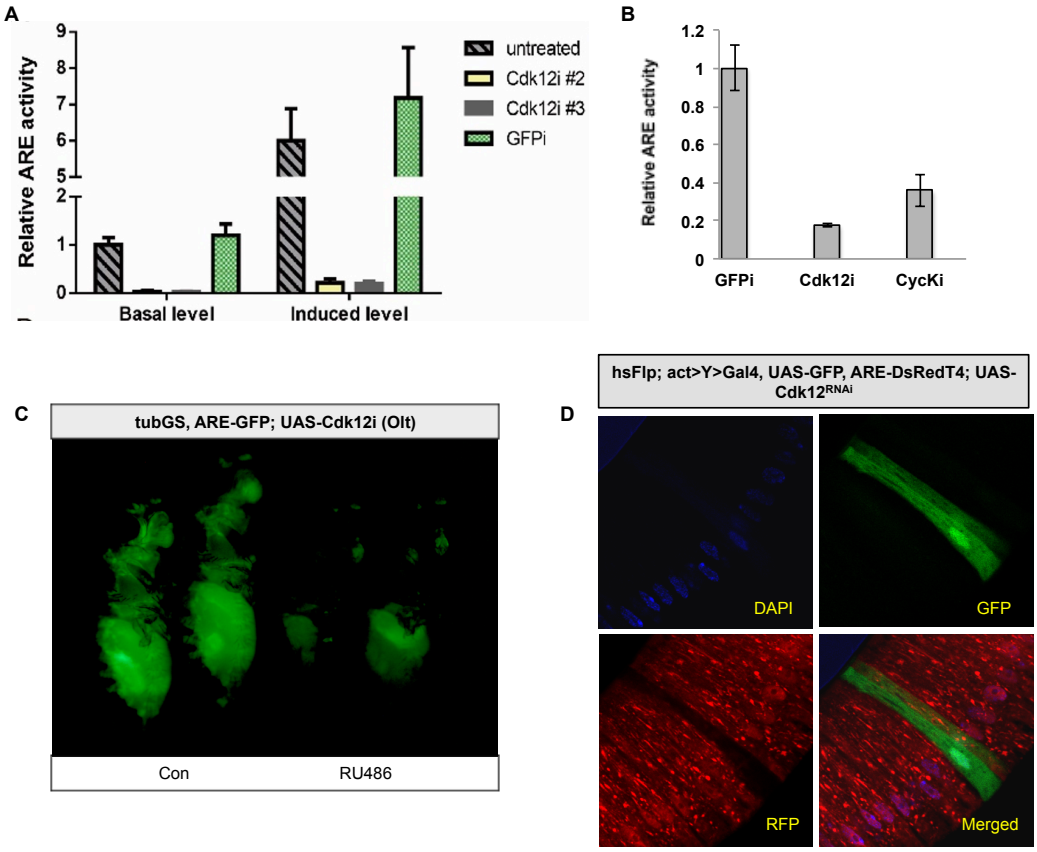


Figure 3.2. Cdk12 is required for CncC-dependent reporter gene activation. (A) Cdk12 knock down abrogated both basal level and oltipraz-induced ARE-fluc activity in S2 cells. (B) Knock down of Cyclin K (CycK), the binding partner of Cdk12, similarly reduced ARE-fluc activity in S2 cells. (C) Ubiquitous knock down of Cdk12 by the expression of a UAS-Cdk12^{RNAi} construct under tubulin gene-switch (tubGS) driver which is inducible by RU486, reduced oltipraz-mediated ARE-GFP activation in adult female *Drosophila*. (D) Cdk12 knock down in hsFlp-induced clones marked with GFP reduced ARE-RFP activity in a cell-autonomous manner in the ejaculatory bulb of adult male *Drosophila*.

In order to characterize the role of Cdk12 in Nrf2 dependent gene activation in *Drosophila*, we examined the effect of Cdk12 knock down on an ARE-GFP reporter transgene in adult flies (Chatterjee & Bohmann, 2012). Cdk12 was ubiquitously knocked

down by expressing Cdk12 specific RNAi under the control of a RU486-inducible Gal4 driver in adult flies carrying the ARE-GFP reporter. It was observed that, unlike the control flies with no RNAi was expressed, oltipraz-mediated induction of ARE-GFP reporter was significantly reduced in Cdk12 knock down condition (Fig. 3.2C). This indicates that Cdk12 is required to the signal-responsive induction of the Nrf2 pathway.

Next, we employed a flp-recombinase-FRT strategy to express the Cdk12 RNAi construct in individual, genetically marked clones to understand the role of Cdk12 in Nrf2 function at the cell level. Clones were generated by a heritable activation of Gal4 expression that was caused by heat shock-induced expression of flp recombinase. Gal4, in turn, activates the expression of the UAS driven RNAi transgene and GFP. Cdk12 knock down in hsFlp-induced clones in the ejaculatory bulb of adult male *Drosophila*, a tissue with a high basal level of Nrf2 reporter activity, significantly reduced the ARE-RFP activity (Fig. 3.2D). In contrast, the ARE activity was not affected in control clones that did not express Cdk12 RNAi (data not shown). This indicates that the effect of Cdk12 on Nrf2 signaling is cell-autonomous. Interestingly, both S2 cells and clones that were partially depleted of the Cdk12 by RNAi, were viable. Moreover, the renilla luciferase activity controlled by *actin5C* promoter in S2 cells was not reduced by Cdk12 knockdown. This supports the finding by Blazek et al that wild type levels of Cdk12 expression are not essential for the all RNA Polymerase II-dependent RNA transcription and Cdk12 has more selective regulatory functions (Blazek et al, 2011).

3.2.2.2. Cdk12 is essential for oxidative stress resistance

Our cell-based and *in vivo* data have shown that Cdk12 is required for Nrf2 target gene activation under various conditions. Since Nrf2 signaling plays a critical role in the oxidative stress responses, we evaluated how Cdk12 influences the oxidative stress sensitivity in flies. We found that ubiquitous knock down of Cdk12 in adult flies make them very sensitive to DEM, which depletes the intracellular glutathione pool and causes oxidative stress (Fig. 3.3). This indicates that Cdk12 is critical for oxidative stress resistance.

These reporter activity and stress resistance studies showed that a putative regulator of Nrf2 signaling identified in the aforementioned screen could be validated by secondary and functional assays. This highlighted the usefulness of the screen in the identification of novel regulators of Nrf2 signaling.

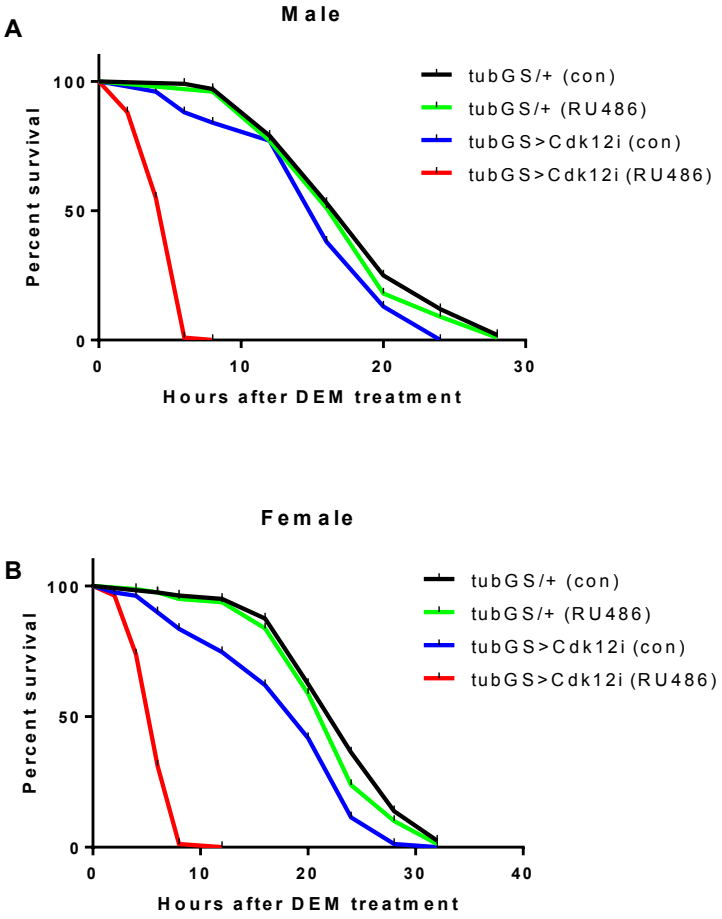


Figure 3.3. Cdk12 is necessary for oxidative stress resistance. Cdk12 was knocked down in adult *Drosophila* by expression of UAS-Cdk12^{RNAi} transgene under the control of the tubGS driver. Exposure of adult flies of this genotype to food containing 300µM RU486 induced the expression of the RNAi construct. The flies containing both tubGS-Gal4 and UAS-Cdk12^{RNAi} that were maintained on RU486 food, showed increased sensitivity to 20 µM DEM compared to those on control food. Flies containing only tubGS-Gal4 did not show heightened sensitivity to DEM irrespective of the presence or absence of RU486 in their food.

Chapter 4

Regulation of Nrf2 Signaling by a BET protein

4.1. Introduction

BET proteins including *Drosophila* Fs(1)h and the members of the mammalian Brd family function as epigenetic readers, as regulators of transcription elongation and of chromatin state (Belkina & Denis, 2012; Kellner et al, 2013; Kockmann et al, 2013; Shi & Vakoc, 2014). BET proteins are characterized by the presence of two bromodomains, adjacent to a so-called extra terminal (ET) domain, which has a putative serine-threonine kinase activity (Florence & Faller, 2001). Through the bromodomains, BET proteins specifically bind to polypeptides carrying acetylated lysine residues, including acetyl-histones (Dey et al, 2003). BET proteins can affect the function of specific transcription factors such as NF-kB, FosL and Twist, but also bind to chromatin and interact with components of the general transcription machinery such as P-TEFb (Devaiah & Singer, 2013; Huang et al, 2009; Jang et al, 2005; Lockwood et al, 2012; Shi et al, 2014; Yang et al, 2005). Mammalian BET proteins, most notably Brd4, have raised broad biomedical interest because they can support several types of cancer, including NUT midline carcinoma, multiple myeloma and acute leukemia (Delmore et al, 2011; French et al, 2003; Loven et al, 2013; Zuber et al, 2011).

Fs(1)h, short for female sterile (on the first chromosome) homeotic, is counted as a member of the heterogeneous group of Trithorax proteins which generally function as epigenetic regulators (Chang et al, 2007; Digan et al, 1986). The product of the *fs(1)h gene* is the sole member of the BET protein family in *Drosophila* (Bagley et al, 2014; Chiang, 2009). The *fs(1)h* gene encodes two different isoforms, a short isoform or Fs(1)h-S and a long isoform or Fs(1)h-L. Fs(1)h-S has previously been reported to transcriptionally activate the *ultrabithorax (ubx)* gene, a function that is not shared by the larger Fs(1)h-L isoform (Chang et al, 2007). The divergence of function between Fs(1)h-S and L is also reflected in their respective genomic binding patterns: A recent genome-wide ChIP-seq study by Kellner et al showed that Fs(1)h-S and Fs(1)h-L are present at different locations of the *Drosophila* genome (Kellner et al, 2013). Whereas Fs(1)h-S was enriched in promoters and enhancers, Fs(1)h-L was predominantly localized to the insulators. These experiments suggest that *fs(1)h* functions in the regulation of gene activity and two isoforms of Fs(1)h carry out different functions.

4.2. Results and Discussion

4.2.1. The long isoform of Fs(1)h is a repressor of Nrf2 activity in *Drosophila*

In a high throughput RNAi screen we found the gene *fs(1)h* to encode a negative regulator of the *Drosophila* Nrf2 homolog, CncC. Multiple independent double stranded RNAs that target *fs(1)h* mRNA, caused a significant and specific increase in the activity of an Nrf2 luciferase reporter gene, identifying the *fs(1)h* gene as a potential inhibitor of Nrf2 function. The function of Fs(1)h as a CncC inhibitor as suggested by its identification in our screen was validated by performing transient transfection assays in *Drosophila* S2 cells. These experiments demonstrated that knock down of Fs(1)h caused Nrf2 reporter gene activation to a similar magnitude as seen upon knock-down of the canonical CncC repressor Keap1 (Fig 4.1B). When, in addition to Keap1 or Fs(1)h, CncC was knocked down, the activation of the Nrf2 reporter was abolished, indicating that the stimulatory effect was, in both cases, dependent on CncC (Fig 4.1B). Quantitative mRNA measurements by RT-qPCR showed that, in accordance with the reporter experiments, endogenous CncC target genes (*gcl-C, gstD1, keap1*) were up-regulated in *fs(1)h* knock down conditions (Fig. 4.1C). These studies are consistent with a strong inhibitory activity of Fs(1)h on CncC target gene expression.

Like some mammalian BET protein genes, the *Drosophila fs(1)h* locus yields alternatively spliced transcripts that give rise to two different polypeptides, one of 120 kDa (Fs(1)h-S) and one of 210 kDa (Fs(1)h-L) molecular mass (Fig 4.1A) (Chang et al, 2007; Floyd et al, 2013). Both alternative splice products contain the two bromodomain and the ET domain. The unique peptide sequences that extend the Fs(1)h-L isoform comprise a C-terminal motif (CTM). To test whether the two splice forms might also differ in their effect on CncC-regulated transcription, we designed dsRNAs that would specifically target only one or the other splice variant (see Materials and Methods).

Selective knock down of the long isoform Fs(1)h-L induced Nrf2 reporter activity. However, knockdown of the short isoform, Fs(1)h-S failed to do so (Fig. 4.1D). We conclude that the repression of CncC activity is a specific function of Fs(1)h-L. Western blot experiments confirmed the efficient and selective knock down of individual isoforms by the respective dsRNAs (Fig. 4.2A).

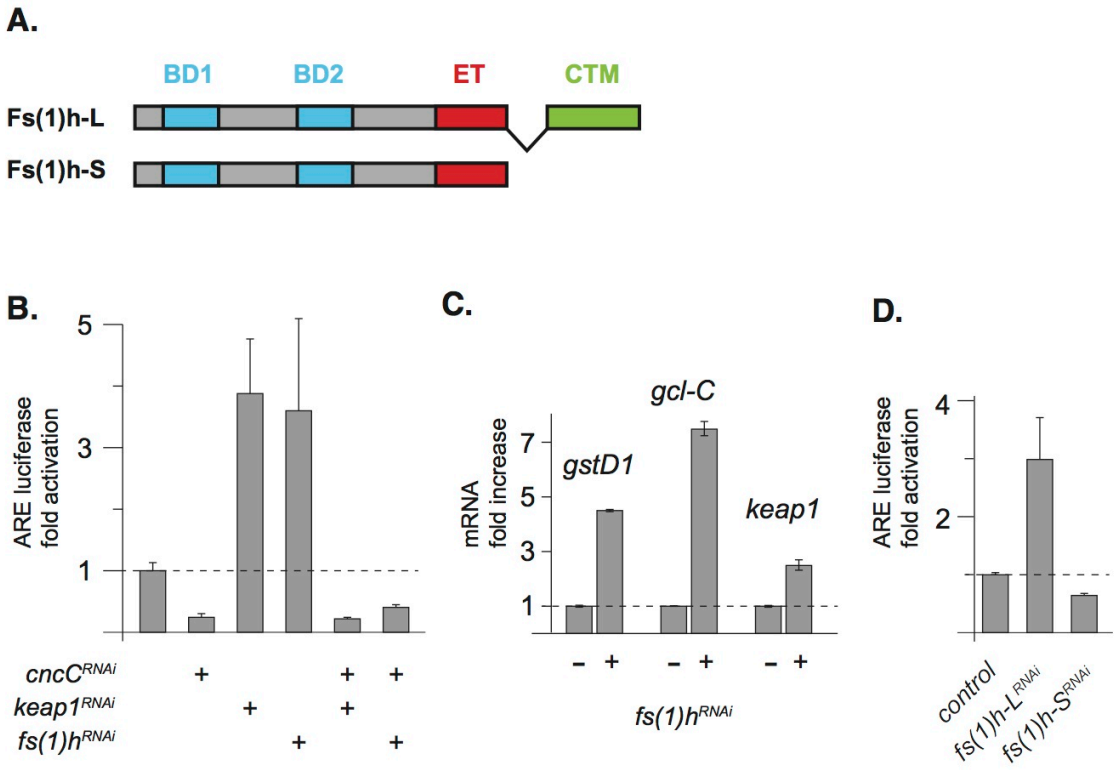


Figure 4.1. Fs(1)h negatively regulates CncC signaling.

(A) The Drosophila *fs(1)h* gene encodes two protein isoforms: the 120kD Fs(1)-S and the 210kD Fs(1)h-L. Both isoforms contain two bromodomains (BD) and an extraterminal (ET) domain. In addition Fs(1)h-L carries a unique C-terminal motif (CTM).

(B) dsRNA mediated knock down of Fs(1)h, like Keap1 knock down, increases the activity of a transiently transfected ARE-fluc reporter in S2 cells. In both cases, this stimulatory effect is abrogated by CncC knock down.

(C) RT-qPCR experiments show that the CncC target genes *gstD1*, *gclC* and *keap1* are activated upon RU486-induced-knock down of Fs(1)h. Measurements of transcript abundance levels were normalized relative to *act5c* transcript levels. Fold activation relative to the mRNA levels in mock treated flies is shown.

(D) Knock down of Fs(1)h-L alone using a specific dsRNA targeting the CTM region was sufficient to induce ARE-fluc activity in S2 cells. However, knock down of Fs(1)h-S alone with dsRNA targeting its 3' UTR did not induce ARE-fluc activity.

Error bars in panels B, C and D signify standard deviation of 3 biological replicates.

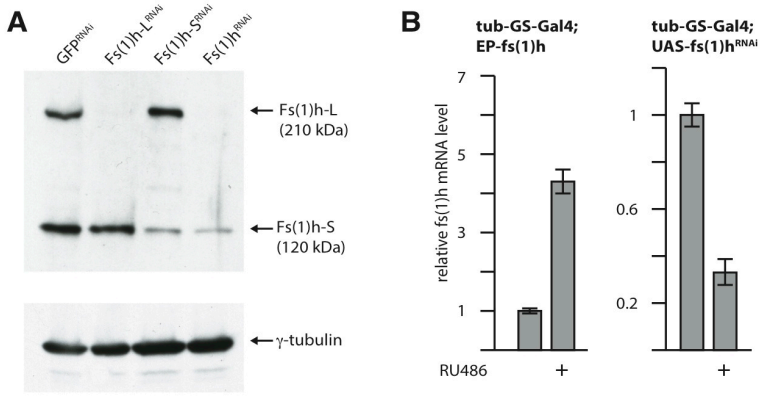


Figure 4.2. Validation of Fs(1)h knock down and overexpression

(A) Both Fs(1)h isoforms can be effectively and selectively knocked down in S2 cells. Treatment of S2 cells with dsRNA targeting the C-terminal motif of the long isoform (Fs(1)h-L^{RNAi}) selectively depleted Fs(1)h-L whereas dsRNA targeting the 3'-UTR of Fs(1)h-S (Fs(1)h^{RNAi}) led to selective depletion of Fs(1)h-S. dsRNA targeting gene region shared by both isoforms (Fs(1)h^{RNAi}) knocked down both isoforms of Fs(1)h.

(B) Validation of Fs(1)h overexpression in P(EP)*fs(1)h*[EP439] and Fs(1)h knockdown in UAS-Fs(1)h^{RNAi} (v51227). qPCR experiments done with primers that can detect both isoforms of Fs(1)h showed that RU486 treatment leads to an induction of Fs(1)h in tubGSGal4>EP-Fs(1)h flies and a knock down of Fs(1)h in tubGSGal4>Fs(1)h^{RNAi} flies . The mRNA levels were normalized to actin5C transcript level and the error bars indicate standard deviation of 3 biological replicates.

To confirm the identification of Fs(1)h as a repressor of CncC also *in vivo*, we conducted experiments with *Drosophila* stocks carrying an Nrf2-GFP reporter gene in which GFP expression is controlled by four consensus ARE sequences (ARE-green, (Chatterjee & Bohmann, 2012)). A UAS *fs(1)h*-RNAi construct was expressed under the control of the RU486-inducible tubulin-GS-Gal4 driver to ubiquitously knock down endogenous *fs(1)h* transcripts in adult flies. This resulted in robust activation of the ARE-green reporter transgene in the animals, recapitulating the effect seen in S2 cells (Fig. 4.3A, 4.2B, 4.3B).

To examine the *in vivo* effect of *fs(1)h* loss-of-function on the Nrf2-GFP reporter at the cell level, we conducted knock down experiments in groups of cells using actin-flipout-Gal4, a driver that can be clonally activated by a short heat-shock induced pulse of flp recombinase expression. Clones of RNAi-expressing cells can be distinguished by the expression of an RFP marker. The thus RFP-labeled $fs(1)h^{RNAi}$-expressing cells showed increased ARE-green reporter activity, consistent with the presumed function of Fs(1)h as a CncC inhibitor. Fig. 4.3B shows such a clone in the crop, a part of the foregut that, together with the anterior midgut, functions as the stomach in *Drosophila* (Singh et al, 2011). We found that the epithelial cells of the crop have low basal and high inducible ARE activity. The stimulatory effect of *fs(1)h* knockdown was restricted to the cells of the clone, demonstrating that the regulatory function of the protein acts by a cell-autonomous mechanism.

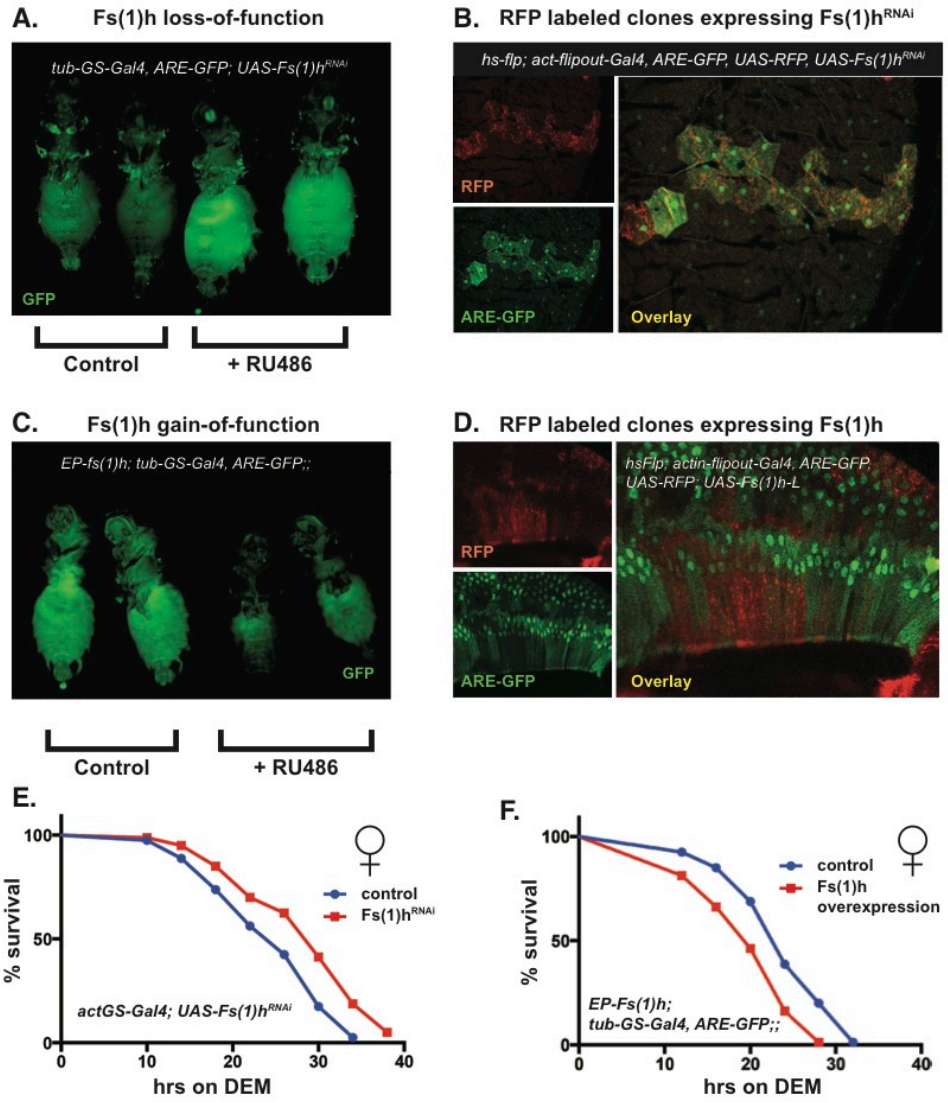


Figure 4.3. Fs(1)h-L cell autonomously inhibits CncC activity and affects oxidative stress resistance in adult flies

(A) Ubiquitous knock down of Fs(1)h (with v51227 RNAi line) in adult flies, using the RU486-inducible tub-GS-Gal4 driver stimulates ARE-GFP reporter activity in most tissues. Two RU486-treated and two mock-treated females are shown in this panel and in panel C.

(B) Knock down of Fs(1)h in actin-flipout-Gal4 clones increased ARE-GFP activity in a cell autonomous manner in the crop of adult *Drosophila* gut. In the cells in which the Fs(1)h is expressed (marked by the expression of RFP, red) ARE-GFP reporter activity (green) is increased.

(C) RU486 induced overexpression form the EP-fs(1)h allele reduces oltipraz stimulated Nrf2 reporter activity in the whole body.

(D) Fs(1)h-L overexpression in actin-flipout-Gal4 clones (labeled by RFP, red), cell autonomously reduces ARE-GFP activity in the ejaculatory bulb of adult males.

(E) Ubiquitous Fs(1)h knock-down in adult *Drosophila* by inducible expression of UAS-Fs(1)h^{RNAi} transgene under the control of the actin-GS-Gal4 driver increases oxidative stress resistance. Survival after exposure to 20µM DEM was recorded and the data were analyzed by Mantel-Cox log-rank test. Female flies incubated on RU486 food, showed significantly increased resistance to DEM (P value < 0.005) compared to those on control food.

(F) Fs(1)h overexpression raises stress sensitivity. Fs(1)h was overexpressed from the EP-fs(1)h allele in female flies by exposing them to food containing 300µM RU486 for 4 days. Lethality after exposure to 20µM DEM was recorded and analyzed by Mantel-Cox log-rank test. The flies that were kept on RU486 food, showed significantly increased sensitivity to DEM (P value < 0.0001) compared to those on control food. Experiments shown in panels E and F were also conducted with males and produced qualitatively the same results (Fig. 4.5).

The results of the loss-of-function experiments described above suggested that Fs(1)h can suppress gene activation by CncC. However, an alternative interpretation would be that the observed increase of CncC activity might be an indirect consequence of possible stress or damage in *fs(1)h* loss-of-function conditions. To rule out the latter mechanism, and to demonstrate the repressive function of Fs(1)h directly, we asked whether its overexpression could decrease CncC target gene activity in adult flies. Fs(1)h overexpression was achieved by two alternative strategies. First, we used an EP-line (P(EP)*fs(1)h*[EP439]), a fly stock in which a Gal4 responsive enhancer was integrated in the 5' genomic region of the *fs(1)h* gene. If combined with the ubiquitously expressed tub-GS-Gal4 driver expression of endogenous *fs(1)h* mRNA was stimulated when flies were exposed to dietary RU486 (Fig. 4.2B). This resulted in a marked reduction of Nrf2 reporter activity (Fig. 4.3C). Second, we generated a transgenic fly line that expresses a

cDNA encoding Fs(1)h-L, the isoform that showed CncC repressor activity *in vitro*. Inducible expression of the Fs(1)-L under the control of tub-GS-Gal4 caused a marked reduction of Nrf2-GFP reporter activity (Fig. 4.4A). In addition, over-expression of Fs(1)h-L in RFP-marked hsFlp-induced clones in the ejaculatory bulb of adult *Drosophila*, a tissue with a high basal level of Nrf2 reporter activity, had a negative effect, confirming that Fs(1)h represses CncC in a cell-autonomous fashion (Fig. 4.3D). Thus, Fs(1)h functions as a repressor of CncC's transcriptional activity both in cell culture and in adult flies.

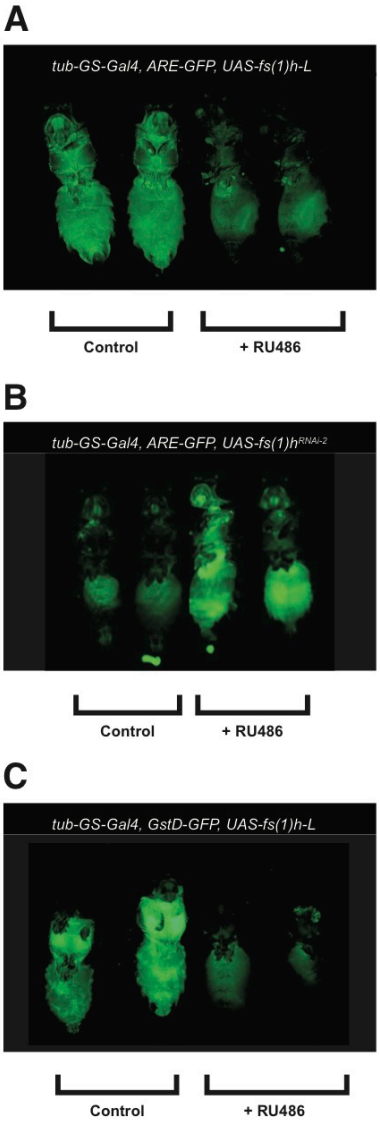


Figure 4.4. Changes in Nrf2 reporter activity with Fs(1)h overexpression and knock down

(A) Transgenic expression of Fs(1)h-L from a UAS-driven transgene in adult *Drosophila* suppresses Nrf2 reporter activity. RU486 induced overexpression of Fs(1)h in tub-GS-Gal4,ARE-GFP;UAS-Fs(1)h-L flies reduced oltipraz induced ARE-GFP reporter activity in the whole body.

(B) Ubiquitous knock down of Fs(1)h with different RNAi line (v108662) that targets a different part of the Fs(1)h gene, using the RU486-inducible tub-GS-Gal4 driver stimulates ARE-GFP reporter activity in most tissues in adult flies.

(C) Transgenic expression of Fs(1)h-L from a UAS-driven transgene in adult *Drosophila* suppresses Nrf2 reporter activity. RU486 induced overexpression of Fs(1)h in tub-GS-Gal4,ARE-GFP;UAS-Fs(1)h-L flies reduced oltipraz induced gstD1-GFP reporter (Sykiotis & Bohmann, 2008) activity in the whole body.

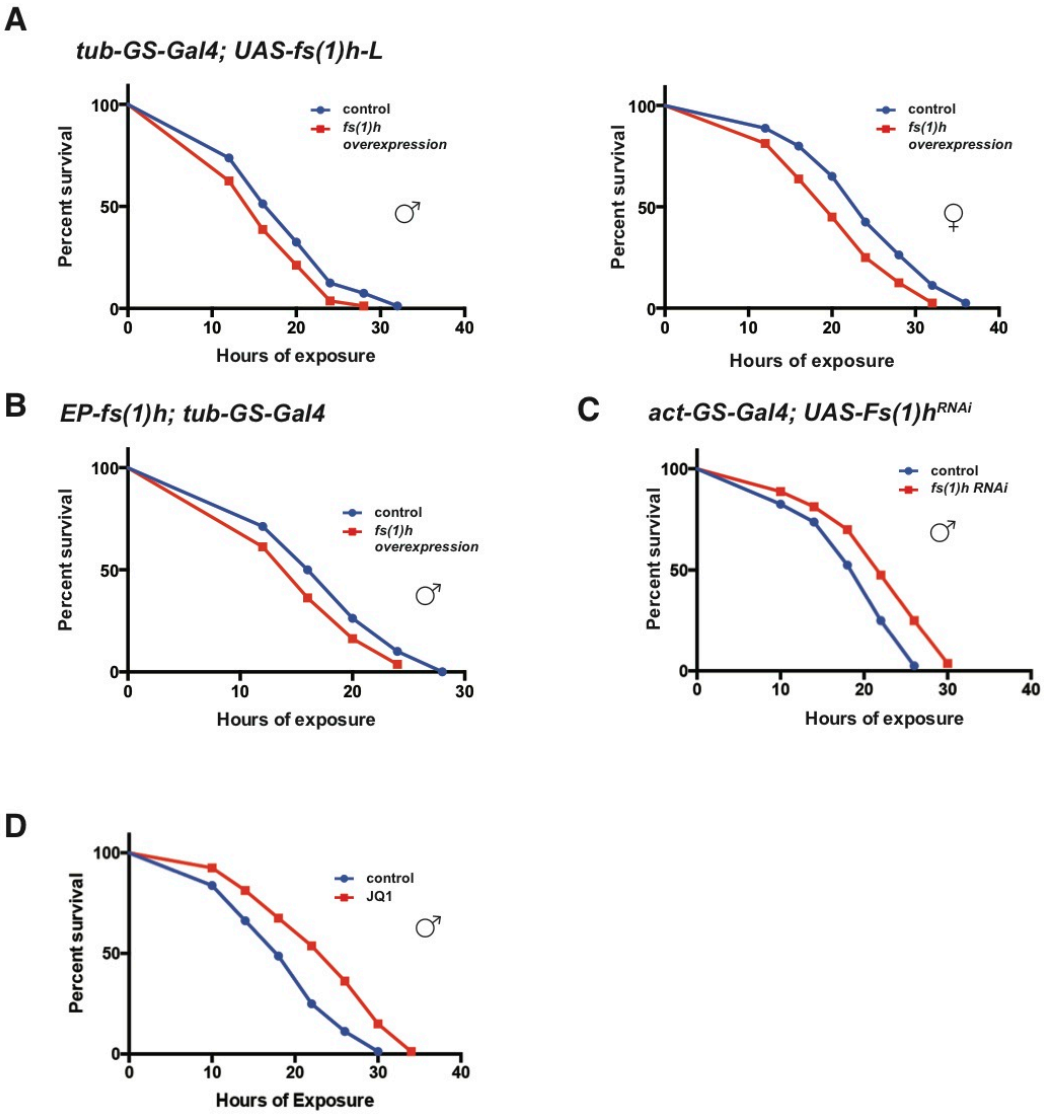


Figure 4.5. fs(1)h gain- and loss-of-function conditions affect oxidative stress resistance in adult flies.

(A) UAS-Fs(1)h-L overexpression in males and female *Drosophila*. Fs(1)h-L was overexpressed in tubGS; UAS-Fs(1)h-L flies by maintaining them on food containing 300µM RU486 for 4 days. Lethality after exposure to 20µM DEM was recorded and analyzed by Mantel-Cox log-rank test. The flies that were kept on RU486 food, showed significantly increased sensitivity to DEM (P value < 0.0023 for females and P value < 0.0214 for males) compared to those on control food.

(B) Fs(1)h was overexpressed in male EP-fs(1)h; tubGS flies by exposing them to food containing 300µM RU486 for 4 days. Lethality after exposure to 20µM DEM was recorded and

analyzed by Mantel-Cox log-rank test. The flies that were maintained on RU486 food, showed significantly increased sensitivity to DEM (P value < 0.041) compared to those on control food.

(C) Ubiquitous Fs(1)h knock-down in young adult males by inducible expression of UAS-Fs(1)h^{RNAi} transgene under the control of the actin-GS-Gal4 driver. Survival after exposure to 20μM DEM was recorded and the data were analyzed by Mantel-Cox log-rank test. Male flies reared on RU486 food, showed significantly increased resistance to DEM (P value < 0.001) compared to those on control food.

(D) w^{1118} male flies were maintained on food containing 0.25mM JQ1 for 48 hrs and then were exposed to 20μM DEM. Survivorship was assessed. Mantel-Cox log-rank test showed that JQ1-treatment extended survival after DEM exposure significantly (P value <0.0001).

4.2.2. Fs(1)h controls oxidative stress defense

The finding that Fs(1)h acts as a repressor of CncC's transcriptional output, predicted that it should modulate the biological functions of the *Drosophila* Nrf2 ortholog. Nrf2-induced gene expression programs can protect organisms against acute oxidative stress. Experimentally such a stress can be generated by dietary exposure to diethyl maleate (DEM), a glutathione depleting agent. We have demonstrated previously that genetically increasing CncC activity can boost the resistance of flies against oxidative insults like DEM exposure (Sykiotis & Bohmann, 2008). To test if Fs(1)h might similarly affect oxidative stress resistance, we measured the DEM sensitivity of flies, in which the activity of Fs(1)h was either suppressed or increased. For loss-of-function experiments, *fs(1)h* mRNA was knocked down in cohorts of young adults flies for 4 days by RU486-induced expression of a corresponding RNAi construct. Subsequently, the animals were transferred to vials containing filter paper laced with a lethal concentration of DEM and the time course of survival was recorded. Cohorts in which *fs(1)h* was depleted displayed

a significantly increased survival time as compared to controls (Fig. 4.3E, Fig. 4.5C). For gain-of-function experiments Fs(1)h was overexpressed by feeding EP-fs(1)h; tub-GS-Gal4 stocks with RU486 for 4 days. DEM exposure resulted in a more rapid demise of the flies in these cohorts compared to matching controls (Fig. 4.3F, Fig. 4.5B). Similarly, inducible overexpression in the UAS-Fs(1)h-L fly line also resulted in sensitization to DEM stress (Fig. 4.5A). These DEM exposure experiments support the conclusion that Fs(1)h is a significant regulator of stress defense mechanisms, presumably through its effect on CncC.

4.2.3. Mechanism of CncC regulation by Fs(1)h

Through their double bromodomains BET proteins like Fs(1)h can bind to polypeptides that carry acetylated lysine residues. To assess whether this acetyl lysine binding activity might contribute to the inhibitory function of the protein towards CncC's transcriptional activity, we conducted experiments with JQ1, a specific inhibitor of BET-domain interactions with acetylated substrates (Filippakopoulos et al, 2010). Treatment of S2 cells with JQ1 caused an approximately 10-fold activation of Nrf2 reporter activity that was dependent on CncC (Fig. 4.6A). Similarly, dietary JQ1 exposure (Fig 4.6B) resulted in a strong enhancement of Nrf2 reporter activity also in flies. This stimulatory effect does not seem to be a stress response, as we did not detect any increased mortality in flies or cells after treatment with JQ1 as they would result from treatment with Nrf2-inducing stressors such as paraquat or DEM.

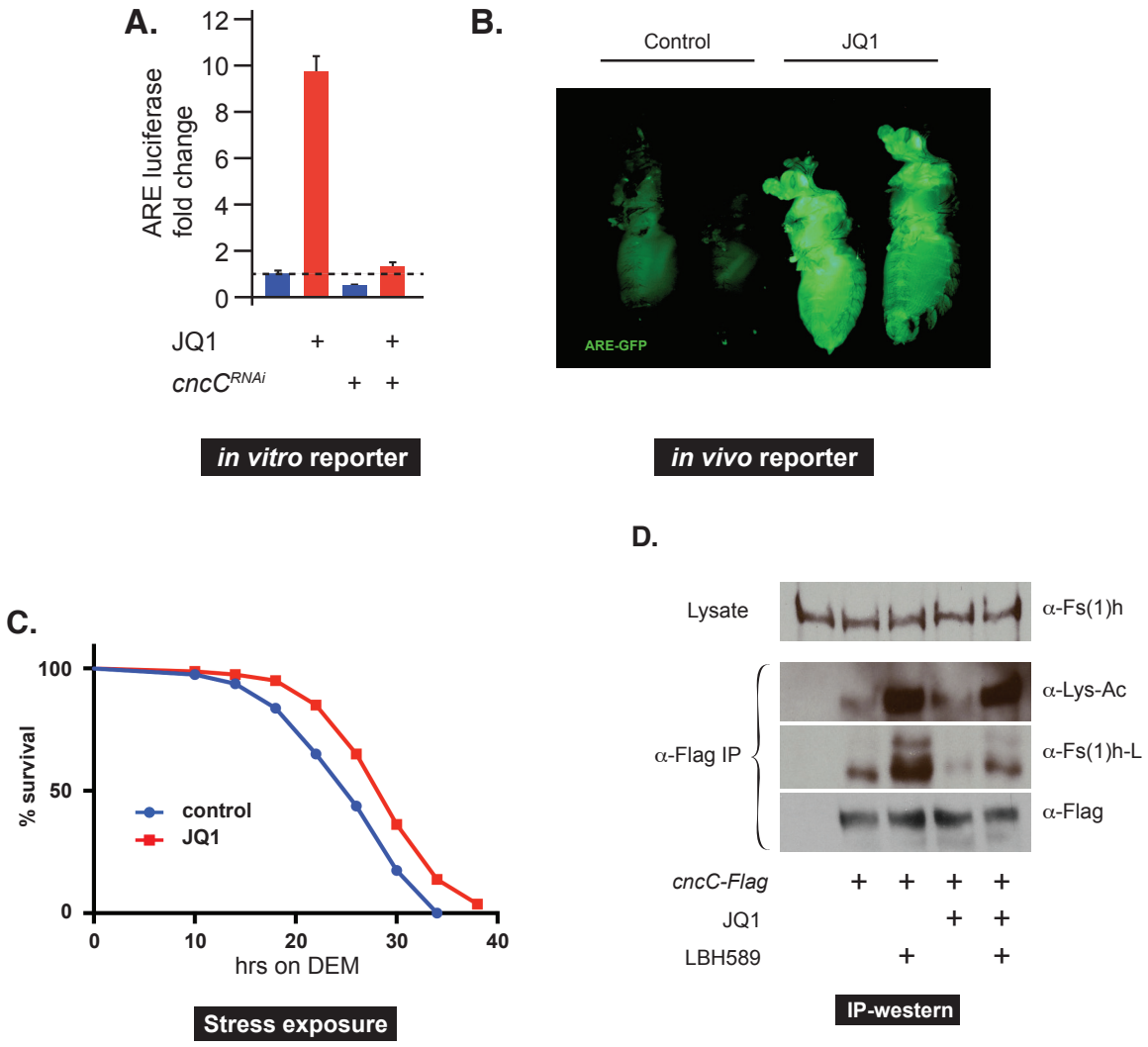


Figure 4.6. Bromodomains mediate the inhibition of CncC by Fs(1)h.

(A) S2 cells transfected with ARE-fluc reporter plasmid were treated with 1μM JQ1 for 24 hrs, which stimulated ARE-fluc activity. This increase was abrogated by CncC knock down. The diagram shows the fold change in luciferase activity relative to controls. Error bars indicate standard deviation of 3 biological replicates.

(B) 5 day old adult ARE-GFP flies were maintained on food containing 0.25mM JQ1 for 2 days. Fluorescence images of adult flies showed a strong induction of ARE-GFP reporter by JQ1. Two randomly chosen JQ1-treated and two solvent-treated female flies are shown.

(C) w^{1118} flies were maintained on food containing 0.25mM JQ1 for 48 hrs and then were exposed to 20μM DEM. Survivorship was assessed. Mantel-Cox log-rank test showed that JQ1-treatment extended survival after DEM exposure significantly (P value <0.0001 for females). Qualitatively identical results were found when males were used (Fig. 4.5)

(D) Co-immunoprecipitation of endogenous Fs(1)h-L with overexpressed CncC-Flag in S2 cells. Immunoprecipitation was performed using anti-Flag antibody followed by immunoblotting with anti-Fs(1)h-L antibody. The cells were treated either with the HDAC inhibitor LBH589 (500nM) as indicated or with 0.01% DMSO (vehicle) for 6 hours before they were processed for IP. 10μM JQ1 was added to the lysate to assess the effect of JQ1 on Fs(1)h-CncC interaction. The acetylation status of CncC was examined in western blots using an antibody against acetylated lysine.

The stimulatory effect of JQ1 on CncC activity as measured in the reporter assays described above, suggested that the drug should activate Nrf2-dependent stress defense and antioxidant gene expression programs. This in turn would be expected to increase the resistance of flies to acute oxidative challenges. To confirm this prediction we exposed adults to a lethal dose of DEM after they had been kept on food containing JQ1 for 4 days. Monitoring the time course of mortality showed increased survival times for the JQ1-pretreated groups relative to controls (Fig. 4.5D, 4.6C). Thus, consistent with its role as a CncC inducer, JQ1 treatment can have stress protective effects *in vivo*.

The experiment described above suggests that JQ1 can relieve the inhibition of CncC by Fs(1)h. Since JQ1 has been shown to block the interaction of BET bromodomains with acetyl lysine residues, we proposed that Fs(1)h would interact with acetylated CncC and that this interaction would be disrupted by JQ1, causing an increase in CncC activity. Indeed, specific acetylated forms of mammalian Nrf2 have been described (Kawai et al,

2011; Sun et al, 2009). To ask whether CncC might also be a substrate for acetylation and could therefore represent a potential binding partner for the Fs(1)h bromodomains, we performed immune precipitation/western blot experiments. S2 cells expressing a Flag-epitope tagged version of CncC were processed for immunoprecipitation with an anti-Flag antibody. Staining Western blots of the resulting immunoprecipitates with a generic anti-acetyl lysine antibody revealed that, like its mammalian counterpart, CncC protein from adult flies is acetylated (Fig. 4.6D). Treatment of the cells with the broad-spectrum HDAC inhibitor LBH589 increases the acetylation of the immuno-precipitated CncC, confirming that the protein is a substrate for acetylation / deacetylation reactions *in vivo*.

Next, we tested whether Fs(1)h might physically interact with CncC and, if so, whether Fs(1)h binding correlates with CncC acetylation. Confirming this idea, we found that Flag-tagged CncC expressed in Schneider cells could be co-immunoprecipitated with endogenous Fs(1)h-L. The yield of the recovered Fs(1)h-L increased in the presence of HDAC inhibitor and decreased in the presence of JQ1 (Fig. 4.6D). These results indicated that Fs(1)h-L and CncC bind to each other through a BET bromodomain – acetyl lysine interaction.

4.2.4. Fs(1)h is a part of a conserved, Keap1-independent axis of Nrf2 regulation

The discovery that Fs(1)h physically interacts with and inhibits CncC raised the question of how this activity relates to the regulatory function of Keap1, the canonical inhibitor of

Nrf2 proteins. Specifically, we wanted to determine whether Fs(1)h and Keap1 act through the same mechanism, or independently, in the control of CncC-dependent gene expression. To address this question we conducted transfection experiments in S2 cells with drugs and RNAi's that specifically interfere with Keap1 or Fs(1)h function (Fig. 4.7A). The experiments described above indicated that JQ1 prevents the inhibitory effect of Fs(1)h on CncC, thereby stimulating ARE-dependent gene expression. Consistent with this interpretation, *fs(1)h* knock down and JQ1 exposure stimulate ARE-luciferase reporter activity to a similar degree (Fig. 4.7A, compare lane 1 with 2 and 3), and combining the *fs(1)h* RNAi with JQ1 treatment does not further enhance this activity (Fig. 4.7A). Likewise, oltipraz exposure and Keap1 knock down activate the reporter to comparable levels, yet the effect of the two treatments is non-additive, confirming that oltipraz acts specifically through inhibition of the Keap1-CncC interaction (lanes 5-7). Interestingly, however, eliminating the function of both Fs(1)h and Keap1 simultaneously by combined RNAi and drug treatments caused a synergistic activation of the reporter (lanes 8 and 9). Similarly, combining JQ1 and Oltipraz treatment causes a synergistic activation of the ARE reporter in S2 cells over a range of concentrations (Fig 4.7E). A synergistic effect also could be observed when the response of endogenous mRNA expression to combined treatment with oltipraz and JQ1 was tested in S2 cells (Fig 4.7C). The expression of four conserved Nrf2 target genes *(gstD1, gstE9, keap1, gclc)* was inducible by treatment with JQ1 or oltipraz and combined treatment had a more than additive effect. These results indicate that Fs(1)h and Keap1 regulate Nrf2 function through independent mechanisms.

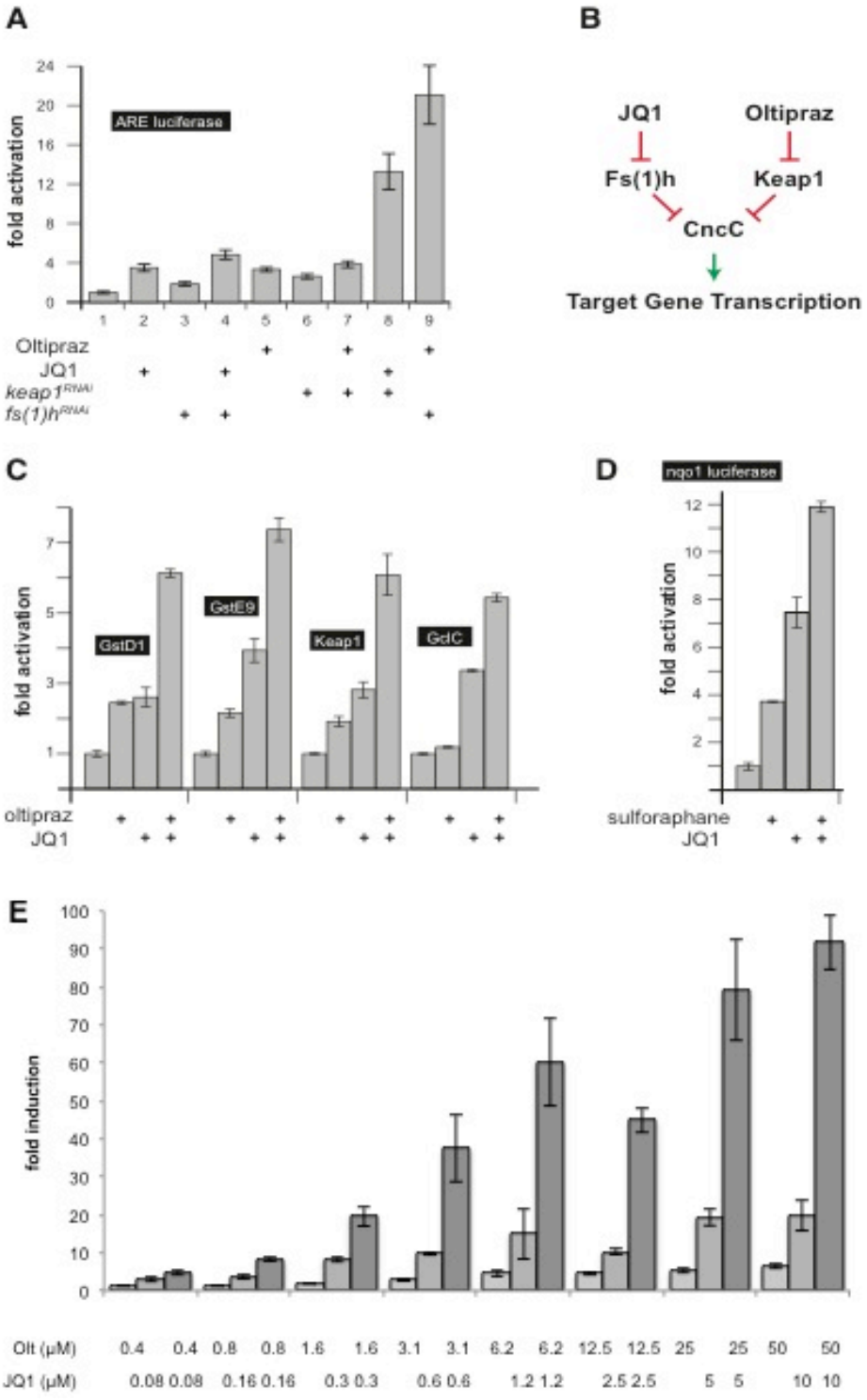

A
ARE luciferase
fold activation
Oltipraz
JQ1
keap1RNAi
fs(1)hRNAi
B
JQ1
Oltipraz
Fs(1)h
Keap1
CncC
Target Gene Transcription
C
GstD1
GstE9
Keap1
GclC
fold activation
oltipraz
JQ1
D
nqo1 luciferase
fold activation
sulforaphane
JQ1
E
fold induction
Olt (μM)
JQ1 (μM)

Figure 4.7. Fs(1)h regulates CncC signaling independently of Keap1.

(A) The graph shows the effect of different combinations of Keap1 and Fs(1)h RNAi with JQ1 or oltipraz, on ARE-fluc activity in S2 cells. Fluc measurements were normalized to actin renilla luciferase expression, which was used as an internal control.

(B) Proposed model for CncC-regulation by Keap1 and Fs(1)h. Treatment of cells with Keap1 RNAi or oltipraz relieves the same mechanism of CncC inhibition and combining both manipulations does not have a stronger effect than either treatment by itself. The same applies to Fs(1)h knock down and JQ1. However, combining Fs(1)h inhibition by RNAi or drug with Keap1 inhibition causes a strong cooperative activation of CncC target genes.

(C) Combined effect of oltipraz and JQ1 on CncC target gene expression. S2 cells were treated with 25μM oltipraz and/or 1μM JQ1 as indicated. *gstD1, gstE9, keap1* and *gclC* mRNAs were quantitated by RT-qPCR. Graphs show fold induction of mRNA levels of CncC target gene after normalization to *actin5C* expression.

(D) Cooperation of Keap1 inhibitor and JQ1 in mammalian cells. HEK293 cells that were transiently transfected with the Nrf2-responisve NQO1-fluc reporter were treated separately with 10μM sulforaphane (a Keap1 inhibitor similar to oltipraz that is suitable for mammalian cell culture) and 0.5μM JQ1 or in combination with both of them. Combined treatment with sulforaphane and JQ1 showed more than additive induction of NQO1-fluc compared to individual treatments. The firefly luciferase activity was normalized to Renilla luciferase activity expressed under the control of a thymidine kinase promoter. Error bars in panels A, C, and D represent standard deviation of 3 biological replicates.

(E) Combination of different concentrations of oltipraz and JQ1 leads to synergistic activation of ARE-Fluc reporter in S2 cells.

The effective and specific pharmacological activation of the Nrf2 response by combined JQ1 and oltipraz treatment might be therapeutically beneficial, provided that the mechanisms that we have characterized in *Drosophila* are conserved and operational in mammals. Interestingly, it has been shown in recent publications (Hussong et al, 2014; Michaeloudes et al, 2014) that mammalian Nrf2 can be stimulated by JQ1. We, therefore, tested if mammalian cells displayed cooperative activation of Nrf2 target genes in response to the combinatorial treatment that we had found effective in *Drosophila*. Confirming this idea, combined treatment of human HEK293 cells with sulforaphane, a

Keap1 inhibitor functionally similar to oltipraz, and JQ1 results in a more than additive activation mammalian Nrf2 reporter when compared to individual treatments (Fig. 4.7D). Combined dosing of these two drugs might, therefore open new avenues for treatment of diseases where increased Nrf2 activity could be beneficial.

4.2.5. Genome-wide analysis of JQ1/Oltipraz (Keap1/Fs(1)h) synergism

To investigate the effect of keap1 and Fs(1)h-mediated regulation of CncC target genes at a genome wide scale, we conducted a transcription profiling experiment. S2 cells were treated with oltipraz (Olt), with JQ1 or with a combination of the two drugs and processed for RNA-seq analysis. The combinatorial effects of oltipraz and JQ1 were assessed by determining which genes were strongly activated (>4 fold) in cells treated with both drugs compared to the effects of single treatments. 26 mRNAs met these criteria. 19 out of these 26 genes (73%) showed a cooperativity index of more than 1 (cooperativity index = fold induction by combined treatment with Olt and JQ1 / (fold induction by treatment with Olt + fold induction by treatment with JQ1)). Gene ontology analysis by DAVID software (Huang da et al, 2009a; Huang da et al, 2009b) showed these synergistically induced genes are significantly enriched in antioxidant and detoxification genes. The ontology groups, that were represented among these genes included: glutathione S-transferases and oxidoreductases (Enrichment scores 10.4 and 1.65 respectively). When we compared these genes with previously reported CncC target genes (Misra et al, 2011), we found a 52.6% (10 out of 19) overlap (Table 4.1).

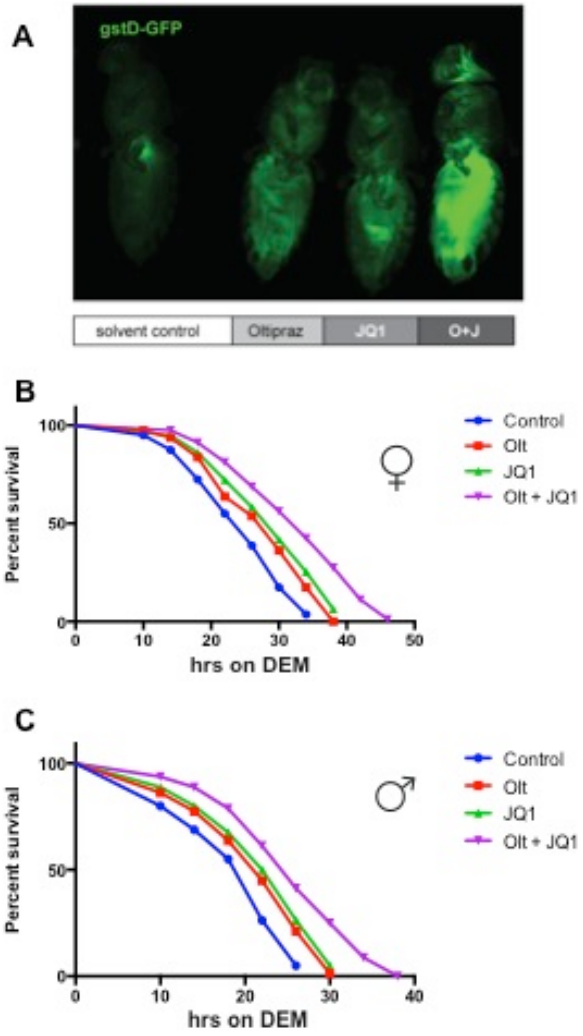


Figure 4.8. Simultaneous inhibition of Keap1 and BET proteins impart enhanced oxidative stress tolerance.

(A) 5 day old gstD-GFP flies were put on food containing 0.4 mM oltipraz and/or 0.1mM JQ1 for 4 days. Fluorescence images of adult flies showed a stronger induction of the gstD-GFP reporter by combined treatment with oltipraz and JQ1. One randomly chosen female fly from each group is shown.

(B) 5 day old female w^{1118} flies were placed on food containing 0.4 mM oltipraz and/or 0.1mM JQ1 for 4 days and then were exposed to 20μM DEM. Survivorship was assessed. Mantel-Cox log-rank test showed that pre-treatment with either oltipraz or JQ1 significantly increased oxidative stress tolerance (P value <0.005 for control/oltipraz comparison and P value <0.001 for control/JQ1 comparison). It was also found that pre-treatment with both oltipraz and JQ1 extended survival after DEM exposure significantly more than pre-treatment with either drug alone (P value <0.001 for olt/combined comparison and P value <0.005 for JQ1/combined comparison).

(C) w^{1118} male flies were maintained on food containing 0.4mM oltipraz and/or 0.1mM JQ1 for 4 days and then were exposed to 20μM DEM. Survivorship was assessed. Mantel-Cox log-rank test showed that combinatorial pre-treatment with oltipraz and JQ1 extended survival after DEM exposure significantly more than pre-treatment with either drug alone (P value <0.001 for oltipraz/combined comparison and P value <0.005 for JQ1/combined comparison). Oxidative stress tolerance was also significantly enhanced by pre-treatment with either oltipraz or JQ1 (P value <0.005 for both control/oltipraz and control/JQ1 comparisons).

Confirming the results from S2 cells, the combined treatment of adult flies with oltipraz and JQ1 lead to stronger induction of gstD-GFP reporter relative to the induction caused

by either oltipraz or JQ1 alone (Fig 4.8A). If a combinatorial treatment with the Keap1 inhibitor oltipraz, and the BET bromodomain inhibitor JQ1 activates Nrf2-dependent stress defense and antioxidant gene expression programs more strongly than oltipraz or JQ1 alone, we would expect a further increase in the resistance of flies to acute oxidative challenges. To confirm this prediction we exposed adults to a lethal dose of DEM after they had been kept on food containing oltipraz, JQ1 or both drugs for 4 days. Monitoring the time course of mortality showed that pretreatment with oltipraz and JQ1 alone enhances the oxidative stress tolerance when compared to the control group. However, pretreatment with a combination of oltipraz and JQ1 increased survival times even further (Fig 4.8B, 4.8C). Thus, combinatorial treatment with oltipraz and JQ1 can yield effective protective effects against oxidative challenges and may also be useful for therapeutic applications in which Nrf2 function is expected to be beneficial.

Table 4.1. Summarized results of an RNA-seq analysis of S2 cells treated with oltipraz alone, with JQ1 alone, or with both drugs combined.

Gene name	Fold induction (OLT)	Fold induction (JQ1)	Fold induction (OLT+JQ1)	Coopertivity index	GO cluster	Induced by CncC
GstD5	1.99	3.37	9.12	1.70	GST	
GstD2	2.14	2.70	7.60	1.57	GST	Yes
GstD8	2.16	2.44	7.14	1.55	GST	Yes
His4:CG33891	1.17	3.04	6.12	1.45		
His4:CG33893	1.08	3.95	7.28	1.45		
ImpL3	1.55	2.27	5.33	1.40	OR	
GstD6	1.90	3.12	6.37	1.27	GST	
CG2065	1.48	2.51	5.03	1.26	OR	Yes
GstE9	1.87	3.10	6.24	1.26	GST	Yes
GstD4	2.01	1.81	4.72	1.24	GST	Yes
CG2064	1.06	2.67	4.52	1.21	OR	Yes
Arc2	1.07	3.12	4.96	1.18		
Xrp1	1.17	2.96	4.67	1.13		
Gclc	1.09	3.89	5.54	1.11		Yes
CG5224	1.30	2.59	4.24	1.09	GST	

CG12868	1.21	3.21	4.74	1.07		
GstE8	1.77	2.41	4.27	1.02	GST	Yes
CG31743	1.27	2.68	4.04	1.02		Yes
GstE7	1.80	2.17	4.04	1.02	GST	Yes
CG12703	1.02	3.05	4.01	0.99		
CG18547	1.04	3.34	4.25	0.97	OR	
CG33641	1.38	2.89	4.14	0.97		
CG18522	1.14	3.52	4.49	0.96	OR	
Adh	1.47	3.66	4.91	0.96	OR	Yes
cv-2	1.15	3.88	4.74	0.94		
fok	1.43	3.68	4.11	0.80		

Genes that activated more than 4-fold by the combined treatment with oltipraz and JQ1 but not with single treatments are shown. The fold induction by different drug treatments, the cooperativity indices, gene ontology classification and reported CncC responsiveness (Misra et al, 2011) are also indicated. GST: Glutathione S-transferase, OR: Oxidoreductase.

Chapter 5:

Final Discussion

The continued integrity of cells and organisms depends on their ability to protect themselves against oxidative stress and fluctuations in redox state. Multiple signaling systems have evolved that can sense oxidative and genotoxic stresses and orchestrate rapid and effective responses in order to deflect or repair potentially harmful damage to cells, tissues and organisms. Conserved stress-inducible signaling systems include: the JNK, p38, p53, NF-kB and Nrf2 pathways (Finkel & Holbrook, 2000). Nrf2 is a bZIP transcription factor that is tethered to its cytoplasmic inhibitor Keap1 in unstressed conditions (Katoh et al, 2005; Motohashi & Yamamoto, 2004). Keap1 limits Nrf2 activity by sequestering it in the cytoplasm and targeting it for degradation by the Proteasome. Upon activation Nrf2 dimerizes with the small leucine zipper protein Maf-S in the nucleus (Blank, 2008) and binds to its cognate promoter elements called AREs (antioxidant response elements). Genes thus regulated encode redox regulators and phase II detoxification gene. The Nrf2 signaling system is functionally and structurally conserved in *Drosophila* (Sykiotis & Bohmann, 2008; Sykiotis & Bohmann, 2010). The fly homolog of Nrf2 is the CncC protein. dKeap1 and a single Maf-S proteins are the counterparts of the mammalian Keap1 and four small Maf proteins in *Drosophila*.

Defects in the Nrf2 pathway cause oxidative stress sensitivity (Kensler et al, 2007; Sykiotis & Bohmann, 2008).

Since oxidative stress is a major contributor to the aging process, Nrf2 signaling also plays important role in aging. Nrf2 is necessary to defend organisms against several age-related diseases like neuro-degeneration, chronic inflammation and cancer (Sykiotis & Bohmann, 2010). Moreover, CncC signaling extends *Drosophila* lifespan and regulates somatic stem cell maintenance in the *Drosophila* intestine (Hochmuth et al, 2011; Sykiotis & Bohmann, 2008). Skn-1, the Nrf2-related protein in *C.elegans*, is necessary for the prolongevity effects of calorie restriction and is negatively regulated by insulin signaling which has been demonstrated to decrease lifespan in different organisms (Bishop & Guarente, 2007; Tullet et al, 2008). In addition, Nrf2 can also respond to endoplasmic reticulum stress (Digaleh et al, 2013; Glover-Cutter et al, 2013). Nrf2 signaling, therefore, serves as the convergence point of multiple stress responses, metabolic signaling and lifespan regulatory mechanisms. A better understanding of Nrf2 signaling would elucidate the connection between oxidative stress and aging. Studies of the genetic and environmental regulation of Nrf2 would shed light on how physiological and environmental cues shape our stress response and longevity.

Development of Nrf2 reporters and identification of putative regulators of Nrf2

To study CncC signaling in *Drosophila* and *Drosophila* cell lines, we generated comparable *in vivo* and cell-based CncC reporters. Since JNK signaling is very similar to CncC in the sense that it responds to oxidative stress, has bZIP transcription factors as its principal effector and have partially overlapping target DNA site with CncC, we generated similar JNK reporters that can be used to determine the specificity of CncC response (Chatterjee & Bohmann, 2012). ΦC31 recombinase mediated site directed transgenesis technique (Groth et al, 2004) was used to integrate the Nrf2 and JNK reporters in two specific docking sites that allowed comparison between the activities of these reporters. Matching cell-based reporters that can be used to carry out, among other experiments, large-scale screens for genetic regulators and chemical modulators of CncC and JNK pathways, were also generated (Chatterjee & Bohmann, 2012).

The integrated reporter system met its intended specifications:

(1) Nrf2 and AP-1 activity can be measured accurately, specifically and sensitively *in vitro*. The *in vivo* reporter lines permitted observation of spatial patterns of JNK and Nrf2 signaling and a semi-quantitative assessment of changes in signaling strength brought about by genetic or environmental factors.

(2) Known or predicted activity patterns, such as the activation of JNK in the leading edge of the dorsally closing embryonic epidermis, or the Nrf2 activity in the digestive tract, were reproduced.

(3) The two insertion sites, attP40 and attP16, were chosen because they were reported to confer little base level activity onto inserted transgenes. Consistently, we saw very low background activity with the mRE control reporter. Swapping insertion points did not change the observed pattern of activity, indicating that the respective chromosomal environments have minimal effect on the reporters and that results from genes inserted in attP16 and attP40 can be directly compared.

(4) Responses are specific: JNK and Nrf2 signaling activates only their respective reporters without "spillover" into the other pathway.

Compared to existing P-element based systems, these reporters generated by ΦC31 recombinase-mediated transgenesis offer several advantages:

(1) The *Drosophila* reporter strains carry DsRed.T4 or eGFP constructs in one of two defined chromosomal positions that were selected for low background activity and neutral responses to the different stress treatments tested so far. This standardized chromosomal integration makes it possible to directly compare the activities of different reporters. This is useful, for example, when comparing ARE or TRE reporters with the mutant version (mRE) that serves as a background control.

(2) The two integration sites chosen are on the two arms of the second chromosome. Pairs of red and green reporters can thus be easily combined, generating stable dual reporter stocks. In this manner, Nrf2 and JNK activity can be tracked independently in the same specimen. It is also possible to combine ARE or TRE reporters with tissue or cell type specific red or green markers or with the control mRE vector. The similar maturation time of eGFP and DsRed.T4 further assists this comparative analysis.

The plasmid constructions are designed in a way that the system can easily accommodate other synthetic or natural promoter or enhancer sequences. Using straightforward recombinant DNA and *Drosophila* transgene technology, the system can be adapted to track other signaling pathways or transcription factors.

Luciferase versions of the same reporter genes show the regulatory characteristics and signal responsiveness in S2 cell transfections that was predicted from the behavior of the *in vivo* reporters. For example, both *in vivo* and *in vitro* assays document that Nrf2 and JNK responses are regulated by independent upstream pathways. The S2 cell system can be used for medium or high throughput RNAi or drug screens. The integrated reporter system allows rapid *in vivo* validation of genes or mechanisms pinpointed by cell based screens.

While JNK, as well as Nrf2, reporters have been constructed for mammals, there are no reports of a comprehensive, standardized and directly comparable system like the one we have generated for *Drosophila*. For example, Tsai et al used a mammalian reporter that contains an ARE-driven luciferase gene (Tsai et al, 2011). However, this construct also contains nucleotides that confer JNK specificity. Huang et al used transgenic mice carrying a TRE-luciferase reporter that is responsive to JNK signaling (Huang et al, 1997). Therefore, the reporter system we generated is suitable for both individual and comparative studies of two major anti-oxidant signaling pathways, namely Nrf2 and JNK. It is also conducive to large-scale screens for the identification of their genetic and pharmacological regulators.

The ARE-fluc plasmid was used in a kinome-wide dsRNA library screen to identify novel regulators of Nrf2 signaling. Several kinases such as PKC-δ, GSK-3β, Fyn, p38, PERK, Casein Kinase 2 (CK2) have been reported to regulate mammalian Nrf2 activity. We identified Sgg, the GSK-3β homolog in *Drosophila*, and CK2-α as putative regulators of CncC signaling in the dsRNA library screen. However, Sgg did not show consistent effect on ARE activity in subsequent validation experiments. We identified 6 inducers and 2 suppressors of Nrf2 signaling in this screen. Cdk12, which was identified as novel putative inducer of CncC signaling in the screen, was found to be required for ARE activity in a cell-autonomous manner. In addition, Cdk12 was shown to be important for oxidative stress resistance. Successful validation of Cdk12 as an inducer of Nrf2 activity

indicated that the dsRNA library screen was useful to identify new regulators of Nrf2 activity.

Regulation of Nrf2 by a BET protein

We identified the *Drosophila* BET protein Fs(1)h as a novel inhibitor of the *Drosophila* Nrf2 homolog CncC in the dsRNA library screen and established that the long isoform or Fs(1)h-L is involved in this regulation. Mammalian BET proteins, notably BRD4, have been implicated in the regulation of gene expression. They are known to bind to chromatin and to interact with components of the transcriptional machinery such as P-TEF B and RNA polymerase II (Jang et al, 2005; Yang et al, 2005). In addition, the activities of specific transcription factors such as NF-κB, and Twist can be regulated by direct interaction with BRD4 (Huang et al, 2009; Lockwood et al, 2012; Shi et al, 2014). Similarly, functional experiments and genome-wide ChIP mapping studies suggest that the *Drosophila* BET protein Fs(1)h functions in the regulation of gene activity (Chang et al, 2007). Fs(1)h gene products have been found associated with transcription control region and genomic insulator elements (Kellner et al, 2013; Kockmann et al, 2013). Interestingly, our analysis of the Fs(1)h ChIP-seq data generated by Kellner et al (Kellner et al, 2013) revealed that Fs(1)h-L was localized to the promoter region of many CncC target genes such as *gstD1, gclC, keap1*. This leads to an interesting possibility that Fs(1)h-L interacts with CncC near its target promoters to prevent the induction of the target genes.

Nrf2 is an attractive drug target. Compounds like oltipraz and sulforaphane which interfere with the Keap1-Nrf2 interaction to activate target genes without causing cell stress, are effective as cancer chemopreventive agents in animal experiments (Cheung & Kong, 2010; Kensler et al, 2005; Wang et al, 1999; Zhang & Munday, 2008). Nrf2-activating drugs can also be beneficial in the treatment of other diseases that are associated with oxidative stress, including neurodegenerative and inflammatory conditions (Crunkhorn, 2012; Garber, 2012). Indeed, an Nrf2 inducer, dimethyl fumarate, marketed under the brand name Tecfidera®, has recently gained FDA approval for treatment of multiple sclerosis (English & Aloi, 2015; Linker et al, 2011). The identification of JQ1 as a CncC activator and, the discovery that JQ1 and oltipraz synergistically activate Nrf2 dependent gene expression programs in *Drosophila* and mammalian cells suggest innovative therapeutic options. Conventional Nrf2 activating drugs like sulforaphane and oltipraz act by inhibiting Keap1. These drugs, though effective in some cases, show moderate induction of Nrf2 and often have off-target effects (Garber, 2012; Zhang & Munday, 2008). Combinations of JQ1 and oltipraz-like drugs might stimulate Nrf2 dependent protective gene expression more efficiently than either compound alone, or could achieve beneficial effects at lower doses, thereby decreasing the risk of unwanted side effects.

Our cell culture and biochemical experiments provide information on the mechanisms by which Fs(1)h can repress CncC function. As opposed to the effect of Keap1, which

targets CncC for cullin 3-mediated proteasomal degradation in the cytoplasm, the inhibitory, acetylation-dependent interaction between Fs(1)h and CncC presumably occur in the nucleus where the BET protein resides. These independent mechanisms of CncC inhibition are consistent with the synergistic effect of combined JQ1 and oltipraz treatment on Nrf2 target gene expression.

The identification of BET proteins as Nrf2 repressors adds another facet to an increasingly complex picture of Nrf2 signaling and biology. Over the last few years several other Keap1-independent pathways of Nrf2 regulation have been described based on experiments in mammalian cell culture: for example it was shown that PKC- δ mediated phosphorylation of Ser40 of mammalian Nrf2 promotes its stabilization and nuclear translocation (Bloom & Jaiswal, 2003; Huang et al, 2002; Niture et al, 2009). The Src-family tyrosine kinase Fyn can phosphorylate Tyr568 of Nrf2 leads to its nuclear export and degradation (Jain & Jaiswal, 2006). Glycogen synthase kinase 3 beta (GSK-3β) can phosphorylate Fyn and increase its nuclear accumulation and thereby promotes nuclear export of Nrf2 and inhibition of Nrf2 signaling (Jain & Jaiswal, 2007). In addition, GSK-3β can phosphorylate the serine residues at the β-TRCP-binding motif ($DSGIS^{338}$) in the Neh6 domain of Nrf2 and promote Cullin 1 dependent proteasomal degradation by β-TRCP (Rada et al, 2011). Future experiments will help to evaluate whether the Fs(1)h and CncC interaction cooperates with any of these more recently described mechanisms of Nrf2 regulation.

Chapter 6

Materials and Methods

Plasmids and fly lines

Synthetic double stranded oligonucleotides, carrying four high affinity AP-1 or Nrf2 binding sites or a mutant control (4 x ARE, 4 x TRE and 4 x mRE, see Fig. 1B) were inserted into modified pGL3-Basic, a plasmid that includes *Drosophila* Hsp70 minimal promoter driving the expression of firefly luciferase gene (DasGupta et al, 2005) (kindly provided by Dr. Michael Boutros) to generate cell-based reporter plasmids. The *in vivo* reporter plasmids were generated in multiple steps. 4 x ARE was transferred into the pGreen H-Pelican vector to generate pGreen-ARE. The attB sequence from piB-GFP (Bateman et al, 2006) plasmid was inserted into pBSKS vector to generate pBSKS-attB plasmid. The ARE-eGFP cassette along with the flanking gypsy insulators was amplified by PCR and inserted into pBSKS-attB to generate pB-ARE-green. The 4 x ARE promoter element and eGFP reporter gene of this plasmid was replaced with other promoter elements (e.g., 4 x TRE, 4 x mRE) and other reporter genes (e.g., DsRed.T4), respectively, to generate plasmids with different combinations of promoters and reporters (Chatterjee & Bohmann, 2012).

The pUAS-HA-attB plasmid was generated by cloning attB sequence amplified with PattB-F and PattB-R primers into pUAS-HA plasmid (Sykiotis & Bohmann, 2008). UAS-Fs(1)hL plasmid was generated in two steps. First the Fs(1)h short isoform cDNA was amplified from LD26482 cDNA clone with PFs(1)hS-UAST-HA-F and PFs(1)hS-UAS-HA-R primers and was cloned into pUAS-HA-attB plasmid to generate pUAS-Fs(1)h-S-HA-attB plasmid. Then the cDNA for the C-terminal motif of Fs(1)h-L was amplified from total *Drosophila* cDNA with PUAST-Fs(1)h-L-int-F and PUAST-Fs(1)hL-int-R primers and was cloned into pUAS-Fs(1)h-S-HA-attB plasmid to generate pUAS-Fs(1)hL-HA-attB plasmid.

ΦC31 recombinase-mediated site-directed transgenesis was used to generate transgenic fly lines with the reporter and overexpression plasmids (service provided by Genetic Services Inc, MA). The reporter plasmids carried by different reporter fly lines and the integration sites of the plasmids are described in Table 6.2. Individual reporter fly lines were then recombined with other reporter fly lines to facilitate the simultaneous study of two different promoter elements in the same organism. For overexpression or RNAi experiments in which genetic effects on reporter activity were analyzed, the reporter lines were also recombined with different driver lines (e.g, arm-Gal4, tubGS-Gal4). EP-Fs(1)h fly line #10097 (Bloomington Stock Center) was used to overexpress Fs(1)h whereas v51227 and v108662 fly lines (Vienna Stock Center) were used to knock down Fs(1)h. v25508 fly line (Vienna Stock Center) was used to knock down Cdk12.

Calcium phosphate transfection

Drosophila S2 cells were transiently transfected with firefly luciferase reporter constructs using the calcium phosphate method. A co-transfected actin-driven renilla luciferase reporter plasmid (pAct-RL) (Armknecht et al, 2005) (kindly provided by Dr. Willis Li) served as an internal reference for normalization. S2 cells grown in M3+BPYE (Sigma-Aldrich) with 10% FBS and supplemented with penicillin and streptomycin were seeded at $2x10^6$ cells/ml concentration 6 hours before transfection. The transfection mix was prepared by combining Solution A (0.25 M $CaCl_2$, 0.5 μg/ml of the firefly luciferase reporter construct, 0.2 μg/ml pAct-RL, 1.3 μg/ml salmon sperm DNA) and Solution B (2 x HBS: 50 mM HEPES-KOH, 1.5 mm Na_2HPO_4, 280 mM NaCl, pH 7.1). Solution A was slowly added drop wise to an equal volume of Solution B with continuous vortexing and the mixture was incubated at room temperature for 30 minutes to allow formation of a fine precipitate. The mixture was briefly vortexed before adding it drop wise to the seeded cells with constant swirling. 1 ml of seeded S2 cells was incubated with 0.5 ml of transfection mix (for a total of 250 ng ARE-fluc/TRE-fluc/mRE-fluc, 100 ng of pAct-RL, 650 ng salmon sperm DNA per well) at 25°C for 16 hrs before the calcium phosphate solution was aspirated, cells were washed with 1x PBS and fresh culture medium was added. The Solution A for CncC and Hep^{act} over expression experiments contained 0.25 M $CaCl_2$, 0.5 μg/ml of the firefly luciferase reporter construct, 0.2 μg/ml pAct-RL, 0.5 μg/ml pAct-Gal4 and 1 μg/ml pUAST-CncC or pUAST-Hep^{act}. pUAST-CncC and pUAST-Hep^{act} were replaced with 1 μg/ml salmon sperm DNA in the control group of these experiments.

Mammalian 293T cells were grown in DMEM (GIBCO) media that is supplemented with 10% FBS and penicillin and streptomycin. $3X10^6$ cells were inoculated 16 hours before transfection. The transfection mix was similarly prepared by combining Solution A (0.25 M $CaCl_2$, 0.2 mg/ml pNQO1-FLuc reporter construct, 0.1 mg/ml pTK-RLuc, 0.7 mg/ml salmon sperm DNA) and Solution B. 1 ml of 293T cells was incubated with 0.2 ml of transfection mix at 37ºC for 16 hrs before the calcium phosphate solution was aspirated, cells were washed with 1XDPBS (GIBCO) and fresh culture medium was added.

Chemical and drug treatments

To study the effect of different oxidative stressors on reporter gene expression in transgenic flies, 1 week old flies that were mated for one day and then separated into males and females, were exposed to different oxidative stressors. The animals were starved for 2 hours and then fed a solution of 5% sucrose ± 20 mM Paraquat (Sigma-Aldrich). To examine the effect of oltipraz on reporter flies, similarly collected reporter flies were fed food supplemented with 1 mM oltipraz (LKT Laboratories Inc.) for 48 hours. 15-20 flies were used in each group for these experiments and 3-5 representative flies were chosen randomly for imaging. Similar results were seen in multiple experiments. To study the effect of JQ1 on reporter gene expression *in vivo*, 5-day-old flies that were mated for one day and then separated into males and females. These were fed food supplemented with 0.25 mM JQ1 (APExBIO) for 48 hours. 15–20 flies were

used in each group for these experiments and 3–5 representative flies were chosen randomly for imaging.

To assess the effect of different oxidative stressors and chemicals on the cell-based reporters S2 cells were transiently transfected with the reporter plasmids by the calcium phosphate method. 8 hours after the PBS wash and medium change, the transfected cells were transferred to 96-well plates and treated with 25 μM oltipraz, 100 μM DEM, 100 μM $NaAsO_2$ (J.T.Baker), 0.5μM JQ1 and were incubated at 25°C for 24 hrs (Chatterjee & Bohmann, 2012). In order to study the effect of different chemicals on the Nrf2 reporter in mammalian cells, transfected 293T cells were treated with 0.5μM JQ1 and 10μM sulforaphane (Enzo Life Sciences) and were incubated at 37°C for 18hrs. DMSO was used as the solvent control in all the drug treatments.

dsRNA synthesis and treatment

dsRNAs were synthesized and purified following the protocol provided by Sandra Steinbrink and Michael Boutros (Steinbrink & Boutros, 2008) using 'T7 RiboMAX Express RNAi System' kit (Promega) and 'RNeasy Kit' (QIAGEN). Predesigned RNA probes obtained through the E-RNAi webservice were used to knock-down CncC, Maf-S, Hep, Bsk, Fs(1)h, Cdk12 and CycK whereas dsRNAs against GFP and firefly luciferase were designed using E-RNAi webservice (Arziman et al, 2005; Horn & Boutros, 2010).

Fs(1)h-L was knocked down using the dsRNA described by Kockmann et al. (Kockmann et al, 2013) whereas dsRNA targeting the unique 3' UTR region of Fs(1)h-S was designed by the 'SnapDragon' webservice. S2 cells ($1x10^6$ cells/ml concentration) were bathed with dsRNAs in 12-well plates (8 μg dsRNA/well) as described (Steinbrink & Boutros, 2008). dsRNA-treated cells were transfected with luciferase reporter plasmids by calcium phosphate method 3 days after dsRNA bathing.

Luciferase assay

The activities of cell-based reporters were measured by 'Dual Glo Luciferase Assay System' kit (Promega). 75 μl of transfected S2 cells (either untreated or treated with dsRNA and/or chemicals) or 293T cells were transferred to wells of 96-well luciferase assay plates and luciferase assay was carried out in triplicate following the protocol provided by the supplier.

Microscopy

To study JNK activity during dorsal closure TRE-red-2R/SGMCA stage 13 embryos were collected on apple plates and were dechorionated manually on double sided tape. Live embryos were mounted in PCTFE oil (HPC#27:HPC#200=1:1, from Halocarbon

Products Corp.). Confocal images were collected using a Leica TCS SP5 system and were processed using Adobe Photoshop.

To study the effect of Fs(1)h knockdown and Fs(1)h-L overexpression on ARE activity in clones of tissue hsFlp; Act>Y>Gal4, UAS-RFP, ARE-GFP; UAS-Fs(1)h-RNAi and hsFlp; Act>Y>Gal4, UAS-RFP, ARE-EGFP; UAS-Fs(1)h-L flies were used. The embryos and larvae were incubated at 18°C before L2 larvae were heat treated at 37°C for 30 minutes and then returned to 18°C. Adult flies were dissected in PBS and crops and ejaculatory bulbs were fixed at room temperature for 30 minutes in 100mM glutamic acid, 25mM KCl, 20mM $MgSO_4$, 4mM sodium phosphate, 1mM $MgCl_2$, and 4% formaldehyde (pH 7.5). DNA was stained with Hoechst dye. Confocal images were collected and processed as above. Cdk12 knock down clones were studied in the ejaculatory bulbs of hsFlp; Act>Y>Gal4, UAS-GFP, ARE-RFP; UAS-Cdk12-RNAi adult male flies.

To study CncC activity tubGS-Gal4, ARE-green-2L/UAS-dKeap1^{RNAi}; UAS-dKeap1^{RNAi}/+ stage 2 larvae were treated with inducer RU486 (Cayman Chemical) by addition of 2 mg/ml RU486 in 50% ethanol to the food. The control group of the same genotype was treated with 50% ethanol. Both the control and treated larvae were dissected at stage 3 in PBS and salivary glands and guts were fixed as described earlier.

DNA was stained with Hoechst dye. Confocal images were collected and processed as above.

Oxidative stress sensitivity assay

Oxidative stress resistance of adult flies of different genotypes was assessed as previously described (Sykiotis & Bohmann, 2008). Newly emerged flies of the specified genotypes were mated for one day, separated into females and males, and at 5 days of age were transferred to RU486-containing (300μM) or control food containing the solvent (ethanol). After 4 days 4 groups of 20 flies from each condition were starved for 3 hours in empty vials, and then fed a solution of 5% sucrose ± a semi-lethal dose of DEM (20mM). Survivors were scored after 36 hours and Log-rank tests were performed on the survivorship data using 'GraphPad Prism' software. In order to study the effect of JQ1 on stress sensitivity, flies raised on food supplemented with 0.25 mM JQ1 for two days were exposed to similar DEM treatment and the survivorship data was collected and analyzed as before. In order to study the synergistic effect of oltipraz and JQ1 on stress sensitivity, flies raised on food supplemented with 0.4mM oltipraz and/or 0.1mM JQ1 for four days were exposed to similar DEM treatment and the survivorship data was collected and analyzed as before.

Co-Immunoprecipitation and Western blot

Co-immunoprecipitation and western blot were carried out as described (Rahman et al, 2013). Briefly, S2 cells transfected with pAct-Gal4 and pUAS-CncC-FLAG plasmids were harvested and washed with cold 1XPBS. The cells were then resuspended in lysis buffer (50mM Tris-HCl, 200mM NaCl, 5mM EDTA, 5% Glycerol, 0.2% NP-40) that contained protease inhibitor complex (Roche) and kept on a nutator at 4°C for 1 hour. The cell debris was removed by centrifugation and the lysate was passed through hypodermic syringe to shear DNA. The protein concentration was estimated using Bradford's reagent and 20-30 µg of protein from each sample was set aside as input. The lysate was pre-cleared with Protein G beads (GE Biosciences) for 1 hour at 4°C. The beads were separated by centrifugation and the cleared lysate was transferred into the fresh chilled tube. Anti-FLAG (Sigma-Aldrich Co) antibody was added at 2 µg per mg lysate protein concentration and was incubated at 4°C for 2 hours. Then 20 µl of 50% Protein G bead slurry was added to the lysate and Incubated overnight on rotating disc at 4°C. The beads were spun down and were washed 5 times with 750 ul of cold lysis buffer. After the final wash, 7.5 µl of sample buffer and 7.5 µl of lysis buffer were added to the beads and incubated at 95°C along with the input samples for 10 minutes. The beads were spun down at the top speed for 5 minutes and the supernatant was loaded for electrophoresis. For LBH treatment, the cells were treated with 2µM of LBH (APExBIO) for 6 hours prior to the cell lysis whereas JQ1 was added to the lysate at 10 µM concentration after the cell lysis. Primary antibody specific to Fs(1)hL isoform (Kindly provided by Dr. Victor Corces) (Kellner et al, 2013) (1:2000 dilution) and secondary

anti-rabbit antibody (1:5000 dilution) (BioRad) were used to probe the western blot membrane to examine the binding of Fs(1)hL protein to CncC-FLAG protein. Antibody that recognizes both isoforms of Fs(1)h (Kindly provided by Dr. Igor Dawid) (Kellner et al, 2013) was used in 1:2000 dilution in western blot experiments to validate the selective knock down of Fs(1)h-S and Fs(1)h-L with isoform-specific dsRNAs.

Quantitative RT-PCR

mRNA was prepared from whole flies or from S2 cells using Trizol reagent (Invitrogen). DNAaseI (NEB) was used to remove contaminating DNA before phenol: chloroform: isoamyl alcohol (25:24:1, Amresco) extraction. Maxima reverse transcriptase (Fermentas) and oligo dT primers were used to generate cDNA. cDNA was diluted 1:50 to serve as template for quantitative real time PCR (qPCR). qPCR reactions were done in triplicates using qPCR super-mix (BioRad) on a BioRad MyIQ thermal cycler. 'Delta-delta Ct' method was used for normalization to actin5c transcript levels. Data shown are averages and standard deviations from at least three biological replicates.

RNA-seq experiment

mRNA extracted from S2 cells treated with DMSO, oltipraz, JQ1, and oltipraz and JQ1 were used in RNA-seq experiments. RNA-seq libraries were generated and sequenced by

the University of Rochester Medical Center Genomics Core Facility using an Illumina HiSeq instrument. ~5 million reads were generated per library and were mapped to *Drosophila* Genome Release 5. Expression values were calculated as reads per kilobase per million mapped reads (RPKM). Gene Ontology enrichment analysis was done using DAVID software (Huang da et al, 2009a; Huang da et al, 2009b).

Bibliography

Adam J, Hatipoglu E, O'Flaherty L, Ternette N, Sahgal N, Lockstone H, Baban D, Nye E, Stamp GW, Wolhuter K, Stevens M, Fischer R, Carmeliet P, Maxwell PH, Pugh CW, Frizzell N, Soga T, Kessler BM, El-Bahrawy M, Ratcliffe PJ, Pollard PJ (2011) Renal cyst formation in Fh1-deficient mice is independent of the Hif/Phd pathway: roles for fumarate in KEAP1 succination and Nrf2 signaling. *Cancer Cell* **20:** 524-537

Aigaki T, Seong KH, Matsuo T (2002) Longevity determination genes in Drosophila melanogaster. *Mech Ageing Dev* **123:** 1531-1541

Akerfelt M, Morimoto RI, Sistonen L (2010) Heat shock factors: integrators of cell stress, development and lifespan. *Nat Rev Mol Cell Biol* **11:** 545-555

Ansell PJ, Espinosa-Nicholas C, Curran EM, Judy BM, Philips BJ, Hannink M, Lubahn DB (2004) In vitro and in vivo regulation of antioxidant response element-dependent gene expression by estrogens. *Endocrinology* **145:** 311-317

Ansell PJ, Lo SC, Newton LG, Espinosa-Nicholas C, Zhang DD, Liu JH, Hannink M, Lubahn DB (2005) Repression of cancer protective genes by 17beta-estradiol: ligand-dependent interaction between human Nrf2 and estrogen receptor alpha. *Mol Cell Endocrinol* **243:** 27-34

Apopa PL, He X, Ma Q (2008) Phosphorylation of Nrf2 in the transcription activation domain by casein kinase 2 (CK2) is critical for the nuclear translocation and transcription activation function of Nrf2 in IMR-32 neuroblastoma cells. *J Biochem Mol Toxicol* **22:** 63-76

Armknecht S, Boutros M, Kiger A, Nybakken K, Mathey-Prevot B, Perrimon N (2005) High-throughput RNA interference screens in Drosophila tissue culture cells. *Methods in enzymology* **392:** 55-73

Arziman Z, Horn T, Boutros M (2005) E-RNAi: a web application to design optimized RNAi constructs. *Nucleic Acids Res* **33:** W582-588

Bagley JA, Yan Z, Zhang W, Wildonger J, Jan LY, Jan YN (2014) Double-bromo and extraterminal (BET) domain proteins regulate dendrite morphology and mechanosensory function. *Genes Dev* **28:** 1940-1956

Baird L, Dinkova-Kostova AT (2011) The cytoprotective role of the Keap1-Nrf2 pathway. *Arch Toxicol* **85:** 241-272

Barolo S, Carver LA, Posakony JW (2000) GFP and beta-galactosidase transformation vectors for promoter/enhancer analysis in Drosophila. *BioTechniques* **29:** 726, 728, 730, 732

Barone MC, Sykiotis GP, Bohmann D (2011) Genetic activation of Nrf2 signaling is sufficient to ameliorate neurodegenerative phenotypes in a Drosophila model of Parkinson's disease. *Dis Model Mech* **4:** 701-707

Bartkowiak B, Liu P, Phatnani HP, Fuda NJ, Cooper JJ, Price DH, Adelman K, Lis JT, Greenleaf AL (2010) CDK12 is a transcription elongation-associated CTD kinase, the metazoan ortholog of yeast Ctk1. *Genes Dev* **24:** 2303-2316

Bartkowiak B, Mackellar AL, Greenleaf AL (2011) Updating the CTD Story: From Tail to Epic. *Genet Res Int* **2011:** 623718

Bateman JR, Lee AM, Wu CT (2006) Site-specific transformation of Drosophila via phiC31 integrase-mediated cassette exchange. *Genetics* **173:** 769-777

Beckman KB, Ames BN (1998) The free radical theory of aging matures. *Physiol Rev* **78:** 547-581

Belkina AC, Denis GV (2012) BET domain co-regulators in obesity, inflammation and cancer. *Nat Rev Cancer* **12:** 465-477

Bishop NA, Guarente L (2007) Two neurons mediate diet-restriction-induced longevity in C. elegans. *Nature* **447:** 545-549

Blackwell TK, Bowerman B, Priess JR, Weintraub H (1994) Formation of a monomeric DNA binding domain by Skn-1 bZIP and homeodomain elements. *Science* **266:** 621-628

Blank V (2008) Small Maf proteins in mammalian gene control: mere dimerization partners or dynamic transcriptional regulators? *J Mol Biol* **376:** 913-925

Blazek D, Kohoutek J, Bartholomeeusen K, Johansen E, Hulinkova P, Luo Z, Cimermancic P, Ule J, Peterlin BM (2011) The Cyclin K/Cdk12 complex maintains genomic stability via regulation of expression of DNA damage response genes. *Genes Dev* **25:** 2158-2172

Bloom DA, Jaiswal AK (2003) Phosphorylation of Nrf2 at Ser40 by protein kinase C in response to antioxidants leads to the release of Nrf2 from INrf2, but is not required for Nrf2 stabilization/accumulation in the nucleus and transcriptional activation of antioxidant response element-mediated NAD(P)H:quinone oxidoreductase-1 gene expression. *J Biol Chem* **278:** 44675-44682

Bluher M, Kahn BB, Kahn CR (2003) Extended longevity in mice lacking the insulin receptor in adipose tissue. *Science* **299:** 572-574

Boutros M, Kiger AA, Armknecht S, Kerr K, Hild M, Koch B, Haas SA, Paro R, Perrimon N, Heidelberg Fly Array C (2004) Genome-wide RNAi analysis of growth and viability in Drosophila cells. *Science* **303:** 832-835

Bowie A, O'Neill LA (2000) Oxidative stress and nuclear factor-kappaB activation: a reassessment of the evidence in the light of recent discoveries. *Biochem Pharmacol* **59:** 13-23

Bowman EA, Kelly WG (2014) RNA polymerase II transcription elongation and Pol II CTD Ser2 phosphorylation: A tail of two kinases. *Nucleus* **5:** 224-236

Boy AL, Zhai Z, Habring-Muller A, Kussler-Schneider Y, Kaspar P, Lohmann I (2010) Vectors for efficient and high-throughput construction of fluorescent drosophila reporters using the PhiC31 site-specific integration system. *Genesis* **48:** 452-456

Bryan HK, Olayanju A, Goldring CE, Park BK (2013) The Nrf2 cell defence pathway: Keap1-dependent and -independent mechanisms of regulation. *Biochem Pharmacol* **85:** 705-717

Budanov AV (2014) The role of tumor suppressor p53 in the antioxidant defense and metabolism. *Subcell Biochem* **85:** 337-358

Burton NC, Kensler TW, Guilarte TR (2006) In vivo modulation of the Parkinsonian phenotype by Nrf2. *Neurotoxicology* **27:** 1094-1100

Castello PR, Drechsel DA, Patel M (2007) Mitochondria are a major source of paraquat-induced reactive oxygen species production in the brain. *J Biol Chem* **282:** 14186-14193

Chan JY, Kwong M, Lu R, Chang J, Wang B, Yen TS, Kan YW (1998) Targeted disruption of the ubiquitous CNC-bZIP transcription factor, Nrf-1, results in anemia and embryonic lethality in mice. *EMBO J* **17:** 1779-1787

Chang YL, King B, Lin SC, Kennison JA, Huang DH (2007) A double-bromodomain protein, FSH-S, activates the homeotic gene ultrabithorax through a critical promoter-proximal region. *Mol Cell Biol* **27:** 5486-5498

Chatterjee N, Bohmann D (2012) A versatile PhiC31 based reporter system for measuring AP-1 and Nrf2 signaling in Drosophila and in tissue culture. *PLoS One* **7:** e34063

Chen L, Kwong M, Lu R, Ginzinger D, Lee C, Leung L, Chan JY (2003) Nrf1 is critical for redox balance and survival of liver cells during development. *Mol Cell Biol* **23:** 4673-4686

Chen PC, Vargas MR, Pani AK, Smeyne RJ, Johnson DA, Kan YW, Johnson JA (2009) Nrf2-mediated neuroprotection in the MPTP mouse model of Parkinson's disease: Critical role for the astrocyte. *Proc Natl Acad Sci U S A* **106:** 2933-2938

Cheung KL, Kong AN (2010) Molecular targets of dietary phenethyl isothiocyanate and sulforaphane for cancer chemoprevention. *AAPS J* **12:** 87-97

Chiang CM (2009) Brd4 engagement from chromatin targeting to transcriptional regulation: selective contact with acetylated histone H3 and H4. *F1000 Biol Rep* **1:** 98

Chowdhry S, Zhang Y, McMahon M, Sutherland C, Cuadrado A, Hayes JD (2013) Nrf2 is controlled by two distinct beta-TrCP recognition motifs in its Neh6 domain, one of which can be modulated by GSK-3 activity. *Oncogene* **32:** 3765-3781

Ciapponi L, Jackson DB, Mlodzik M, Bohmann D (2001) Drosophila Fos mediates ERK and JNK signals via distinct phosphorylation sites. *Genes & development* **15:** 1540-1553

Clancy DJ, Gems D, Harshman LG, Oldham S, Stocker H, Hafen E, Leevers SJ, Partridge L (2001) Extension of life-span by loss of CHICO, a Drosophila insulin receptor substrate protein. *Science* **292:** 104-106

Crunkhorn S (2012) Deal watch: Abbott boosts investment in NRF2 activators for reducing oxidative stress. *Nat Rev Drug Discov* **11:** 96

Cullinan SB, Gordan JD, Jin J, Harper JW, Diehl JA (2004) The Keap1-BTB protein is an adaptor that bridges Nrf2 to a Cul3-based E3 ligase: oxidative stress sensing by a Cul3-Keap1 ligase. *Mol Cell Biol* **24:** 8477-8486

Cullinan SB, Zhang D, Hannink M, Arvisais E, Kaufman RJ, Diehl JA (2003) Nrf2 is a direct PERK substrate and effector of PERK-dependent cell survival. *Mol Cell Biol* **23:** 7198-7209

Dai Q, Lei T, Zhao C, Zhong J, Tang YZ, Chen B, Yang J, Li C, Wang S, Song X, Li L, Li Q (2012) Cyclin K-containing kinase complexes maintain self-renewal in murine embryonic stem cells. *J Biol Chem* **287:** 25344-25352

Daniels SB, McCarron M, Love C, Clark SH, Chovnick A (1986) The underlying bases of gene expression differences in stable transformants of the rosy locus in Drosophila melanogaster. *Genetics* **113:** 265-285

DasGupta R, Kaykas A, Moon RT, Perrimon N (2005) Functional genomic analysis of the Wnt-wingless signaling pathway. *Science (New York, NY* **308:** 826-833

Davis RJ (2000) Signal transduction by the JNK group of MAP kinases. *Cell* **103:** 239-252

Delmore JE, Issa GC, Lemieux ME, Rahl PB, Shi J, Jacobs HM, Kastritis E, Gilpatrick T, Paranal RM, Qi J, Chesi M, Schinzel AC, McKeown MR, Heffernan TP, Vakoc CR, Bergsagel PL, Ghobrial IM, Richardson PG, Young RA, Hahn WC, Anderson KC, Kung AL, Bradner JE, Mitsiades CS (2011) BET bromodomain inhibition as a therapeutic strategy to target c-Myc. *Cell* **146:** 904-917

DeNicola GM, Karreth FA, Humpton TJ, Gopinathan A, Wei C, Frese K, Mangal D, Yu KH, Yeo CJ, Calhoun ES, Scrimieri F, Winter JM, Hruban RH, Iacobuzio-Donahue C, Kern SE, Blair IA, Tuveson DA (2011) Oncogene-induced Nrf2 transcription promotes ROS detoxification and tumorigenesis. *Nature* **475:** 106-109

Devaiah BN, Singer DS (2013) Two faces of brd4: mitotic bookmark and transcriptional lynchpin. *Transcription* **4:** 13-17

Dey A, Chitsaz F, Abbasi A, Misteli T, Ozato K (2003) The double bromodomain protein Brd4 binds to acetylated chromatin during interphase and mitosis. *Proc Natl Acad Sci U S A* **100:** 8758-8763

Digaleh H, Kiaei M, Khodagholi F (2013) Nrf2 and Nrf1 signaling and ER stress crosstalk: implication for proteasomal degradation and autophagy. *Cell Mol Life Sci* **70:** 4681-4694

Digan ME, Haynes SR, Mozer BA, Dawid IB, Forquignon F, Gans M (1986) Genetic and molecular analysis of fs(1)h, a maternal effect homeotic gene in Drosophila. *Dev Biol* **114:** 161-169

Dinkova-Kostova AT, Holtzclaw WD, Cole RN, Itoh K, Wakabayashi N, Katoh Y, Yamamoto M, Talalay P (2002) Direct evidence that sulfhydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants. *Proc Natl Acad Sci U S A* **99:** 11908-11913

English C, Aloi JJ (2015) New FDA-Approved Disease-Modifying Therapies for Multiple Sclerosis. *Clin Ther* **37:** 691-715

Filippakopoulos P, Qi J, Picaud S, Shen Y, Smith WB, Fedorov O, Morse EM, Keates T, Hickman TT, Felletar I, Philpott M, Munro S, McKeown MR, Wang Y, Christie AL, West N, Cameron MJ, Schwartz B, Heightman TD, La Thangue N, French CA, Wiest O, Kung AL, Knapp S, Bradner JE (2010) Selective inhibition of BET bromodomains. *Nature* **468:** 1067-1073

Finkel T, Holbrook NJ (2000) Oxidants, oxidative stress and the biology of ageing. *Nature* **408:** 239-247

Florence B, Faller DV (2001) You bet-cha: a novel family of transcriptional regulators. *Front Biosci* **6:** D1008-1018

Floyd SR, Pacold ME, Huang Q, Clarke SM, Lam FC, Cannell IG, Bryson BD, Rameseder J, Lee MJ, Blake EJ, Fydrych A, Ho R, Greenberger BA, Chen GC, Maffa A, Del Rosario AM, Root DE, Carpenter AE, Hahn WC, Sabatini DM, Chen CC, White FM, Bradner JE, Yaffe MB (2013) The bromodomain protein Brd4 insulates chromatin from DNA damage signalling. *Nature* **498:** 246-250

Fontana L, Klein S, Holloszy JO (2010) Effects of long-term calorie restriction and endurance exercise on glucose tolerance, insulin action, and adipokine production. *Age (Dordr)* **32:** 97-108

French CA, Miyoshi I, Kubonishi I, Grier HE, Perez-Atayde AR, Fletcher JA (2003) BRD4-NUT fusion oncogene: a novel mechanism in aggressive carcinoma. *Cancer Res* **63:** 304-307

Furukawa M, Xiong Y (2005) BTB protein Keap1 targets antioxidant transcription factor Nrf2 for ubiquitination by the Cullin 3-Roc1 ligase. *Mol Cell Biol* **25:** 162-171

Garber K (2012) Biochemistry: A radical treatment. *Nature* **489:** S4-6

Glise B, Bourbon H, Noselli S (1995) hemipterous encodes a novel Drosophila MAP kinase kinase, required for epithelial cell sheet movement. *Cell* **83:** 451-461

Glise B, Noselli S (1997) Coupling of Jun amino-terminal kinase and Decapentaplegic signaling pathways in Drosophila morphogenesis. *Genes & development* **11:** 1738-1747

Glover-Cutter KM, Lin S, Blackwell TK (2013) Integration of the unfolded protein and oxidative stress responses through SKN-1/Nrf. *PLoS Genet* **9:** e1003701

Gounder SS, Kannan S, Devadoss D, Miller CJ, Whitehead KJ, Odelberg SJ, Firpo MA, Paine R, 3rd, Hoidal JR, Abel ED, Rajasekaran NS (2012) Impaired transcriptional activity of Nrf2 in age-related myocardial oxidative stress is reversible by moderate exercise training. *PLoS One* **7:** e45697

Grimberg KB, Beskow A, Lundin D, Davis MM, Young P (2011) Basic leucine zipper protein Cnc-C is a substrate and transcriptional regulator of the Drosophila 26S proteasome. *Mol Cell Biol* **31:** 897-909

Groth AC, Fish M, Nusse R, Calos MP (2004) Construction of transgenic Drosophila by using the site-specific integrase from phage phiC31. *Genetics* **166:** 1775-1782

Harding KW, Gellon G, McGinnis N, McGinnis W (1995) A screen for modifiers of Deformed function in Drosophila. *Genetics* **140:** 1339-1352

Harman D (1956) Aging: a theory based on free radical and radiation chemistry. *J Gerontol* **11:** 298-300

Hayes JD, Dinkova-Kostova AT (2014) The Nrf2 regulatory network provides an interface between redox and intermediary metabolism. *Trends Biochem Sci* **39:** 199-218

Hayes JD, McMahon M (2009) NRF2 and KEAP1 mutations: permanent activation of an adaptive response in cancer. *Trends Biochem Sci* **34:** 176-188

Hess J, Angel P, Schorpp-Kistner M (2004) AP-1 subunits: quarrel and harmony among siblings. *Journal of cell science* **117:** 5965-5973

Hochmuth CE, Biteau B, Bohmann D, Jasper H (2011) Redox regulation by Keap1 and Nrf2 controls intestinal stem cell proliferation in Drosophila. *Cell Stem Cell* **8:** 188-199

Homsy JG, Jasper H, Peralta XG, Wu H, Kiehart DP, Bohmann D (2006) JNK signaling coordinates integrin and actin functions during Drosophila embryogenesis. *Dev Dyn* **235:** 427-434

Horn T, Boutros M (2010) E-RNAi: a web application for the multi-species design of RNAi reagents--2010 update. *Nucleic Acids Res* **38:** W332-339

Huang B, Yang XD, Zhou MM, Ozato K, Chen LF (2009) Brd4 coactivates transcriptional activation of NF-kappaB via specific binding to acetylated RelA. *Mol Cell Biol* **29:** 1375-1387

Huang C, Ma WY, Dawson MI, Rincon M, Flavell RA, Dong Z (1997) Blocking activator protein-1 activity, but not activating retinoic acid response element, is required for the antitumor promotion effect of retinoic acid. *Proc Natl Acad Sci U S A* **94:** 5826-5830

Huang da W, Sherman BT, Lempicki RA (2009a) Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res* **37:** 1-13

Huang da W, Sherman BT, Lempicki RA (2009b) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* **4:** 44-57

Huang HC, Nguyen T, Pickett CB (2002) Phosphorylation of Nrf2 at Ser-40 by protein kinase C regulates antioxidant response element-mediated transcription. *J Biol Chem* **277:** 42769-42774

Hur W, Gray NS (2011) Small molecule modulators of antioxidant response pathway. *Curr Opin Chem Biol* **15:** 162-173

Hussong M, Borno ST, Kerick M, Wunderlich A, Franz A, Sultmann H, Timmermann B, Lehrach H, Hirsch-Kauffmann M, Schweiger MR (2014) The bromodomain protein BRD4 regulates the KEAP1/NRF2-dependent oxidative stress response. *Cell Death Dis* **5:** e1195

Hutson MS, Tokutake Y, Chang MS, Bloor JW, Venakides S, Kiehart DP, Edwards GS (2003) Forces for morphogenesis investigated with laser microsurgery and quantitative modeling. *Science (New York, NY* **300:** 145-149

Hwang YP, Jeong HG (2008) The coffee diterpene kahweol induces heme oxygenase-1 via the PI3K and p38/Nrf2 pathway to protect human dopaminergic neurons from 6-hydroxydopamine-derived oxidative stress. *FEBS Lett* **582:** 2655-2662

Hwangbo DS, Gershman B, Tu MP, Palmer M, Tatar M (2004) Drosophila dFOXO controls lifespan and regulates insulin signalling in brain and fat body. *Nature* **429:** 562-566

Inami Y, Waguri S, Sakamoto A, Kouno T, Nakada K, Hino O, Watanabe S, Ando J, Iwadate M, Yamamoto M, Lee MS, Tanaka K, Komatsu M (2011) Persistent activation of Nrf2 through p62 in hepatocellular carcinoma cells. *J Cell Biol* **193:** 275-284

Ito K, Barnes PJ (2009) COPD as a disease of accelerated lung aging. *Chest* **135:** 173-180

Itoh K, Chiba T, Takahashi S, Ishii T, Igarashi K, Katoh Y, Oyake T, Hayashi N, Satoh K, Hatayama I, Yamamoto M, Nabeshima Y (1997) An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. *Biochem Biophys Res Commun* **236:** 313-322

Jain AK, Jaiswal AK (2006) Phosphorylation of tyrosine 568 controls nuclear export of Nrf2. *J Biol Chem* **281:** 12132-12142

Jain AK, Jaiswal AK (2007) GSK-3beta acts upstream of Fyn kinase in regulation of nuclear export and degradation of NF-E2 related factor 2. *J Biol Chem* **282:** 16502-16510

Jakel RJ, Townsend JA, Kraft AD, Johnson JA (2007) Nrf2-mediated protection against 6-hydroxydopamine. *Brain Res* **1144:** 192-201

Jang MK, Mochizuki K, Zhou M, Jeong HS, Brady JN, Ozato K (2005) The bromodomain protein Brd4 is a positive regulatory component of P-TEFb and stimulates RNA polymerase II-dependent transcription. *Mol Cell* **19:** 523-534

Jaramillo MC, Zhang DD (2013) The emerging role of the Nrf2-Keap1 signaling pathway in cancer. *Genes Dev* **27:** 2179-2191

Jasper H, Benes V, Schwager C, Sauer S, Clauder-Munster S, Ansorge W, Bohmann D (2001) The genomic response of the Drosophila embryo to JNK signaling. *Developmental cell* **1:** 579-586

Jiang T, Xing B, Rao J (2008) Recent developments of biological reporter technology for detecting gene expression. *Biotechnol Genet Eng Rev* **25:** 41-75

Johnson TE (1990) Increased life-span of age-1 mutants in Caenorhabditis elegans and lower Gompertz rate of aging. *Science* **249:** 908-912

Junger MA, Rintelen F, Stocker H, Wasserman JD, Vegh M, Radimerski T, Greenberg ME, Hafen E (2003) The Drosophila forkhead transcription factor FOXO mediates the reduction in cell number associated with reduced insulin signaling. *J Biol* **2:** 20

Kansanen E, Kuosmanen SM, Leinonen H, Levonen AL (2013) The Keap1-Nrf2 pathway: Mechanisms of activation and dysregulation in cancer. *Redox Biol* **1:** 45-49

Katoh Y, Iida K, Kang MI, Kobayashi A, Mizukami M, Tong KI, McMahon M, Hayes JD, Itoh K, Yamamoto M (2005) Evolutionary conserved N-terminal domain of Nrf2 is essential for the Keap1-mediated degradation of the protein by proteasome. *Arch Biochem Biophys* **433:** 342-350

Katoh Y, Itoh K, Yoshida E, Miyagishi M, Fukamizu A, Yamamoto M (2001) Two domains of Nrf2 cooperatively bind CBP, a CREB binding protein, and synergistically activate transcription. *Genes Cells* **6:** 857-868

Kawai Y, Garduno L, Theodore M, Yang J, Arinze IJ (2011) Acetylation-deacetylation of the transcription factor Nrf2 (nuclear factor erythroid 2-related factor 2) regulates its transcriptional activity and nucleocytoplasmic localization. *J Biol Chem* **286:** 7629-7640

Kellner WA, Van Bortle K, Li L, Ramos E, Takenaka N, Corces VG (2013) Distinct isoforms of the Drosophila Brd4 homologue are present at enhancers, promoters and insulator sites. *Nucleic Acids Res* **41:** 9274-9283

Kensler TW, Chen JG, Egner PA, Fahey JW, Jacobson LP, Stephenson KK, Ye L, Coady JL, Wang JB, Wu Y, Sun Y, Zhang QN, Zhang BC, Zhu YR, Qian GS, Carmella SG, Hecht SS, Benning L, Gange SJ, Groopman JD, Talalay P (2005) Effects of glucosinolate-rich broccoli sprouts on urinary levels of aflatoxin-DNA adducts and phenanthrene tetraols in a randomized clinical trial in He Zuo township, Qidong, People's Republic of China. *Cancer Epidemiol Biomarkers Prev* **14:** 2605-2613

Kensler TW, Groopman JD, Sutter TR, Curphey TJ, Roebuck BD (1999) Development of cancer chemopreventive agents: oltipraz as a paradigm. *Chemical research in toxicology* **12:** 113-126

Kensler TW, Wakabayashi N, Biswal S (2007) Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. *Annu Rev Pharmacol Toxicol* **47:** 89-116

Kenyon C (2005) The plasticity of aging: insights from long-lived mutants. *Cell* **120:** 449-460

Kenyon C, Chang J, Gensch E, Rudner A, Tabtiang R (1993) A C. elegans mutant that lives twice as long as wild type. *Nature* **366:** 461-464

Kerppola TK, Curran T (1994) Maf and Nrl can bind to AP-1 sites and form heterodimers with Fos and Jun. *Oncogene* **9:** 675-684

Keum YS, Choi BY (2014) Molecular and chemical regulation of the Keap1-Nrf2 signaling pathway. *Molecules* **19:** 10074-10089

Keum YS, Owuor ED, Kim BR, Hu R, Kong AN (2003) Involvement of Nrf2 and JNK1 in the activation of antioxidant responsive element (ARE) by chemopreventive agent phenethyl isothiocyanate (PEITC). *Pharm Res* **20:** 1351-1356

Keum YS, Yu S, Chang PP, Yuan X, Kim JH, Xu C, Han J, Agarwal A, Kong AN (2006) Mechanism of action of sulforaphane: inhibition of p38 mitogen-activated protein kinase isoforms contributing to the induction of antioxidant response element-mediated heme oxygenase-1 in human hepatoma HepG2 cells. *Cancer Res* **66:** 8804-8813

Ki SH, Cho IJ, Choi DW, Kim SG (2005) Glucocorticoid receptor (GR)-associated SMRT binding to C/EBPbeta TAD and Nrf2 Neh4/5: role of SMRT recruited to GR in GSTA2 gene repression. *Mol Cell Biol* **25:** 4150-4165

Kim EK, Choi EJ (2010) Pathological roles of MAPK signaling pathways in human diseases. *Biochim Biophys Acta* **1802:** 396-405

Kitteringham NR, Abdullah A, Walsh J, Randle L, Jenkins RE, Sison R, Goldring CE, Powell H, Sanderson C, Williams S, Higgins L, Yamamoto M, Hayes J, Park BK (2010) Proteomic analysis of Nrf2 deficient transgenic mice reveals cellular defence and lipid metabolism as primary Nrf2-dependent pathways in the liver. *J Proteomics* **73:** 1612-1631

Kobayashi A, Kang MI, Okawa H, Ohtsuji M, Zenke Y, Chiba T, Igarashi K, Yamamoto M (2004) Oxidative stress sensor Keap1 functions as an adaptor for Cul3-based E3 ligase to regulate proteasomal degradation of Nrf2. *Mol Cell Biol* **24:** 7130-7139

Kockel L, Homsy JG, Bohmann D (2001) Drosophila AP-1: lessons from an invertebrate. *Oncogene* **20:** 2347-2364

Kockel L, Zeitlinger J, Staszewski LM, Mlodzik M, Bohmann D (1997) Jun in Drosophila development: redundant and nonredundant functions and regulation by two MAPK signal transduction pathways. *Genes & development* **11:** 1748-1758

Kockmann T, Gerstung M, Schlumpf T, Xhinzhou Z, Hess D, Beerenwinkel N, Beisel C, Paro R (2013) The BET protein FSH functionally interacts with ASH1 to orchestrate global gene activity in Drosophila. *Genome Biol* **14:** R18

Kratschmar DV, Calabrese D, Walsh J, Lister A, Birk J, Appenzeller-Herzog C, Moulin P, Goldring CE, Odermatt A (2012) Suppression of the Nrf2-dependent antioxidant response by glucocorticoids and 11beta-HSD1-mediated glucocorticoid activation in hepatic cells. *PLoS One* **7:** e36774

L'Episcopo F, Tirolo C, Testa N, Caniglia S, Morale MC, Impagnatiello F, Pluchino S, Marchetti B (2013) Aging-induced Nrf2-ARE pathway disruption in the subventricular zone drives neurogenic impairment in parkinsonian mice via PI3K-Wnt/beta-catenin dysregulation. *J Neurosci* **33:** 1462-1485

Li MH, Cha YN, Surh YJ (2006) Peroxynitrite induces HO-1 expression via PI3K/Akt-dependent activation of NF-E2-related factor 2 in PC12 cells. *Free Radic Biol Med* **41:** 1079-1091

Linker RA, Lee DH, Ryan S, van Dam AM, Conrad R, Bista P, Zeng W, Hronowsky X, Buko A, Chollate S, Ellrichmann G, Bruck W, Dawson K, Goelz S, Wiese S, Scannevin RH, Lukashev M, Gold R (2011) Fumaric acid esters exert neuroprotective effects in neuroinflammation via activation of the Nrf2 antioxidant pathway. *Brain* **134:** 678-692

Lockwood WW, Zejnullahu K, Bradner JE, Varmus H (2012) Sensitivity of human lung adenocarcinoma cell lines to targeted inhibition of BET epigenetic signaling proteins. *Proc Natl Acad Sci U S A* **109:** 19408-19413

Lopez-Bergami P, Lau E, Ronai Z (2010) Emerging roles of ATF2 and the dynamic AP1 network in cancer. *Nat Rev Cancer* **10:** 65-76

Loven J, Hoke HA, Lin CY, Lau A, Orlando DA, Vakoc CR, Bradner JE, Lee TI, Young RA (2013) Selective inhibition of tumor oncogenes by disruption of super-enhancers. *Cell* **153:** 320-334

Magesh S, Chen Y, Hu L (2012) Small molecule modulators of Keap1-Nrf2-ARE pathway as potential preventive and therapeutic agents. *Med Res Rev* **32:** 687-726

Manseau L, Baradaran A, Brower D, Budhu A, Elefant F, Phan H, Philp AV, Yang M, Glover D, Kaiser K, Palter K, Selleck S (1997) GAL4 enhancer traps expressed in the embryo, larval brain, imaginal discs, and ovary of Drosophila. *Dev Dyn* **209:** 310-322

Marini MG, Chan K, Casula L, Kan YW, Cao A, Moi P (1997) hMAF, a small human transcription factor that heterodimerizes specifically with Nrf1 and Nrf2. *J Biol Chem* **272:** 16490-16497

Markstein M, Pitsouli C, Villalta C, Celniker SE, Perrimon N (2008) Exploiting position effects and the gypsy retrovirus insulator to engineer precisely expressed transgenes. *Nat Genet* **40:** 476-483

Martin-Blanco E, Gampel A, Ring J, Virdee K, Kirov N, Tolkovsky AM, Martinez-Arias A (1998) puckered encodes a phosphatase that mediates a feedback loop regulating JNK activity during dorsal closure in Drosophila. *Genes & development* **12:** 557-570

Michaeloudes C, Mercado N, Clarke C, Bhavsar PK, Adcock IM, Barnes PJ, Chung KF (2014) Bromodomain and extraterminal proteins suppress NF-E2-related factor 2-mediated antioxidant gene expression. *J Immunol* **192:** 4913-4920

Misra JR, Horner MA, Lam G, Thummel CS (2011) Transcriptional regulation of xenobiotic detoxification in Drosophila. *Genes Dev* **25:** 1796-1806

Mitsuishi Y, Taguchi K, Kawatani Y, Shibata T, Nukiwa T, Aburatani H, Yamamoto M, Motohashi H (2012) Nrf2 redirects glucose and glutamine into anabolic pathways in metabolic reprogramming. *Cancer Cell* **22:** 66-79

Mohler J, Mahaffey JW, Deutsch E, Vani K (1995) Control of Drosophila head segment identity by the bZIP homeotic gene cnc. *Development* **121:** 237-247

Moi P, Chan K, Asunis I, Cao A, Kan YW (1994) Isolation of NF-E2-related factor 2 (Nrf2), a NF-E2-like basic leucine zipper transcriptional activator that binds to the tandem NF-E2/AP1 repeat of the beta-globin locus control region. *Proc Natl Acad Sci U S A* **91:** 9926-9930

Motohashi H, Katsuoka F, Engel JD, Yamamoto M (2004) Small Maf proteins serve as transcriptional cofactors for keratinocyte differentiation in the Keap1-Nrf2 regulatory pathway. *Proc Natl Acad Sci U S A* **101:** 6379-6384

Motohashi H, O'Connor T, Katsuoka F, Engel JD, Yamamoto M (2002) Integration and diversity of the regulatory network composed of Maf and CNC families of transcription factors. *Gene* **294:** 1-12

Motohashi H, Yamamoto M (2004) Nrf2-Keap1 defines a physiologically important stress response mechanism. *Trends Mol Med* **10:** 549-557

Murakami S, Johnson TE (1996) A genetic pathway conferring life extension and resistance to UV stress in Caenorhabditis elegans. *Genetics* **143:** 1207-1218

Namani A, Li Y, Wang XJ, Tang X (2014) Modulation of NRF2 signaling pathway by nuclear receptors: implications for cancer. *Biochim Biophys Acta* **1843:** 1875-1885

Nioi P, Nguyen T, Sherratt PJ, Pickett CB (2005) The carboxy-terminal Neh3 domain of Nrf2 is required for transcriptional activation. *Mol Cell Biol* **25:** 10895-10906

Niture SK, Jain AK, Jaiswal AK (2009) Antioxidant-induced modification of INrf2 cysteine 151 and PKC-delta-mediated phosphorylation of Nrf2 serine 40 are both required for stabilization and nuclear translocation of Nrf2 and increased drug resistance. *J Cell Sci* **122:** 4452-4464

Niture SK, Khatri R, Jaiswal AK (2014) Regulation of Nrf2-an update. *Free Radic Biol Med* **66:** 36-44

Noselli S (1998) JNK signaling and morphogenesis in Drosophila. *Trends Genet* **14:** 33-38

Ogg S, Paradis S, Gottlieb S, Patterson GI, Lee L, Tissenbaum HA, Ruvkun G (1997) The Fork head transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in C. elegans. *Nature* **389:** 994-999

Ogryzko VV, Schiltz RL, Russanova V, Howard BH, Nakatani Y (1996) The transcriptional coactivators p300 and CBP are histone acetyltransferases. *Cell* **87:** 953-959

Ohta T, Iijima K, Miyamoto M, Nakahara I, Tanaka H, Ohtsuji M, Suzuki T, Kobayashi A, Yokota J, Sakiyama T, Shibata T, Yamamoto M, Hirohashi S (2008) Loss of Keap1 function activates Nrf2 and provides advantages for lung cancer cell growth. *Cancer Res* **68:** 1303-1309

Ohtsuji M, Katsuoka F, Kobayashi A, Aburatani H, Hayes JD, Yamamoto M (2008) Nrf1 and Nrf2 play distinct roles in activation of antioxidant response element-dependent genes. *J Biol Chem* **283:** 33554-33562

Ooi A, Wong JC, Petillo D, Roossien D, Perrier-Trudova V, Whitten D, Min BW, Tan MH, Zhang Z, Yang XJ, Zhou M, Gardie B, Molinie V, Richard S, Tan PH, Teh BT, Furge KA (2011) An antioxidant response phenotype shared between hereditary and sporadic type 2 papillary renal cell carcinoma. *Cancer Cell* **20:** 511-523

Osburn WO, Kensler TW (2008) Nrf2 signaling: an adaptive response pathway for protection against environmental toxic insults. *Mutation research* **659:** 31-39

Pearson KJ, Lewis KN, Price NL, Chang JW, Perez E, Cascajo MV, Tamashiro KL, Poosala S, Csiszar A, Ungvari Z, Kensler TW, Yamamoto M, Egan JM, Longo DL,

Ingram DK, Navas P, de Cabo R (2008) Nrf2 mediates cancer protection but not prolongevity induced by caloric restriction. *Proc Natl Acad Sci U S A* **105:** 2325-2330

Peverali FA, Isaksson A, Papavassiliou AG, Plastina P, Staszewski LM, Mlodzik M, Bohmann D (1996) Phosphorylation of Drosophila Jun by the MAP kinase rolled regulates photoreceptor differentiation. *The EMBO journal* **15:** 3943-3950

Phatnani HP, Greenleaf AL (2006) Phosphorylation and functions of the RNA polymerase II CTD. *Genes Dev* **20:** 2922-2936

Pitoniak A, Bohmann D (2015) Mechanisms and Functions of Nrf2 Signaling in Drosophila. *Free Radic Biol Med*

Pratico D (2008) Evidence of oxidative stress in Alzheimer's disease brain and antioxidant therapy: lights and shadows. *Ann N Y Acad Sci* **1147:** 70-78

Przybysz AJ, Choe KP, Roberts LJ, Strange K (2009) Increased age reduces DAF-16 and SKN-1 signaling and the hormetic response of Caenorhabditis elegans to the xenobiotic juglone. *Mech Ageing Dev* **130:** 357-369

Puig O, Tjian R (2005) Transcriptional feedback control of insulin receptor by dFOXO/FOXO1. *Genes Dev* **19:** 2435-2446

Rada P, Rojo AI, Chowdhry S, McMahon M, Hayes JD, Cuadrado A (2011) SCF/{beta}-TrCP promotes glycogen synthase kinase 3-dependent degradation of the Nrf2 transcription factor in a Keap1-independent manner. *Mol Cell Biol* **31:** 1121-1133

Rahman MM, Sykiotis GP, Nishimura M, Bodmer R, Bohmann D (2013) Declining signal dependence of Nrf2-MafS-regulated gene expression correlates with aging phenotypes. *Aging Cell* **12:** 554-562

Ramos-Gomez M, Kwak MK, Dolan PM, Itoh K, Yamamoto M, Talalay P, Kensler TW (2001) Sensitivity to carcinogenesis is increased and chemoprotective efficacy of enzyme inducers is lost in nrf2 transcription factor-deficient mice. *Proceedings of the National Academy of Sciences of the United States of America* **98:** 3410-3415

Riesgo-Escovar JR, Jenni M, Fritz A, Hafen E (1996) The Drosophila Jun-N-terminal kinase is required for cell morphogenesis but not for DJun-dependent cell fate specification in the eye. *Genes & development* **10:** 2759-2768

Rizvi F, Shukla S, Kakkar P (2014) Essential role of PH domain and leucine-rich repeat protein phosphatase 2 in Nrf2 suppression via modulation of

Akt/GSK3beta/Fyn kinase axis during oxidative hepatocellular toxicity. *Cell Death Dis* **5:** e1153

Rogers WA, Williams TM (2011) Quantitative Comparison of cis-Regulatory Element (CRE) Activities in Transgenic Drosophila melanogaster. *J Vis Exp*

Roth SY, Denu JM, Allis CD (2001) Histone acetyltransferases. *Annu Rev Biochem* **70:** 81-120

Safdar A, deBeer J, Tarnopolsky MA (2010) Dysfunctional Nrf2-Keap1 redox signaling in skeletal muscle of the sedentary old. *Free Radic Biol Med* **49:** 1487-1493

Salih DA, Brunet A (2008) FoxO transcription factors in the maintenance of cellular homeostasis during aging. *Curr Opin Cell Biol* **20:** 126-136

Shen G, Hebbar V, Nair S, Xu C, Li W, Lin W, Keum YS, Han J, Gallo MA, Kong AN (2004) Regulation of Nrf2 transactivation domain activity. The differential effects of mitogen-activated protein kinase cascades and synergistic stimulatory effect of Raf and CREB-binding protein. *J Biol Chem* **279:** 23052-23060

Shi J, Vakoc CR (2014) The mechanisms behind the therapeutic activity of BET bromodomain inhibition. *Mol Cell* **54:** 728-736

Shi J, Wang Y, Zeng L, Wu Y, Deng J, Zhang Q, Lin Y, Li J, Kang T, Tao M, Rusinova E, Zhang G, Wang C, Zhu H, Yao J, Zeng YX, Evers BM, Zhou MM, Zhou BP (2014) Disrupting the interaction of BRD4 with diacetylated Twist suppresses tumorigenesis in basal-like breast cancer. *Cancer Cell* **25:** 210-225

Shibata T, Kokubu A, Gotoh M, Ojima H, Ohta T, Yamamoto M, Hirohashi S (2008) Genetic alteration of Keap1 confers constitutive Nrf2 activation and resistance to chemotherapy in gallbladder cancer. *Gastroenterology* **135:** 1358-1368, 1368 e1351-1354

Shin S, Wakabayashi J, Yates MS, Wakabayashi N, Dolan PM, Aja S, Liby KT, Sporn MB, Yamamoto M, Kensler TW (2009) Role of Nrf2 in prevention of high-fat diet-induced obesity by synthetic triterpenoid CDDO-imidazolide. *Eur J Pharmacol* **620:** 138-144

Singh A, Misra V, Thimmulappa RK, Lee H, Ames S, Hoque MO, Herman JG, Baylin SB, Sidransky D, Gabrielson E, Brock MV, Biswal S (2006) Dysfunctional KEAP1-NRF2 interaction in non-small-cell lung cancer. *PLoS Med* **3:** e420

Singh SR, Zeng X, Zheng Z, Hou SX (2011) The adult Drosophila gastric and stomach organs are maintained by a multipotent stem cell pool at the foregut/midgut junction in the cardia (proventriculus). *Cell Cycle* **10:** 1109-1120

Sporn MB, Liby KT (2012) NRF2 and cancer: the good, the bad and the importance of context. *Nat Rev Cancer* **12:** 564-571

Steinbrink S, Boutros M (2008) RNAi screening in cultured Drosophila cells. *Methods Mol Biol* **420:** 139-153

Stielow B, Sapetschnig A, Kruger I, Kunert N, Brehm A, Boutros M, Suske G (2008) Identification of SUMO-dependent chromatin-associated transcriptional repression components by a genome-wide RNAi screen. *Mol Cell* **29:** 742-754

Suh JH, Shenvi SV, Dixon BM, Liu H, Jaiswal AK, Liu RM, Hagen TM (2004) Decline in transcriptional activity of Nrf2 causes age-related loss of glutathione synthesis, which is reversible with lipoic acid. *Proc Natl Acad Sci U S A* **101:** 3381-3386

Sun Z, Chin YE, Zhang DD (2009) Acetylation of Nrf2 by p300/CBP augments promoter-specific DNA binding of Nrf2 during the antioxidant response. *Mol Cell Biol* **29:** 2658-2672

Sykiotis GP, Bohmann D (2007) Stress-Activated Protein Kinase Signaling in Drosophila. *Topics in Current Genetics* **20:** 225-241

Sykiotis GP, Bohmann D (2008) Keap1/Nrf2 signaling regulates oxidative stress tolerance and lifespan in Drosophila. *Dev Cell* **14:** 76-85

Sykiotis GP, Bohmann D (2010) Stress-activated cap'n'collar transcription factors in aging and human disease. *Sci Signal* **3:** re3

Sykiotis GP, Habeos IG, Samuelson AV, Bohmann D (2011) The role of the antioxidant and longevity-promoting Nrf2 pathway in metabolic regulation. *Curr Opin Clin Nutr Metab Care* **14:** 41-48

Tatar M, Kopelman A, Epstein D, Tu MP, Yin CM, Garofalo RS (2001) A mutant Drosophila insulin receptor homolog that extends life-span and impairs neuroendocrine function. *Science* **292:** 107-110

Trachootham D, Lu W, Ogasawara MA, Nilsa RD, Huang P (2008) Redox regulation of cell survival. *Antioxid Redox Signal* **10:** 1343-1374

Trinh K, Moore K, Wes PD, Muchowski PJ, Dey J, Andrews L, Pallanck LJ (2008) Induction of the phase II detoxification pathway suppresses neuron loss in Drosophila models of Parkinson's disease. *J Neurosci* **28:** 465-472

Tsai CW, Lin CY, Wang YJ (2011) Carnosic acid induces the NAD(P)H: quinone oxidoreductase 1 expression in rat clone 9 cells through the p38/nuclear factor erythroid-2 related factor 2 pathway. *J Nutr* **141:** 2119-2125

Tullet JM, Hertweck M, An JH, Baker J, Hwang JY, Liu S, Oliveira RP, Baumeister R, Blackwell TK (2008) Direct inhibition of the longevity-promoting factor SKN-1 by insulin-like signaling in C. elegans. *Cell* **132:** 1025-1038

Ungvari Z, Bailey-Downs L, Sosnowska D, Gautam T, Koncz P, Losonczy G, Ballabh P, de Cabo R, Sonntag WE, Csiszar A (2011) Vascular oxidative stress in aging: a homeostatic failure due to dysregulation of NRF2-mediated antioxidant response. *Am J Physiol Heart Circ Physiol* **301:** H363-372

Ungvari Z, Buffenstein R, Austad SN, Podlutsky A, Kaley G, Csiszar A (2008) Oxidative stress in vascular senescence: lessons from successfully aging species. *Front Biosci* **13:** 5056-5070

Urano F, Wang X, Bertolotti A, Zhang Y, Chung P, Harding HP, Ron D (2000) Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1. *Science* **287:** 664-666

Veraksa A, McGinnis N, Li X, Mohler J, McGinnis W (2000) Cap 'n' collar B cooperates with a small Maf subunit to specify pharyngeal development and suppress deformed homeotic function in the Drosophila head. *Development (Cambridge, England)* **127:** 4023-4037

Wakabayashi N, Dinkova-Kostova AT, Holtzclaw WD, Kang MI, Kobayashi A, Yamamoto M, Kensler TW, Talalay P (2004) Protection against electrophile and oxidant stress by induction of the phase 2 response: fate of cysteines of the Keap1 sensor modified by inducers. *Proc Natl Acad Sci U S A* **101:** 2040-2045

Wang H, Liu K, Geng M, Gao P, Wu X, Hai Y, Li Y, Li Y, Luo L, Hayes JD, Wang XJ, Tang X (2013) RXRalpha inhibits the NRF2-ARE signaling pathway through a direct interaction with the Neh7 domain of NRF2. *Cancer Res* **73:** 3097-3108

Wang JS, Shen X, He X, Zhu YR, Zhang BC, Wang JB, Qian GS, Kuang SY, Zarba A, Egner PA, Jacobson LP, Munoz A, Helzlsouer KJ, Groopman JD, Kensler TW (1999) Protective alterations in phase 1 and 2 metabolism of aflatoxin B1 by oltipraz in residents of Qidong, People's Republic of China. *J Natl Cancer Inst* **91:** 347-354

Wang L, Chen Y, Sternberg P, Cai J (2008a) Essential roles of the PI3 kinase/Akt pathway in regulating Nrf2-dependent antioxidant functions in the RPE. *Invest Ophthalmol Vis Sci* **49:** 1671-1678

Wang Q, Carmichael GG (2004) Effects of length and location on the cellular response to double-stranded RNA. *Microbiol Mol Biol Rev* **68:** 432-452, table of contents

Wang R, An J, Ji F, Jiao H, Sun H, Zhou D (2008b) Hypermethylation of the Keap1 gene in human lung cancer cell lines and lung cancer tissues. *Biochem Biophys Res Commun* **373:** 151-154

Wang XJ, Hayes JD, Henderson CJ, Wolf CR (2007) Identification of retinoic acid as an inhibitor of transcription factor Nrf2 through activation of retinoic acid receptor alpha. *Proc Natl Acad Sci U S A* **104:** 19589-19594

Wang Y, Zhou Y, Graves DT (2014) FOXO transcription factors: their clinical significance and regulation. *Biomed Res Int* **2014:** 925350

Weber U, Paricio N, Mlodzik M (2000) Jun mediates Frizzled-induced R3/R4 cell fate distinction and planar polarity determination in the Drosophila eye. *Development (Cambridge, England)* **127:** 3619-3629

Weston CR, Davis RJ (2007) The JNK signal transduction pathway. *Current opinion in cell biology* **19:** 142-149

Yang L, Calingasan NY, Thomas B, Chaturvedi RK, Kiaei M, Wille EJ, Liby KT, Williams C, Royce D, Risingsong R, Musiek ES, Morrow JD, Sporn M, Beal MF (2009) Neuroprotective effects of the triterpenoid, CDDO methyl amide, a potent inducer of Nrf2-mediated transcription. *PLoS One* **4:** e5757

Yang Z, Yik JH, Chen R, He N, Jang MK, Ozato K, Zhou Q (2005) Recruitment of P-TEFb for stimulation of transcriptional elongation by the bromodomain protein Brd4. *Mol Cell* **19:** 535-545

Yao Y, Brodie AM, Davidson NE, Kensler TW, Zhou Q (2010) Inhibition of estrogen signaling activates the NRF2 pathway in breast cancer. *Breast Cancer Res Treat* **124:** 585-591

Zehring WA, Lee JM, Weeks JR, Jokerst RS, Greenleaf AL (1988) The C-terminal repeat domain of RNA polymerase II largest subunit is essential in vivo but is not

required for accurate transcription initiation in vitro. *Proc Natl Acad Sci U S A* **85:** 3698-3702

Zhang DD, Hannink M (2003) Distinct cysteine residues in Keap1 are required for Keap1-dependent ubiquitination of Nrf2 and for stabilization of Nrf2 by chemopreventive agents and oxidative stress. *Mol Cell Biol* **23:** 8137-8151

Zhang DD, Lo SC, Cross JV, Templeton DJ, Hannink M (2004) Keap1 is a redox-regulated substrate adaptor protein for a Cul3-dependent ubiquitin ligase complex. *Mol Cell Biol* **24:** 10941-10953

Zhang Y, Munday R (2008) Dithiolethiones for cancer chemoprevention: where do we stand? *Mol Cancer Ther* **7:** 3470-3479

Zhang Y, Nicholatos J, Dreier JR, Ricoult SJ, Widenmaier SB, Hotamisligil GS, Kwiatkowski DJ, Manning BD (2014a) Coordinated regulation of protein synthesis and degradation by mTORC1. *Nature* **513:** 440-443

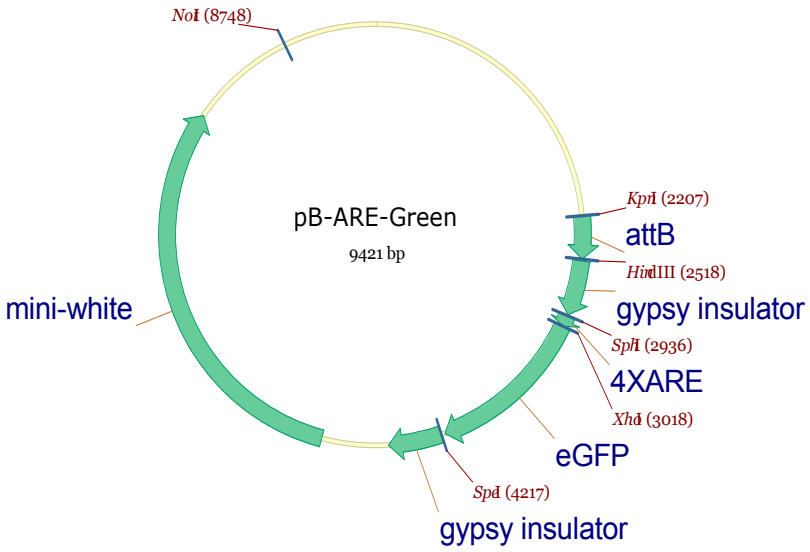
Zhang Y, Ren Y, Li S, Hayes JD (2014b) Transcription factor Nrf1 is topologically repartitioned across membranes to enable target gene transactivation through its acidic glucose-responsive domains. *PLoS One* **9:** e93458

Zhu M, Fahl WE (2001) Functional characterization of transcription regulators that interact with the electrophile response element. *Biochem Biophys Res Commun* **289:** 212-219

Zuber J, Shi J, Wang E, Rappaport AR, Herrmann H, Sison EA, Magoon D, Qi J, Blatt K, Wunderlich M, Taylor MJ, Johns C, Chicas A, Mulloy JC, Kogan SC, Brown P, Valent P, Bradner JE, Lowe SW, Vakoc CR (2011) RNAi screen identifies Brd4 as a therapeutic target in acute myeloid leukaemia. *Nature* **478:** 524-528

Appendix

The plasmid maps of pB-ARE-green and pB-TRE-red.



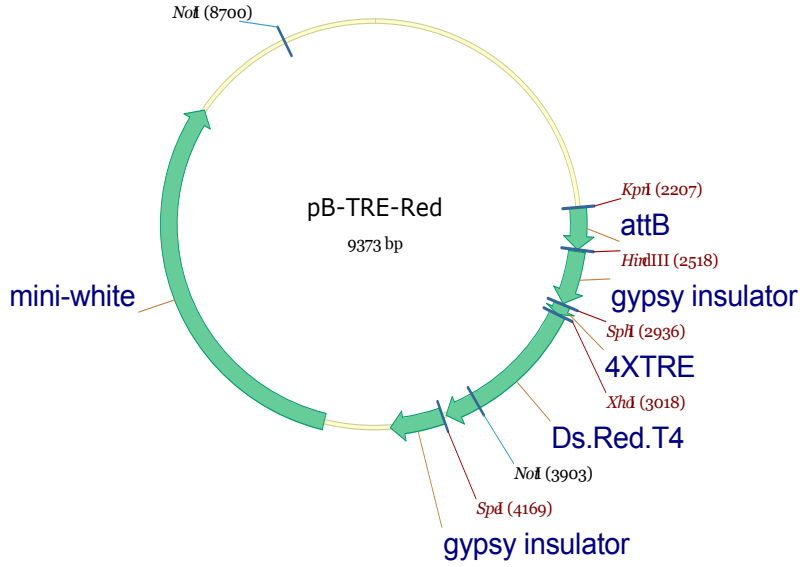


Table: Primers used in pUAS-Fs(1)h-S-HA-attB and pUAS-Fs(1)hL-HA-attB cloning

Primer Name	Primer sequence
PattB-F	GGAATTCCATATGATTACGCCAAGCGCGCA
PattB-R	GGAATTCCATATGCAGCCCAAGCTTATCGATAC
PFs(1)h-S-UAST-HA-F	CGGAATTCTGAAGCACCAGAACCACCAC
PFs(1)h-S-UAST-HA-R	GAAGATCTATACCTGCTTCACTGTCGCTCGA
PUAST-Fs(1)h-L-int-F	TGGAGAAGCGCCTGCAGGACGTC
PUAST-Fs(1)h-L-int-R	GAAGATCTATCAGCGTCTCCTCAAAGGC

Table: Primers used in qPCR experiments

Primer name	Primer sequence
Fs(1)h-FP	AACAAGTTGCCAGGGGACAA
Fs(1)h-RP	GATTGGAGTCACGCAGCGAT
GclC-FP	CGCTTTATCCAGAAGCGTGCCG
GclC-RP	AATCCTGCTTGTAATCCGGGTGGC
GstD1-FP	GGCCGCCTTCGAGTTCCTGA
GstD1-RP	CGGTTGCCACCAGGGCAATG
GstE9-FP	GGTGCCGCGTTCCCAGATTG
GstE9-RP	GACCGGTCCGCAGAGGTAAGC
Keap1-FP	TGGCCAGCGTGGAGTGCTAC
Keap1-RP	TTGCAGCAACACCCGCTCCA
Act5C-FP	CCTGGCATCGCCGACCGTAT
Act5C-RP	AGTACTTGCGCTCTGGCGGG

Table: Primers used to generate amplicons for dsRNA synthesis

Primer name	Primer sequence
Fs(1)hi-F	TTCCTCATCCGAGTTGGATT
Fs(1)hi-R	TGAACAAGGAGAAGCTGTCGG
Fs(1)h-Si-F	GCGTGGCTTGAGTCAGAGA
Fs(1)hSi-R	CTCTTGGGCGTGCTAATGGT
Fs(1)h-Li-F	GGGCAGCAGCACAACAAGAAT
Fs(1)h-Li-R	GGTAGAACCGGCGCTGAACT
Keap1i-F	CGTGTTGTGGTACTCCATGC
Keap1i-R	CCAACTTCCTCAAGGAGCAG
CncCi-F	TCAATGTGCTACCTTATTGACT
CncCi-R	TTATCTTGTTGAAGCTCCTCC
MafSi-F	GATTTGGTGAGCATTTCGGT
MafSi-R	AAACAGGAATGTGACTGGGC
Hepi-F	GTGGTCCCGGTGGTGGA
Hepi-R	ACCTTGCCCAGGATCTGTT
Bski-F	AGCATCCACTTCCTCAGCAT
Bski-R	GGTACACCATCACCCTCGTT
GFPi-F	CACATGAAGCAGCACGACTT
GFPi-R	TGTTCTGCTGGTAGTGGTCG
Fluci-F	GGAGAGCAACTGCATAAGGC
Fluci-R	ACATCGACTGAAATCCCTGG
Cdk12i2-F	TAGTGTGCAGCACTCACGAA
Cdk12i2-R	TTTACCAGCTCGGCAAATAGG
Cdk12i3-F	TATCAATGCCTTACTGGACCG
Cdk12i3-R	TTGATGCGCACTTACATCCTC
CycKi-F	TTTGGAAACATCATTTCAGCA
CycKi-R	GTGGATACTACAACGCCGGT

Each dsRNA primer sequence is preceded by a T7 promoter (sequence TAATACGACTCACTATAGG) at the 5’ end.