

**Epigenetic regulation of chromatin structure by dKeap1 and
CncC in *Drosophila melanogaster***

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Chapter 1: Introduction

Living organisms are frequently exposed to oxidative stress and xenobiotic compounds (drugs or environmental compounds that are not native to a given biological system) that can be toxic or carcinogenic if not detoxified. Correlational studies have indicated that exposure to environmental toxins or oxidative stress can result in epigenetic modifications (i.e. remodeling the chromatin to express or repress different genes) which can have important implications for the development of an organism (Marsit 2015). For example, arsenic is a metalloid compound that is often found in groundwater and industrial plants. Long term exposure to arsenic has been found to correlate with alterations in histone modification (Chervona et al. 2012) and DNA methylation (Pilsner et al. 2007, 2012). These epigenetic alterations may be responsible for the toxic effects of arsenic, but the mechanisms that mediate these epigenetic alterations remain unknown (Marsit 2015).

The Nrf2/Keap1 signaling pathway is one detoxification mechanism employed by many organisms (Itoh, Ishii, et al. 1999). Regulation of gene expression via the Nrf2/Keap1 pathway provides a mechanism for both (1) short-term response to oxidative stress and xenobiotic factors (Itoh, Ishii, et al. 1999) and (2) regulation of normal development and other cellular processes (Deng and Kerppola 2013). Although the short-term xenobiotic response pathway is well understood, the longer-term effects of xenobiotics on development at the molecular level remain unknown. Elucidating the mechanisms by which Nrf2/Keap1 signaling regulates normal development is the first step towards understanding the mechanisms by which xenobiotics affect development. Recently, it was found that *Drosophila* Nrf2 and Keap1 (CncC and dKeap1) regulate normal development by regulating ecdysone biosynthesis and response (Deng and Kerppola 2013). The goal of this research was to elucidate other mechanisms by which Nrf2 and Keap1 might regulate normal development. Since epigenetic regulation is important in development, I investigated whether Nrf2 and Keap1 regulate chromatin structure.

Understanding the developmental and epigenetic roles of the Nrf2/Keap1 pathway can have several important implications. First, regulating epigenetics may represent another mechanism by which the Nrf2/Keap1 pathway can regulate development. Second, since Nrf2 and Keap1 regulate toxin response, epigenetics, and normal development the Nrf2/Keap1 pathway may mediate the effects of toxins on epigenetic status and/or the effects of toxins on development. Finally, elucidating the functions of Nrf2 and Keap1 in normal development can further our understanding of the complex role of Nrf2/Keap1 in human disease and potentially lead to the development of therapies that target these genes in the treatments of diseases and developmental disorders.

Response to Xenobiotic Factors

Nrf2 is a transcription factor that activates the transcription of genes encoding detoxification and antioxidant genes (which encode many cytochrome P450 enzymes) in response to xenobiotic compounds and oxidative stress, respectively. Under non-stress conditions (**Figure 1A**), cytoplasmic Nrf2 is bound by Keap1, which

Nrf2

- Nuclear factor erythroid 2-related factor 2
- bZIP family transcription factor
- bZIP domain is the DNA binding domain
- 605 amino acids
- Localized to 2nd chromosome, in mice
- ETGE and DLG domains (in Nrf2) are the binding sites for Keap1

targets Nrf2 for ubiquitination and subsequent degradation (Furukawa and Xiong 2005). This degradation of Nrf2 prohibits Nrf2 from accumulating in the nucleus, thereby prohibiting Nrf2 from activating xenobiotic response genes. When xenobiotic factors or oxidative stress is present (**Figure 1B**), the Keap1-Nrf2 interaction is disrupted, thereby allowing Nrf2 to enter the nucleus and activate xenobiotic response genes (Slocum and Kensler 2011). Nrf2 knockout mice are viable but they are more sensitive to chemical carcinogens and are more susceptible to diseases caused by (or aggravated by) oxidative stress (e.g. cancer, inflammatory diseases, and neurodegenerative diseases such as Parkinson's disease) (Burton, Kensler, and Guilarte 2006; Hayes and McMahon 2009; Osburn and Kensler 2008).

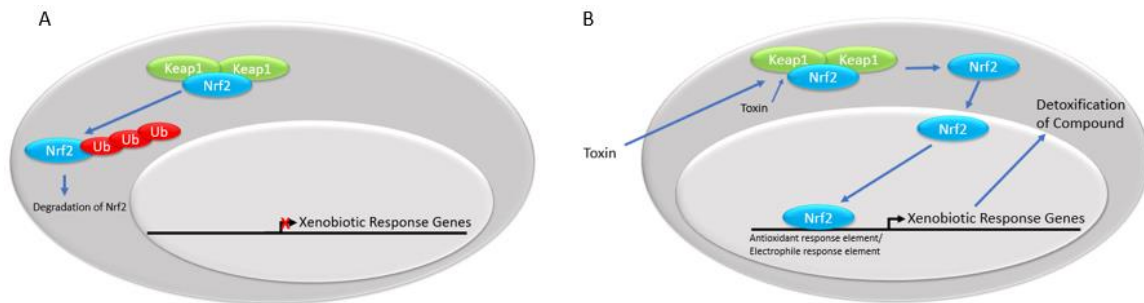


Figure 1. Xenobiotic Response. (A) Under non-stress conditions, Keap1 binds to Nrf2 in the cytoplasm and targets it for ubiquitination and subsequent degradation. This inhibits Nrf2 from activating expression of xenobiotic response genes. (B) Xenobiotic factors, however, disrupt the binding of Keap1 to Nrf2. This frees Nrf2 to move to the nucleus and activate transcription of xenobiotic response genes.

CncC and dKeap1 are the Drosophila homologs of Nrf2 and Keap1, respectively

CncC is the *Drosophila* homolog of Nrf2 and they are largely conserved at both the molecular and functional level. Both contain a bZIP domain for DNA binding and the N-terminal ETGE and DLG motifs for Keap1 binding (Sykiotis and Bohmann 2008). They also have conserved functions in xenobiotic response. However, while Nrf2 knockout mice are viable, CncC knockout flies die as larvae. This may be because mammals have three Nrf family members (Nrf1, Nrf2, and Nrf3) and Nrf1 and Nrf3 can partially compensate for the loss of Nrf2. However, Nrf2 and Nrf3 cannot compensate for the loss of Nrf1 and Nrf1 knockout mice die early in development. In *Drosophila*,

CncC performs both the functions of Nrf2 (antioxidant response) and Nrf1 (regulation of proteasomal activity) (Pitoniak and Bohmann 2015). Like Nrf2, CncC functions in xenobiotic and oxidative stress

<p><u>CncC</u></p> <ul style="list-style-type: none"> • <i>cap 'n' collar</i>, isoform C • bZIP family transcription factor • 1383 amino acids • 101 kDa • Localized to 3rd chromosome, region 94E in <i>Drosophila</i> (Mohler et al. 1991) <p><u>dKeap1</u></p> <ul style="list-style-type: none"> • <i>Drosophila</i> Kelch-like ECH-Associated Protein 1 • Kelch family protein (Itoh, Wakabayashi, et al. 1999) • 776 amino acids • 87 kDa • Localized to 3rd chromosome • Domains: <ul style="list-style-type: none"> ○ CTD – required for dKeap1 binding to chromatin ○ BTB Domain – CuI3 binding/Dimerization ○ DC/Kelch Domain – CncC binding

response and is regulated by dKeap1 (the *Drosophila* homolog of Keap1). Mammalian Keap1 and *Drosophila* Keap1 both contain a BTB dimerization domain as well a Kelch domain that functions as an Nrf2/CncC binding site (Sykiotis and Bohmann 2008). Both dKeap1 knockdown and exposure of flies to xenobiotic compounds will increase the activity of CncC, thereby increasing the transcription of xenobiotic response genes. Knockout of Keap1 in mice and knockout of dKeap1 in *Drosophila* both result in an early lethal phenotype – Keap1 knockout mice die within 3 weeks of birth and dKeap1 knockout *Drosophila* die as larvae.

Regulation of Development by CncC and dKeap1

Nrf2 and Keap1 have also been found to play important roles in the regulation of development in mice and *Drosophila*. In mice, Nrf2 and Keap1 regulate developmental genes involved in adipogenesis (Kim et al. 2018) as well as genes involved in regulating neuronal stem cell differentiation (Khacho et al. 2016). In *Drosophila*, CncC and dKeap1 play an important role in metamorphosis (Deng and Kerppola 2013). The *Drosophila* life cycle involves a 1-day embryo stage followed by three larval stages, during which the larvae grow. The first two larval stages (1st instar and 2nd instar) each last approximately 1 day while the 3rd instar larval stage lasts approximately 2-3 days. The larvae then enter the pupa stage which lasts for 3.5 – 4.5 days before the fly ecloses as an adult (Ong et al. 2015). Metamorphosis is regulated by several hormones including ecdysone (the active form of ecdysone is 20-hydroxyecdysone, 20E) and Juvenile hormone (JH). Juvenile hormone represses metamorphosis, whereas 20E promotes molting. When both JH and 20E are present, the insect will molt to the next larval instar stage. When only 20E is present, the insect will undergo metamorphosis to the adult stage. While dKeap1 acts antagonistically on CncC to inhibit transcription of xenobiotic response genes, dKeap1 acts in concert with CncC to promote metamorphosis. To promote metamorphosis, CncC and dKeap1 activate expression of juvenile hormone epoxide hydrolases, which degrade juvenile hormone in the salivary glands (Sun et al. 2017). Within the prothoracic gland, CncC and dKeap1 bind

cooperatively to the chromatin to activate ecdysone biosynthetic genes (Deng and Kerppola 2013; **Figure 2**). Once ecdysone has been synthesized, it is secreted to the hemolymph (the insect equivalent of blood) and transported to the salivary glands and other peripheral tissues where it is converted to 20-hydroxyecdysone (20E) (Ono 2014). Within the salivary glands, 20E enters the nucleus, binds to its receptor EcR, and activates the expression of ecdysone response genes. CncC and dKeap1 also localize to ecdysone puffs and activate ecdysone-response genes (Deng and Kerppola 2013).

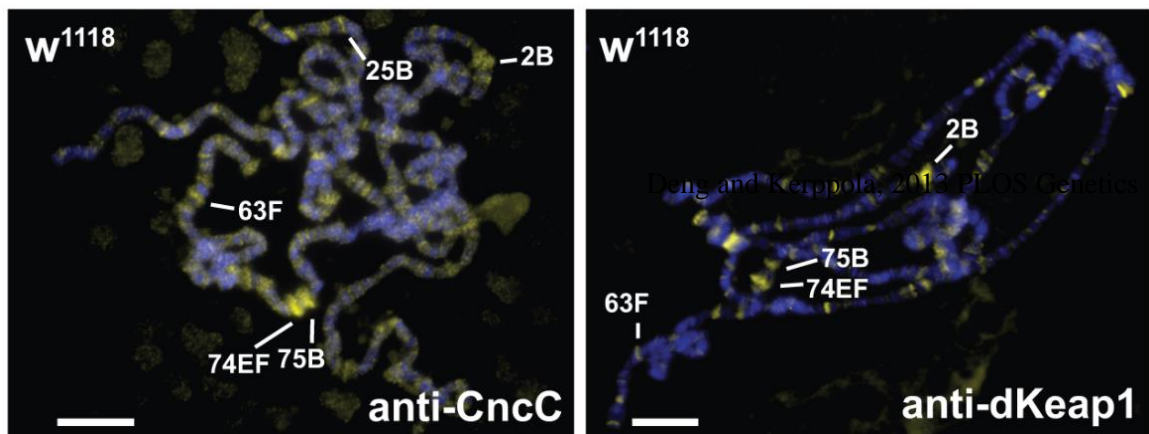


Figure 2. CncC and dKeap1 bind to chromatin at ecdysone-regulated early puffs. In wild type larval salivary glands, CncC and dKeap1 (yellow) bind to many loci on polytene chromosomes (blue). These loci include both early ecdysone-regulated puffs (labeled in white) as well as non-puff regions. The scale bar is 10µm. This figure was originally published in (Deng and Kerppola 2013).

Research goals and hypotheses

Given the importance of epigenetics in development (Boland, Nazor, and Loring 2014) and the evidence that CncC and dKeap1 bind to chromatin (Deng and Kerppola 2013, 2014), the goal of this research was to investigate whether epigenetics may be a novel mechanism by which CncC and dKeap1 may regulate development. Specifically, this research aimed to determine whether CncC and dKeap1 regulate chromatin structure. Previous studies showed that CncC and dKeap1 localize to many decondensed chromatin loci (Deng and Kerppola 2013). However, we did not know whether CncC and/or dKeap1 promoted the opening and/or maintenance of open chromatin

structure or if these proteins just bound to chromatin regions once they were de-condensed. Three preliminary studies indicated that CncC and dKeap1 may play a role in chromatin de-condensation. First, CncC overexpression resulted in the de-condensation of the chromatin in telomere regions. Second, somatic knockout of dKeap1 in the salivary glands resulted in cells with more condensed polytene chromosomes compared to control cells. Third, the polytene chromosomes of dKeap1- Δ CTD truncation flies (Δ CTD is required for dKeap1 to bind to chromatin) had more condensed arms (all three of these are based on unpublished data from the Deng Lab). Two additional studies suggested that it is likely that CncC and dKeap1 may act cooperatively to regulate developmental genes. First, both CncC and dKeap1 bind to and activate ecdysone response genes (Deng and Kerppola 2013). Second, for some xenobiotic compounds, dKeap1-CncC complexes are necessary to activate transcription of response genes (Deng and Kerppola 2014). Thus, we hypothesized that dKeap1 and CncC function cooperatively to open the chromatin.

Chapter 2: Regulation of Position Effect Variegation at Pericentric Heterochromatin by *Drosophila* Keap1-Nrf2 Xenobiotic Response Factors

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Introduction

Epigenetic modifications are thought to be important for mediating long-term developmental adaptations to constitutive changes in the environment. Environmental epigenetic studies have revealed broad influences of environmental toxins on human epigenomes (Marsit 2015).

However, the molecular mechanisms that mediate these influences are less understood.

Elucidating the roles of stress response factors in epigenetic regulation will provide insights into the mechanisms that mediate the effects of environmental stimuli on development and human health.

Keap1-Nrf2 stress response signaling is an essential pathway that protects cells from both endogenous reactive oxygen species and foreign toxic chemicals (xenobiotics) (Kensler, Wakabayashi, and Biswal 2007). Nrf2 (NF-E2-Related Factor 2) is a bZIP family transcription factor that can bind to and activate a cassette of genes that encode antioxidant and detoxifying enzymes (Malhotra et al. 2010; Zhang 2006). Keap1 (kelch-like ECH-associated protein 1) is a Kelch family protein that can interact with Nrf2 in the cytoplasm and induce Nrf2 ubiquitination and degradation (Itoh et al. 1999; McMahon et al. 2006). Selective modifications of cysteine residues on Keap1 by oxidative and xenobiotic compounds disrupt the Keap1-Nrf2 interaction, resulting in the nuclear accumulation of Nrf2 and the activation of detoxifying genes (Egler et al. 2005). Mis-regulations of Keap1 and Nrf2 are associated with many diseases, including

cancer, neurodegeneration, and cardiovascular dysfunctions (Sykiotis and Bohmann 2010; Taguchi, Motohashi, and Yamamoto 2011).

Recent studies in mice and *Drosophila* have revealed that Keap1-Nrf2 can regulate developmental genes and programs independent of oxidative/xenobiotic responses. *Drosophila* dKeap1 and CncC (cap-n-collar C) proteins (the homologs of Keap1 and Nrf2, respectively) control metamorphosis through transcriptional regulation of ecdysone signaling (Deng and Kerppola 2013). In mice, Nrf2 binds to and activates genes involved in adipogenesis and lipid metabolism in specific tissues (Huang et al. 2010; Pi et al. 2010). Nrf2 can also promote cell proliferation through activation of genes that encode glucose metabolic enzymes (Mitsuishi et al. 2012). It is thought that the multiple developmental functions of Keap1 and Nrf2 account for at least some of their complicated roles in pathogenesises.

Our previous studies in *Drosophila* have indicated that the mechanism by which dKeap1 and CncC regulate developmental genes is different from the established mechanism according to which Keap1-Nrf2 controls detoxifying genes. Interestingly, dKeap1 can directly bind to chromatin and function as a transcriptional coactivator with CncC (Deng and Kerppola 2014). Both dKeap1 and CncC occupy ecdysone-response early puffs on polytene chromosomes and activate ecdysone-induced genes located at these puffs (Deng and Kerppola 2013). The specific localization of dKeap1 and CncC at the polytene chromosome puffs, which represent highly decondensed chromatin regions, indicate a potential role for dKeap1 and CncC in chromatin remodeling.

In this study, we employed position effect variegation (PEV) assays to examine the effects of *cncC* and *dKeap1* mutants on the PEV expression of alleles at euchromatin/heterochromatin borders. Surprisingly, both *cncC* and *dKeap1* loss-of-function mutations suppress the variegations of *w^{m4}* and *Sb^V* pericentric PEV alleles. Moreover, depletion of CncC or dKeap1 reduces the level of the heterochromatin marker H3K9me2. These results suggest that CncC and

dKeap1 might play positive roles in the establishment or maintenance of pericentric heterochromatin.

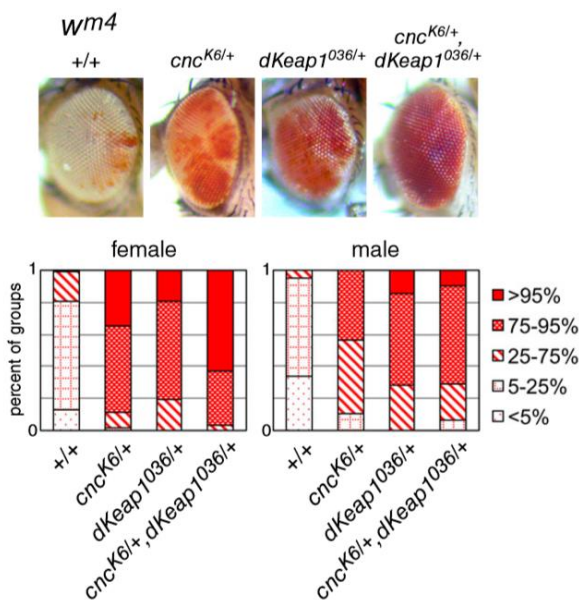
Results and Discussion

To determine the potential roles of CncC and dKeap1 in chromatin remodeling, we examined the effects of their loss-of-function mutations on the PEV of the w^{m4} allele. The *In(1)w^{m4}* X chromosome contains an inversion that relocates the euchromatic *white* gene to the pericentric region (Schultz 1936). Spreading of pericentric heterochromatin to the w^{m4} allele results in somatic variegation of *white* gene expression, causing white and red ommatidia in the compound eyes of adult flies (Elgin and Reuter 2013). Genetic studies of this PEV allele suggest that depletion of heterochromatin factors can reduce the spreading of heterochromatin, therefore suppressing the variegation of w^{m4} expression (Su(var)) and increasing red ommatidia. In contrast, depletion of euchromatic factors is predicted to increase the spreading of heterochromatin at the w^{m4} locus, thus enhancing the variegation (E(var)) and increasing white ommatidia (Elgin and Reuter 2013). The loss-of-function alleles of *cncC* and *dKeap1* are homozygous lethal at an early larval stage (Sykiotis and Bohmann 2008; Veraksa et al. 2000). We performed the PEV assay by introducing heterozygous mutations of *cncC^{k6}* and *dKeap1⁰³⁶* into the w^{m4} background. Surprisingly, both *cnc^{k6/+}* and *dKeap1^{036/+}* heterozygous mutants had significantly suppressed variegation of w^{m4} , represented by the observation of more flies containing a higher percentage of red ommatidia (**Figure 3**). The Su(var) effects were observed in both male and female populations, although females showed stronger suppression. These results suggest that reduction of either CncC or dKeap1 causes a decrease of heterochromatic silencing at the w^{m4} locus.

Given that Keap1/dKeap1 act as Nrf2/CncC inhibitors (Sykiotis and Bohmann 2008; Taguchi et al. 2011), we tested whether reducing dKeap1 can antagonize the PEV effect of CncC depletion by introducing a *cnc^{k6/+},dKeap1^{036/+}* double heterozygous mutant into the w^{m4} background.

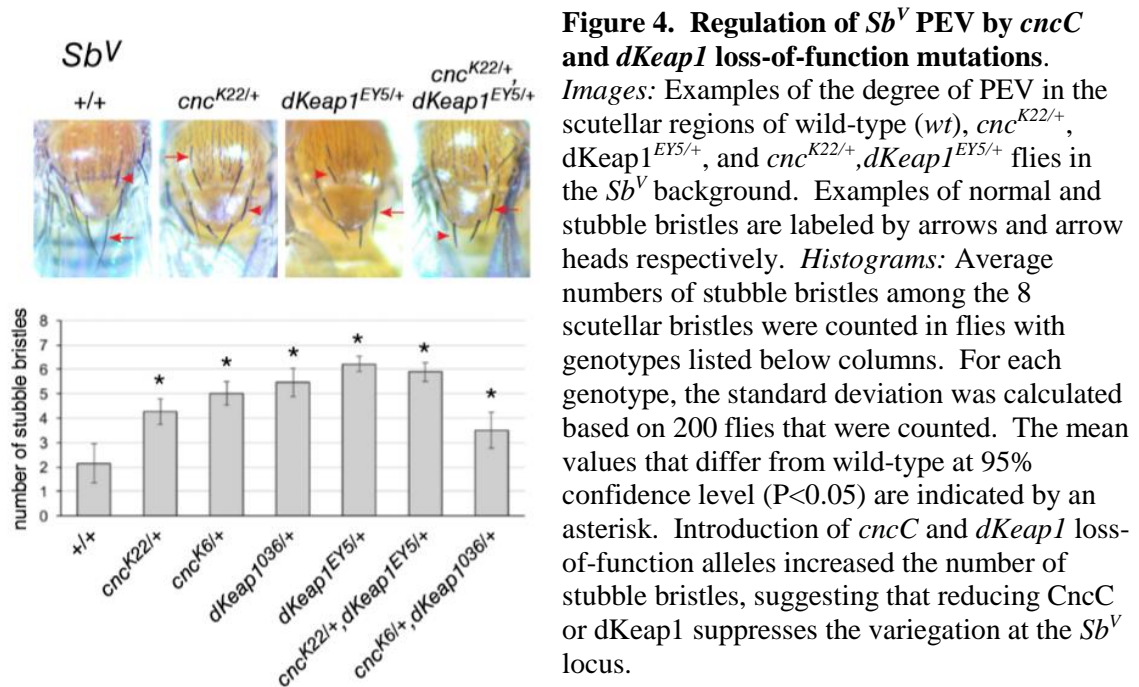
Combinatory reduction of dKeap1 and CncC enhanced PEV suppression more than CncC depletion alone in both females and males, suggesting that dKeap1 and CncC have an additive effect when regulating w^{m4} PEV (**Figure 3**).

To exclude the possibility that unknown mutations in the $cncC^{k6}$ and $dKeap1^{036}$ flies caused the observed PEV effects, we examined other $cncC$ and $dKeap1$ mutations that are generated separately in different genetic backgrounds. Fly lines containing $cncC^{K22}$ and $dKeap1^{EY5}$ alleles have red eyes and cannot be used for w^{m4} PEV assay. Instead, we tested the effects of the $cncC^{K22}$ and $dKeap1^{EY5}$ alleles on the PEV of the Sb^V locus (**Figure 4**). The dominant Sb gene gives rise to shorter and thicker bristles. The $T(2:3)Sb^V$ rearrangement results in pericentric localization of Sb , leading to the partial silencing of Sb by heterochromatin spreading and hence, the restoration of wild-type size of some scutellar bristles (Sinclair, Lloyd, and Grigliatti 1989). We genetically combined Sb^V with $cncC^{K22}$, $cncC^{K6}$, $dKeap1^{EY5}$, or $dKeap1^{036}$ heterozygous mutations as well as relevant $cncC/dKeap1$ double heterozygous mutants. All of the mutants had an increased number of short bristles in the scutellar region, indicating significant suppression of Sb^V PEV (**Figure 4**). These results further support the Su(var) effects of $cncC$ and $dKeap1$ loss-of-function mutations on PEV alleles at the pericentric heterochromatic loci.



To determine if CncC and dKeap1 regulate PEV at other heterochromatic loci, we examined the effects of the *cnc*^{K6/+} and *dKeap1*^{036/+} mutations and the *cnc*^{K6/+}, *dKeap1*^{036/+} double mutant on a PEV locus that is not in the pericentric region. The 39C-5 line was generated through the insertion of a P-element containing a *hsp70-white* reporter gene into the telomeric area of chromosome 2L, which causes the variegation of *white* reporter expression (Wallrath and Elgin 1995). Introduction of *cnc*^{K6/+} or *dKeap1*^{036/+} caused no significant effect on 39C-5 PEV. However, moderate suppression of 39C-5 PEV was observed in the females of the *cnc*^{K6/+}, *dKeap1*^{036/+} double mutant. We speculate that CncC and dKeap1 likely control PEV at telomeric heterochromatin, but the effects were too weak to be detected in the heterozygous mutations, and only the additive effects of the *cncC/dKeap1* double mutant made the PEV effect visible in our experiment. The effects of *cncC* and *dKeap1* mutations on PEV at telomeric and intercalary heterochromatin regions remain to be fully characterized.

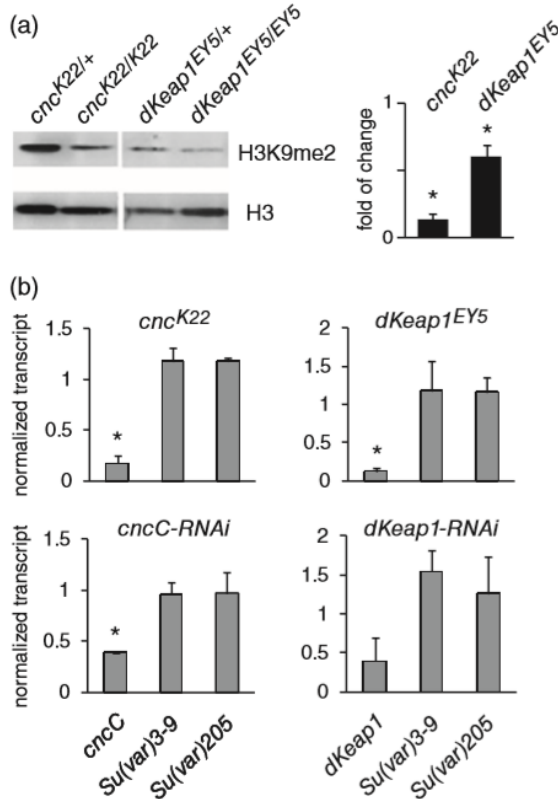
The observation that *cncC* and *dKeap1* loss-of-function mutations act as suppressors of PEV at pericentric loci suggests that CncC and dKeap1 could facilitate heterochromatin formation. To test this hypothesis, we examined the effect of CncC or dKeap1 depletion on the level of the



major heterochromatin marker histone H3K9me2 (Ebert et al. 2004). Using western blotting, we detected and quantified the levels of H3K9me2 in embryos of *cnc*^{K22} or *dKeap1*^{EY5} homozygous null mutations and in the heterozygous embryos used as controls (**Figure 5A**). The levels of H3K9me2 significantly decreased in both *cncC* and *dKeap1* null embryos, suggesting that both CncC and dKeap1 are required for the maintenance of a normal H3K9me2 level.

Su(var)3-9 histone methyltransferase and Heterochromatin Protein 1 (HP1a; encoded by the *Su(var)205* gene) are the key factors that promote heterochromatin formation in *Drosophila* (James and Elgin 1986; Schotta et al. 2002; Tschiersch et al. 1994). Given that CncC targets numerous genes (Misra et al. 2011), it is possible that CncC and dKeap1 regulate heterochromatin through controlling Su(var)3-9 or HP1a at the transcriptional level. To assess this possibility, we examined expression levels of the *Su(var)3-9* and *Su(var)205* genes when CncC and dKeap1 were knocked out in embryos or were RNAi-knocked down in salivary glands (**Figure 5B**). No significant changes in *Su(var)3-9* or *Su(var)205* transcripts were detected in any of the *cncC* or *dKeap1* mutants (**Figure 5B**). Therefore, CncC and dKeap1 likely regulate heterochromatin formation at the post-transcriptional level.

Results of this study revealed a novel function of the Keap1-Nrf2 family proteins in the regulation of heterochromatin-induced gene silencing. Interestingly, both dKeap1 and CncC depletions suppress PEV at pericentric heterochromatin (**Figure 3, 4**), indicating a cooperative



role of dKeap1 and CncC in the regulation of heterochromatic silencing. It has been well established that Keap1 acts as an inhibitor of Nrf2 in the transcription of antioxidant/detoxifying genes (Taguchi et al. 2011). Studies in *Drosophila* have confirmed that dKeap1 counteracts CncC in the regulation of lifespan and xenobiotic responses (Deng and Kerppola 2014; Sykiotis and Bohmann 2008). On the other hand, we have revealed that dKeap1 can bind chromatin and activate ecdysone biosynthetic and response

Figure 5. Regulation of heterochromatin level by CncC and dKeap1. *A. Effects of cncC and dKeap1 null mutations on H3K9me2 heterochromatin marker.* Histone H3K9me2 and histone H3 (loading control) were detected in protein extractions from embryos with the genotypes labeled above using immunoblotting. The levels of H3K9me2, represented by the intensity of the western bands, were measured and normalized to H3 levels. The histogram shows the average fold of change of the H3K9me2 levels in *cnc^{K22}* and *dKeap1^{EY5}* homozygous embryos relative to those in heterozygous embryos. Loss of CncC or dKeap1 results in ~80% or ~40% decrease of H3K9me2 level, respectively. The error bars indicate s.d. from three experiments using different biological replicates (*, P<0.05). *B. Effects of CncC and dKeap1 depletions on transcripts.* RT-qPCR assays were used to measure transcript levels of the genes labeled below in the embryos of *cnc^{K22}* and *dKeap1^{EY5}* null mutations (upper panels) or in the salivary glands that express shRNA targeting *cncC* or *dKeap1* using *Sgs3-GAL4* (lower panels). The corresponding heterozygous embryos and salivary glands containing the GAL4 driver only were used as controls. All transcript levels were normalized with the levels of *Rp49* transcripts. Histograms show the fold of change of transcript levels in the mutants relative to the transcript levels of the same gene in the controls. Error bars represent the s.d. from two experiments using different biological replicates (*, P<0.05). In all the mutants, *cncC* or *dKeap1* transcripts were efficiently abolished, while the transcript levels of *Su(var)3-9* and *Su(var)205* (encoding HP1a) were not significantly altered.

genes in cooperation with CncC), and that dKeap1-CncC form complexes on chromatin and co-activate *Jheh* and *dKeap1* genes (Deng and Kerppola 2014). The discovery that dKeap1 and CncC co-regulate heterochromatic silencing provides additional evidence in support of the model that the chromatin-bound dKeap1 cooperates with CncC when regulating chromatin structure and developmental genes, while the cytosolic dKeap1 inhibits CncC when controlling antioxidant and detoxifying genes (**Figure 6**).

The current finding that sufficient dKeap1 and CncC proteins are required for the maintenance of heterochromatic silencing and the H3K9me2 heterochromatin marker contrasts with our previous hypothesis that dKeap1 and CncC facilitate euchromatin structure and transcriptional activation. Further studies are needed to determine the roles of dKeap1 and CncC in euchromatin architecture, especially at specific genomic loci such as ecdysone-induced puffs. We now hypothesize that dKeap1 and CncC may regulate both euchromatin and heterochromatin, probably through interactions with different chromatin modifiers (**Figure 6**). Such multiple functions in chromatin remodeling have been identified for some chromatin modifiers. The essential heterochromatin component HP1a can occupy several euchromatic loci and activate transcription (Cryderman et al. 2005; Piacentini et al. 2003, 2009). JIL-1, a histone H3S10 kinase that mainly occupies and maintains euchromatin regions, can also function in pericentric heterochromatin through the creation of H3S10phK9me2 mark and the interaction with Su(var)3-9 (Boeke et al. 2010; Wang et al. 2014). It will be interesting to elucidate whether dKeap1 and CncC directly interact with these chromatin modifiers. It also remains to be determined whether mammalian Keap1-Nrf2 can regulate heterochromatic silencing similarly.

Taken together, our study provides preliminary evidence in support of a novel function of the Keap1-Nrf2 oxidative/xenobiotic response factors in heterochromatin architecture. Full characterization of the epigenetic functions of the Keap1/Nrf2 family proteins will help elucidate

the molecular mechanisms that mediate influences of environmental toxins on development and the epigenome, as well as the full range of roles of Keap1 and Nrf2 in human diseases.

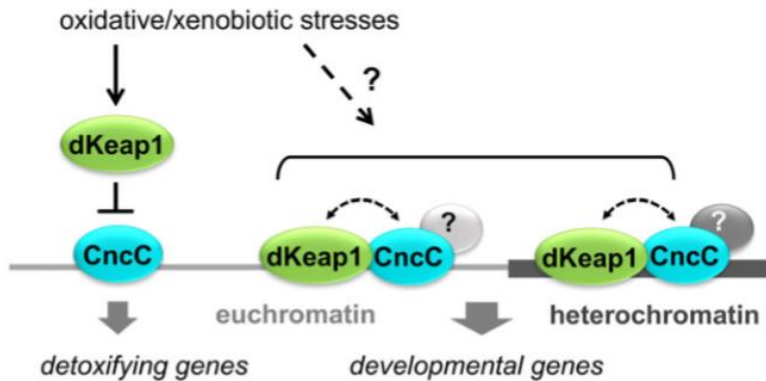


Figure 6. Model for multiple dKeap1-CncC functions on chromatin. The dKeap1-CncC complex regulates different sets of genes using distinct mechanisms: dKeap1-CncC interactions in the cytoplasm or nucleoplasm suppress CncC activity and the activation of antioxidant/detoxifying genes (left). In addition, dKeap1 can bind to chromatin and cooperate with CncC when regulating genes that are independent of stress-responses, such as developmental genes (middle, right), probably through a mechanism of epigenetic chromatin remodeling at least at some chromatic loci. The potential function of dKeap1-CncC in heterochromatin architecture was supported by the current study (right).

Materials and Methods

Drosophila stocks

All fly stocks were maintained using standard protocols. Strains containing *dKeap1*⁰³⁶, *dKeap1*^{EY5}, *cnc*^{K22}, *UAS-dKeap1-RNAi*, and *UAS-cncC-RNAi* were provided by Dirk Bohmann (Sykiotis and Bohmann 2008). Strains containing *cnc*^{K6}, *In(1)w^{m4}*, and *39C-5* were obtained from Osamu Shimmi, Kristen Johansen, and Lori Wallrath, respectively. Salivary gland expression of RNAi was obtained using the *Sgs3-GAL4* driver. *T(2:3)Sb^V* and *Sgs3-GAL4* were from the Bloomington Stock Center. All studies were conducted at 25°C with the exception of larvae carrying the RNAi transgenes, which were maintained at 29°C to improve the efficiency of CncC and dKeap1 depletions. All *cncC* and *dKeap1* mutations were combined with *TM6,Tb,Hu,e* or

TM6, Tb, Sb, Hu, e, Dfd-YFP balancers. Appropriate progenies were selected based on the *Hu* marker in adults or *Dfd-YFP* fluorescence marker in embryos.

PEV assays

PEV assays were performed as previously described (Deng et al. 2010; Gandhi et al. 2015). Different mutations were introduced into *In(1)w^{m4}*, *T(2:3)Sb^V*, or *39C-5* backgrounds using standard genetic crossing. Newly eclosed adults were collected and aged for 4 days. For *w^{m4}* assays, the number of flies in each of 5 different classes were counted based on the percentage of red color in the compound eyes. For *Sb^V* assays, the number of stubble bristles among 8 major scutellar bristles in each fly were counted, and the significance of the differences in average numbers was evaluated using the t-test. Eyes and scutellar regions from representative individuals were photographed using an AmScope SM1-BX stereomicroscope with a digital camera.

Immunoblotting

Since the null mutations of *cncC* and *dKeap1* are homozygous lethal at early 1st instar larval stage, late (12-16 hours) embryos were used for protein extraction and immunoblotting assays (Deng and Kerppola 2013). Homozygous and heterozygous embryos were collected using apple juice plates and sorted based on the *Dfd-YFP* marker under a Leica MZ10 F fluorescence stereomicroscope. Embryos (~15 in each sample) were homogenized in 50 μ l of ice-cold Buffer (20 mM Tris-HCl pH8.0, 0.2% NP-40, 0.2% Triton X-100, 150 mM NaCl, 5 mM EDTA, 1 mM EGTA, 2 mM NaVO₃, 1 mM PMSF, and 1.5 μ g/ml aprotinin) and centrifuged at 10,000 g for 3 minutes to remove precipitates. The supernatant samples were resolved using a NuPAGE 4-12% Bis-Tris gel (Invitrogen). The proteins were transferred to a nitrocellulose membrane (Bio-Rad) and probed using H3K9me2 mouse monoclonal antibody (1:400; Abcam) or H3 rabbit polyclonal antibody (1:3000; Proteintech), followed by HRP-conjugated secondary antibodies (1:3000, Bio-

Rad). Intensities of western bands were measured using ImageJ software. The levels of H3K9me2 were calculated by normalizing the intensities of the H3K9me2 bands with those of the H3 bands. Three replicates of this experiment were performed using independent biological samples. Statistical tests were conducted using a one-way ANOVA.

Transcript quantitation

Around 50 late stage (12-16 hours) embryos that were collected using apple juice plates and sorted based on the *Dfd-YFP* marker or 10 pairs of salivary glands that were dissected from early wandering 3rd instar larvae were used for each mRNA extraction using the RNeasy kit (Qiagen). Isolated mRNA was quantified using a NanoDrop Spectrophotometer and the purity was evaluated based on A260/A280. Next, mRNA was treated with RQ1 RNase-Free DNase (Promega) and reverse transcribed using the iScript™ cDNA Synthesis kit (Bio-Rad). Real-time PCR was performed using the GoTaq qPCR kit (Bio-Rad) in an Eppendorf Mastercycler. The relative transcript levels were calculated by assuming that they were proportional to 2^{-C_p} , and normalized by the levels of *Rp49* transcripts. Two replicates using separate biological samples were performed and statistical tests were conducted using a one-way ANOVA. Primer sequences producing intron-spanning amplicons were designed using Universal ProbeLibrary software (Roche) and are listed in Table S1.

Chapter 3: Loss of CncC and dKeap1 may reduce H3K9me2 levels on polytene chromosomes.

Histone H3K9 dimethylation (H3K9me2) is prevalent in pericentromeric heterochromatin in *Drosophila* (Deng et al. 2010). Given the results from the PEV assay (i.e. CncC and dKeap1 function as suppressors of variegation), I expected that knocking out CncC and/or dKeap1 would reduce the levels of H3K9me2. Before using a western blot to quantitatively measure H3K9me2 levels, I used immunofluorescence staining to test whether the loss of CncC and/or dKeap1 would reduce the levels of H3K9me2. Since complete knockout of dKeap1 or CncC in *Drosophila* results in larval lethality, I used Mosaic Analysis with a Repressible Cell Marker (i.e. MARCM) to produce mosaic flies in which to study dKeap1 and CncC function (Wu and Luo 2006). The MARCM method produces flies that have some somatic cells with wild type *dKeap1* and *cncC* and other cells with either *dKeap1* or *cncC* (or both) null mutations. Here, I will describe the MARCM method for producing dKeap1 knockout mosaic flies. The MARCM method consists of several components. First, flies with the genotypes in **Figure 7** were crossed.

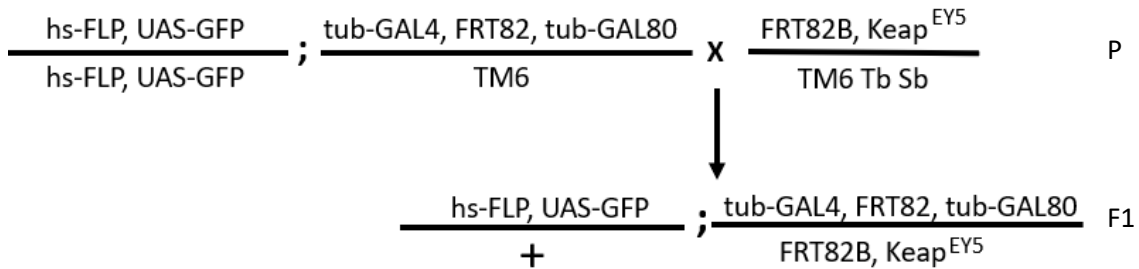


Figure 7. MARCM Cross. This cross was used to generate somatic mosaic flies in which to study dKeap1 function. Cells that express GFP have the Keap^{EY5} mutation. Abbreviations: hs = heat shock promoter, FLP = Flippase, FRT = Flippase Recombination Target Site, Keap^{EY5} – dKeap1 null mutation; for descriptions of UAS, GAL4, and GAL80 see Figure 8. This figure is modified from (Wu and Luo 2006).

The F1 generation contains a heat shock (hs) induced promoter that induces the expression of *flippase* (FLP). After S phase, FLP facilitates a crossover between the two FRT sites (flippase recombination target sites), resulting in two chromosomes with tub-GAL80 (an inhibitor of tub-

GAL4) and FRT and two chromosomes with tub-GAL4, FRT, and Keap^{EY5} (**Figure 9**). (The UAS-GAL4 expression system is described in **Figure 8**.) The chromosomes are divided between the two daughter cells such that both tub-GAL80 chromosomes end up in one cell and both Keap^{EY5} chromosomes end up in the other cell. Since the cells with the Keap^{EY5} do not have tub-GAL80, tub-GAL4 is not inhibited so tub-GAL4 can induce the expression of the marker gene GFP. This produces a mosaic insect where half of the cells are Keap1 null (i.e. they have the Keap^{EY5} mutation) and are recognized by GFP expression and half of the cells express Keap1 but do not express GFP (i.e. because tub-GAL80 inhibits tub-GAL4 so it cannot induce the expression of GFP).

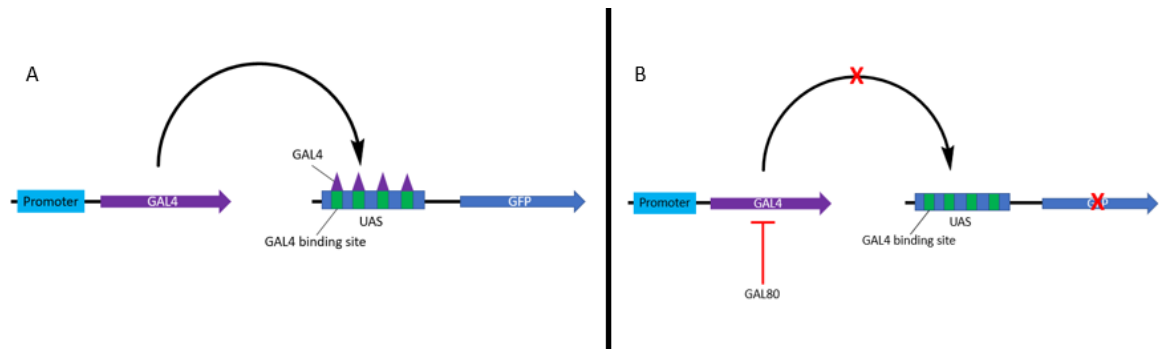


Figure 8. UAS-GAL4 Expression System. (A) A tissue specific promoter is used to drive the expression of GAL4. GAL4 then binds to UAS and drives the expression of the gene of interest (GFP in this case). (B) When GAL80 is expressed, it inhibits the expression of GAL4. Thus, the UAS promoter is not activated and GFP is not expressed. Part A of this figure was modified from (Phelps and Brand 1998).

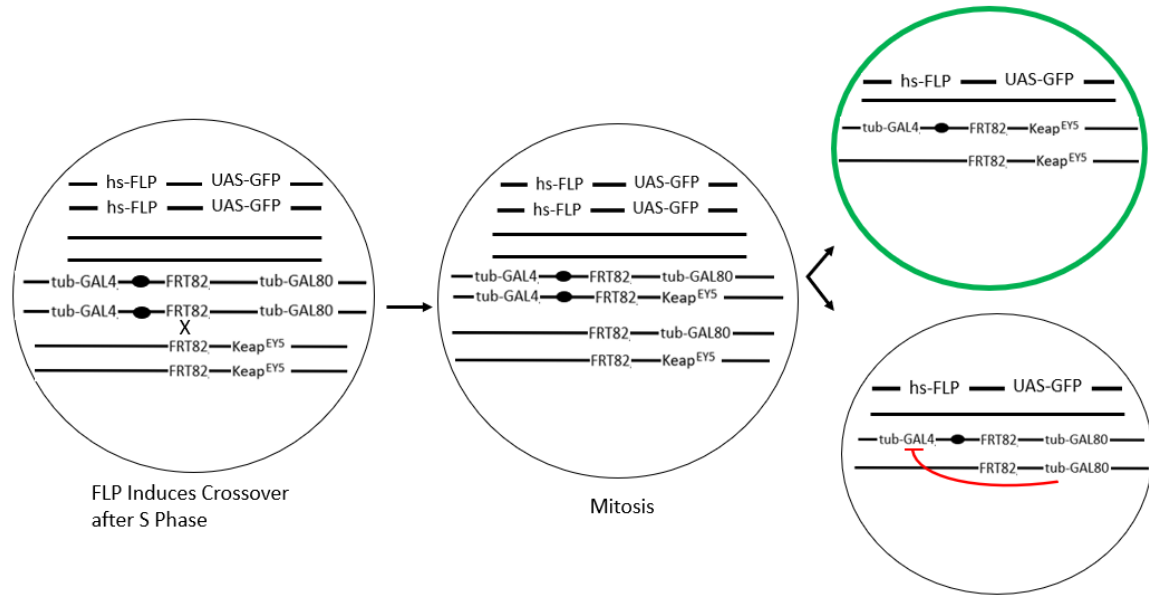


Figure 9. Mitotic generation of mosaic dKeap1 knockout flies. The cells in the flies produced from the cross in Figure 7 will undergo mitosis. During mitosis, FLP induces a crossover at the FRT sites. The cell then divides, and the chromosomes are divided between the two daughter cells. The daughter cell that does not express tub-GAL80 will be able to express tub-GAL4 which, in turn, will lead to expression of GFP (green circle). In the other daughter cell (black circle), tub-GAL80 will inhibit tub-GAL4 and the cell will not express GFP. This figure is modified from (Wu and Luo 2006).

Since the salivary glands cells stop mitotically dividing early in development, the F1 generation were heat-shocked as embryos. Once the flies reached 3rd instar, the salivary glands were dissected and probed with H3K9me2 mouse monoclonal antibody (1:200; Abcam, Cambridge, MA, USA) followed by a fluorescent goat anti-mouse secondary antibody (1:1000, ThermoFisher scientific, goat anti-mouse 594, A11032).

Materials and Methods

The fly stocks were all maintained according to standard protocols. The *hs-FLP*, *UAS-GFP*; *tub-GAL4*, *FRT82*, *tub-GAL80/TM6* stocks and the *FRT82B*, *Keap1^{EY5}/TM6* *Tb Sb Hu e Dfd-YFP* and the *FRT82B*, *Cnc^{K22}/TM6* *Tb Sb Hu e Dfd-YFP* stocks came from Dirk Bohmann's lab. Virgin females from *hs-FLP*, *UAS-GFP*; *tub-GAL4*, *FRT82*, *tub-GAL80/TM6* were crossed with males

from either *FRT82B*, *Keap1^{EY5}/TM6 Tb Sb Hu e Dfd-YFP* or *FRT82B*, *Cnc^{K22}/TM6 Tb Sb Hu e Dfd-YFP* and the F1 embryos with the genotype *hs-FLP*, *UAS-GFP/+*; *tub-GAL4*, *FRT82*, *tub-GAL80/FRT82B*, *Keap1^{EY5}* or *hs-FLP*, *UAS-GFP/+*; *tub-GAL4*, *FRT82*, *tub-GAL80/FRT82B*, *Cnc^{K22}* (identified by not having the Tb marker) were collected with apple juice plates and heat-shocked at 37°C as 8-hour embryos for 30 minutes. The embryos were then allowed to develop to 3rd instar larvae and then the salivary glands were dissected and fixed with para-formaldehyde (3.8% para-formaldehyde in 1X PBS and 1X Triton X-100) for 2 minutes and then washed in PBS-0.2% Triton X-100. The salivary glands were then blocked with 1% NGS for 1 hour at room temperature, treated with H3K9me2 antibody (2.5 µg/mL, ab1220, Abcam) at 4°C overnight, washed with 0.2% PBST and then treated with Alexa Fluor 594 goat anti-mouse secondary antibody (Invitrogen) for 2 hours at room temperature. The salivary glands were washed with 1X PBS, stained with Hoechst stain (1 µg/mL, Hoechst 33342, Cell Signaling) for 10 minutes, and

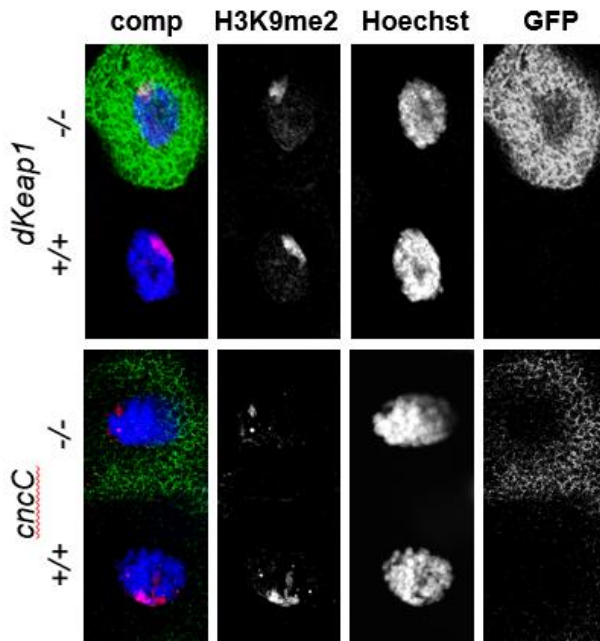


Figure 10: Knockout of *cncC* or *dKeap1* may reduce heterochromatin levels on polytene chromosomes. *cncC* and *dKeap1* knockout cells (green; -/-) appear to have lower levels of H3K9me2 (pink) than wild type cells (+/+).

then mounted in 90% glycerol containing 0.5% n-propyl gallate. The cells were visualized under a Zeiss LSM710 confocal microscope.

Results and Discussion: If CncC and dKeap1 are involved in heterochromatin maintenance or formation, then knocking out either of these genes should result in more open chromatin (i.e. loss of heterochromatin), as represented by a reduction in heterochromatin markers (e.g. H3K9me2) at the pericentric regions of the polytene chromosomes. It was difficult to

quantify the immunofluorescence intensity, but the null mutations *cncC^{K6}* and *dKeap1^{EY5}* visually appeared to result in minimal reduction of heterochromatin markers (**Figure 10**). There are a few potential reasons why there was not a greater reduction of H3K9me2. First, staining the salivary glands as whole mounts does not provide as clear levels of chromatin staining as would a chromosome squash. It is possible that the levels of H3K9me2 staining would have been more clearly reduced if a chromosome squash had been done, staining with anti-dKeap1 and/or anti-CncC as well as heterochromatin markers and using as controls the chromosomes to which anti-Keap1 and anti-CncC antibodies are bound. Second, the H3K9me2 levels may be tissue and developmental stage specific. The immunofluorescent experiment was done using salivary glands from 3rd instar larvae whereas the PEV assays and western blots were done using adult flies and late embryos, respectively. Finally, it is possible that the results from the dKeap1 knockout may have been partially masked by CncC overexpression (since dKeap1 would not be inhibiting it in the cytoplasm). Two approaches could be used to eliminate this problem. First, a dKeap1- Δ CTD mutant could be used instead of a dKeap1 knockout. The CTD domain is required for dKeap1 binding to chromatin, but it is not necessary for CncC inhibition (dKeap1 binds to CncC via its Kelch domain). Thus, a dKeap1- Δ CTD mutant will still be able to inhibit CncC, but it will not be able to bind to the chromatin. A second approach that could be used is to knockout both dKeap1 and CncC simultaneously via the MARCM method. This will eliminate the dKeap1-CncC complex and allow us to visualize the chromosomal effects of this complex.

Future studies using the MARCM method

The MARCM analysis is a powerful tool for investigating the effects of CncC and/or dKeap1 knockout on chromatin structure because it not only allows us to (1) circumvent the early lethal phenotype of larvae, it also (2) allows the knockout cells and control cells to be compared directly in the same animal, thereby avoiding any unknown differences between larvae. Thus, the MARCM method will allow us to investigate the functions of CncC and dKeap1 in many

different programs in different tissues at different developmental stages. For example, MARCM will be used in future studies to (1) visualize the effects on chromatin structure when CncC and/or dKeap1 are knocked out and (2) observe binding loci of possible interacting partners in CncC/dKeap1 null and wild type cells. If chromatin modifiers are found that bind to the same loci as CncC and dKeap1, then bimolecular fluorescence complementation (BiFC) analysis and co-immunoprecipitation experiments can be used to further test for any physical interaction.

Chapter 4: Conclusions and Future Directions

This study supported the hypothesis that CncC and dKeap1 function cooperatively as suppressors of variegation at pericentromeric regions of chromosomes, likely acting at the post-transcriptional level. This was shown by three lines of evidence. First, results from PEV assays, using two separate reporter genes in two different genetic backgrounds with two different *cncC* and *dKeap1* mutations, indicated that CncC and dKeap1 function as suppressors of variegation (i.e. when *cncC* and/or *dKeap1* are mutated, the chromatin in the region of the reporter gene is less tightly compacted) at the pericentromeric regions. Second, results from the western blots showed that reduction of CncC or dKeap1 leads to reduction of H3K9 dimethylation (H3K9me₂; a pericentromeric heterochromatin marker). Finally, the RT-qPCR results showed that there was no significant change in the expression levels of *Su(var)3-9* and *Su(var)205*, which encode Su(var)3-9 and HP1 – two essential proteins for heterochromatin formation. This indicates that CncC and dKeap1 are probably not regulating heterochromatin by regulating the transcription of heterochromatin promoting genes, but rather, are regulating heterochromatin at the post-transcriptional level.

Future studies on the regulation of heterochromatin by CncC and dKeap1

The mechanism by which CncC and dKeap1 regulate chromatin structure remains unknown. To elucidate the mechanism by which CncC and dKeap1 regulate pericentromeric heterochromatin, an initial step is to identify chromatin modifiers that CncC and/or dKeap1 interact with.

Pericentromeric heterochromatin is established by the HP1/SU(VAR)3-9 complex. In the classic model, SU(VAR)3-9 methylates the chromatin at histone H3K9 (Demakova et al. 2007) and then HP1 binds to the methylated lysine residue and maintains heterochromatin structure (Zeng, Ball, and Yokomori 2010). Given the central role of the HP1/SU(VAR)3-9 complex in

heterochromatin establishment and maintenance, these proteins are reasonable candidate interaction partners of CncC and dKeap1.

Future studies investigating whether CncC and dKeap1 in regulate euchromatin

It is likely that CncC and dKeap1 also regulate euchromatin structure. CncC and dKeap1 are both known to bind to chromosomal puff regions (i.e. decondensed chromatin where transcription can occur) (Deng and Kerppola 2013). CncC overexpression results in a more open chromatin structure at the telomeres. Somatic knockout of dKeap1 via MARCM results in salivary gland knockout cells with more condensed polytene chromosomes compared to control cells. Larvae with the dKeap1- Δ CTD truncation (which prevents dKeap1 from binding to chromatin) had more condensed polytene chromosome arms. Thus, in the future, the roles of CncC and dKeap1 in regulating euchromatin structure should be investigated. Two initial experiments to test this would be generating somatic knockouts of dKeap1 and CncC and then measuring the levels of (1) heterochromatin markers, such as H3K27me3 and HP1, and (2) euchromatin markers, such as H3K4me3 and H3K9ac).

Broader impacts

Investigating the complex roles of Nrf2/CncC and Keap1/dKeap1 in development and xenobiotic response can have several implications. First, understanding the roles of Nrf2 and CncC can lead to a better understanding of how epigenetic status is affected by the environment. Several studies have found correlations between exposure to toxins (xenobiotics or oxidative stress) and epigenetic modification. Some of these environmental factors directly impact Nrf2 interaction with Keap1. Other environmental factors can epigenetically regulate Nrf2 expression (Kang and Hyun 2017). Still others, may act via Nrf2 to alter the epigenetic status of specific chromatin regions. Understanding how environmental factors can affect epigenetic status could help us predict the effects of these factors on the health of humans and other animals. Second, it can lead to a better

understanding of how drugs other xenobiotic factors affect development. For example, phenobarbital has two effects in *Drosophila* larval salivary glands. (1) Phenobarbital disrupts the interaction between dKeap1 and CncC in the cytoplasm, releasing CncC to bind to, and activate expression of, xenobiotic response genes. (2) Phenobarbital also induces dKeap1 and CncC to act cooperatively to increase expression of dKeap1 and juvenile hormone epoxide hydrolase (*Jheh*) genes. Since JHEH proteins degrade juvenile hormone and promote metamorphosis (Deng and Kerppola 2014), phenobarbital may disrupt developmental timing in *Drosophila*. Understanding how drugs affect development, could help us predict physiological effects before drugs are used. Finally, understanding the interaction between the xenobiotic response and developmental pathways can help us understand the complex role of Nrf2/Keap1 in human disease. For example, Nrf2 knockout has been associated with cancer and several neurological diseases (e.g. Alzheimer's disease and Parkinson's disease). However, Nrf2 knockdown and Nrf2 overexpression can both lead to tumorigenesis. One possible reason for this is that when Nrf2 is knocked down, the animal is more susceptible to carcinogens (since Nrf2 cannot activate xenobiotic response genes), but when Nrf2 is overexpressed it activates xenobiotic response genes which hinder cancer treatments by inhibiting anti-cancer drugs (Taguchi et al. 2011). Understanding the roles of Nrf2 and Keap1 could lead to the development of therapies that target Nrf2 and/or Keap1 in the treatments of diseases and developmental disorders.

Bibliography

- Boeke, Joern, Catherine Regnard, Weili Cai, Jørgen Johansen, Kristen M. Johansen, Peter B. Becker, and Axel Imhof. 2010. "Phosphorylation of SU(VAR)3-9 by the Chromosomal Kinase JIL-1." *PLoS ONE* 5(4).
- Boland, Michael J., Kristopher L. Nazor, and Jeanne F. Loring. 2014. "Epigenetic Regulation of Pluripotency and Differentiation." *Circulation Research* 115(2):311-24.
- Burton, Neal C., Thomas W. Kensler, and Tomas R. Guilarte. 2006. "In Vivo Modulation of the Parkinsonian Phenotype by Nrf2." *NeuroToxicology* 27:1094-1100.
- Chervona, Yana, Megan N. Hall, Adriana Arita, Fen Wu, Hong Sun, Hsiang-chi Tseng, Eunus Ali, Mohammad Nasir Uddin, Xinhua Liu, Maria Antonietta Zoroddu, V. Mary, and Max Costa. 2012. "Association Between Arsenic Exposure and Global Post-Translational Histone Modifications among Adults in Bangladesh." *Cancer Epidemiol Biomarkers and Prevention* 21(12):2252-60.
- Cryderman, Diane E., Stephanie K. Grade, Yuhong Li, Laura Fanti, Sergio Pimpinelli, and Lori L. Wallrath. 2005. "Role of Drosophila HP1 in Euchromatic Gene Expression." *Developmental Dynamics* 232:767-74.
- Demakova, Olga V, Galina V Pokholkova, Tatyana D. Kolesnikova, Sergey A. Demakov, Eugenia N. Andreyeva, Elena S. Belyaeva, and Igor F. Zhimulev. 2007. "The SU(VAR)3-9/HP1 Complex Differentially Regulates the Compaction State and Degree of Underreplication of X Chromosome Pericentric Heterochromatin in Drosophila Melanogaster." *Genetics* 175(February):609-20.
- Deng, H. and T. K. Kerppola. 2014. "Visualization of the Drosophila DKeap1-CncC Interaction on Chromatin Illumines Cooperative, Xenobiotic-Specific Gene Activation." *Development* 141(16):3277-88.
- Deng, Huai, Weili Cai, Chao Wang, Stephanie Lerach, Marion Delattre, Jack Girton, Jørgen Johansen, and Kristen M. Johansen. 2010. "JIL-1 and Su(Var)3-7 Interact Genetically and Counteract Each Other's Effect on Position-Effect Variegation in Drosophila." *Genetics* 185:1183-92.
- Deng, Huai and Tom K. Kerppola. 2013. "Regulation of Drosophila Metamorphosis by Xenobiotic Response Regulators." *PLoS Genetics* 9(2):e1003263.
- Ebert, Anja, Gunnar Schotta, Sandro Lein, Stefan Kubicek, Veiko Krauss, Thomas Jenuwein, and Gunter Reuter. 2004. "Su (Var) Genes Regulate the Balance between Euchromatin and Heterochromatin in Drosophila." *Genes and Development* 18:2973-83.
- Eggler, Aimee L., Guowen Liu, John M. Pezzuto, Richard B. Van Breemen, and Andrew D. Mesecar. 2005. "Modifying Specific Cysteines of the Electrophile-Sensing Human Keap1 Protein Is Insufficient to Disrupt Binding to the Nrf2 Domain Neh2." *PNAS* 102(29):10070-75.
- Elgin, Sarah C. R. and Gunter Reuter. 2013. "Position-Effect Variegation, Heterochromatin Formation, and Gene Silencing in Drosophila." *Cold Spring Harbor Perspectives in Biology* 5:a017780.
- Furukawa, Manabu and Yue Xiong. 2005. "BTB Protein Keap1 Targets Antioxidant Transcription Factor Nrf2 for Ubiquitination by the Cullin 3-Roc1 Ligase." *Molecular and*

Cellular Biology 25(1):162–71.

- Gandhi, Sumit G., Indira Bag, Saswati Sengupta, Manika Pal-bhadra, and Utpal Bhadra. 2015. “Drosophila Oncogene Gas41 Is an RNA Interference Modulator That Intersects Heterochromatin and the Small Interfering RNA Pathway.” *Federation of European Biochemical Societies* 282(1):153–73.
- Hayes, John D. and Michael McMahon. 2009. “NRF2 and KEAP1 Mutations : Permanent Activation of an Adaptive Response in Cancer.” *Trends in Biochemical Sciences* 34(4):176–88.
- Huang, Jiansheng, Imene Tabbi-anneni, Viswanath Gunda, and Li Wang. 2010. “Transcription Factor Nrf2 Regulates SHP and Lipogenic Gene Expression in Hepatic Lipid Metabolism.” *American Journal of Physiology-Gastrointestinal and Liver Physiology* 299:1211–21.
- Itoh, Ken, Tetsuro Ishii, Nobunao Wakabayashi, and Masayuki Yamamoto. 1999. “Regulatory Mechanisms of Cellular Response to Oxidative Stress.” *Free Radical Research* 31:319–24.
- Itoh, Ken, Nobunao Wakabayashi, Yasutake Katoh, Tetsuro Ishii, Kazuhiko Igarashi, James Douglas Engel, and Masayuki Yamamoto. 1999. “Keap1 Represses Nuclear Activation of Antioxidant Responsive Elements by Nrf2 through Binding to the Amino-Terminal Neh2 Domain.” *Genes and Development* 13(1):76–86.
- James, Tharappel C. and Sarah C. R. Elgin. 1986. “Identification of a Nonhistone Chromosomal Protein Associated with Heterochromatin in Drosophila Melanogaster and Its Gene.” *Molecular and Cellular Biology* 6(11):3862–72.
- Kang, Kyoung Ah and Jin Won Hyun. 2017. “Oxidative Stress , Nrf2 , and Epigenetic Modification Contribute to Anticancer Drug Resistance.” *Toxicol. Res.* 33(1):1–5.
- Kensler, Thomas W., Nobunao Wakabayashi, and Shyam Biswal. 2007. “Cell Survival Responses to Environmental Stresses Via the Keap1-Nrf2-ARE Pathway.” *Annual Review of Pharmacology and Toxicology* 47:89–116.
- Khacho, Mireille, Alysen Clark, Devon S. Svoboda, Mary-ellen Harper, David S. Park, Ruth S. Slack, Mireille Khacho, Alysen Clark, Devon S. Svoboda, Joelle Azzi, Jason G. Maclaurin, Cynthia Meghaizel, Hiromi Sesaki, Diane C. Lagace, Marc Germain, Mary-ellen Harper, David S. Park, and Ruth S. Slack. 2016. “Mitochondrial Dynamics Impacts Stem Cell Identity and Fate Decisions by Regulating a Nuclear Transcriptional Program.” *Cell Stem Cell* 19:232–47.
- Kim, Bo-rahm, Gha Young, Hyeree Yu, Hyo Jin, Tae Jung, Kyoung Min, Jae Hoon, Soo Lim, Hak Chul, and Sung Hee. 2018. “Suppression of Nrf2 Attenuates Adipogenesis and Decreases FGF21 Expression through PPAR Gamma in 3T3-L1 Cells.” *Biochemical and Biophysical Research Communications* 497:1149–53.
- Malhotra, Deepti, Elodie Portales-casamar, Anju Singh, Siddhartha Srivastava, David Arenillas, Christine Happel, Casper Shyr, Nobunao Wakabayashi, Thomas W. Kensler, Wyeth W. Wasserman, and Shyam Biswal. 2010. “Global Mapping of Binding Sites for Nrf2 Identifies Novel Targets in Cell Survival Response through ChIP-Seq Profiling and Network Analysis.” *Nucleic Acids Research* 38(17):5718–34.
- Marsit, Carmen J. 2015. “Influence of Environmental Exposure on Human Epigenetic Regulation.” *The Journal of Experimental Biology* 218:71–79.
- McMahon, Michael, Nerys Thomas, Ken Itoh, Masayuki Yamamoto, and John D. Hayes. 2006.

- “Dimerization of Substrate Adaptors Can Facilitate Cullin-Mediated Ubiquitylation of Proteins by a ‘ Tethering ’ Mechanism.” *Journal of Biological Chemistry* 281(34):24756–68.
- Misra, Jyoti R., Michael A. Horner, Geanette Lam, and Carl S. Thummel. 2011. “Transcriptional Regulation of Xenobiotic Detoxification in *Drosophila*.” *Genes and Development* 25:1796–1806.
- Mitsuishi, Yoichiro, Keiko Taguchi, Yukie Kawatani, Tatsuhiro Shibata, Toshihiro Nukiwa, and Hiroyuki Aburatani. 2012. “Nrf2 Redirects Glucose and Glutamine into Anabolic Pathways in Metabolic Reprogramming.” *Cancer Cell* 22:66–79.
- Mohler, Jym, Kodela Vani, Sophia Leung, and Alyse Epstein. 1991. “Segmentally Restricted, Cephalic Expression of a Leucine Zipper Gene during *Drosophila* Embryogenesis.” *Mechanisms of Development* 34:3–10.
- Ong, Cynthia, Lin-yue Lanry Yung, Yu Cai, Boon-huat Bay, and Gyeong-hun Baeg. 2015. “*Drosophila Melanogaster* as a Model Organism to Study Nanotoxicity.” *Nanotoxicology* 9(3):396–403.
- Ono, Hajime. 2014. “Ecdysone Differentially Regulates Metamorphic Timing Relative to 20-Hydroxyecdysone by Antagonizing Juvenile Hormone in *Drosophila Melanogaster*.” *Developmental Biology* 391:32–42.
- Osburn, William O. and Thomas W. Kensler. 2008. “Nrf2 Signaling: An Adaptive Response Pathway for Protection against Environmental Toxic Insults.” *Mutation Research* 659(1–2):31–39.
- Phelps, Chris B. and Andrea H. Brand. 1998. “Ectopic Gene Expression in *Drosophila* Using GAL4 System.” *Methods: A Companion to Methods in Enzymology* 14:367–79.
- Pi, Jingbo, Laura Leung, Peng Xue, Weiping Wang, Yongyong Hou, Dianxin Liu, Einav Yehuda-shnaidman, Candy Lee, Jackie Lau, Theodore W. Kurtz, and Jefferson Y. Chan. 2010. “Deficiency in the Nuclear Factor E2-Related Factor-2 Transcription Factor Results in Impaired Adipogenesis and Protects against Diet-Induced Obesity.” *Journal of Biological Chemistry* 285(12):9292–9300.
- Piacentini, Lucia, Laura Fanti, Maria Berloco, Barbara Perrini, Sergio Pimpinelli, Istituto Pasteur, Fondazione Cenci Bolognetti, Biologia Molecolare, Roma La, Anatomia Patologica, and Università Bari. 2003. “Heterochromatin Protein 1 (HP1) Is Associated with Induced Gene Expression in *Drosophila* Euchromatin.” *Journal of Cell Biology* 161:707–14.
- Piacentini, Lucia, Laura Fanti, Rodolfo Negri, Valerio Del Vescovo, Alessandro Fatica, Fabio Altieri, and Sergio Pimpinelli. 2009. “Heterochromatin Protein 1 (HP1a) Positively Regulates Euchromatic Gene Expression through RNA Transcript Association and Interaction with HnRNPs in *Drosophila*.” *PLoS Genetics* 5(10):e1000670.
- Pilsner, J. Richard, Megan N. Hall, Xinhua Liu, Vesna Ilievski, Vesna Slavkovich, Diane Levy, Pam Factor-litvak, Mahammad Yunus, Mahfuzar Rahman, Joseph H. Graziano, and Mary V Gamble. 2012. “Influence of Prenatal Arsenic Exposure and Newborn Sex on Global Methylation of Cord Blood DNA.” *PLoS One* 7(5):e37147.
- Pilsner, J. Richard, Xinhua Liu, Habibul Ahsan, Vesna Ilievski, Vesna Slavkovich, Diane Levy, Pam Factor-litvak, Joseph H. Graziano, and Mary V Gamble. 2007. “Genomic Methylation of Peripheral Blood Leukocyte DNA : Influences of Arsenic and Folate in Bangladeshi

- Adults.” *American Journal of Clinical Nutrition* 86:1179–86.
- Pitoniak, Andrew and Dirk Bohmann. 2015. “Mechanisms and Functions of Nrf2 Signaling in *Drosophila*.” *Free Radic Biol Med* 88((Pt B)):302–13.
- Schotta, Gunnar, Anja Ebert, Veiko Krauss, Andreas Fischer, Jan Hoffmann, Stephen Rea, Thomas Jenuwein, Rainer Dorn, and Gunter Reuter. 2002. “Central Role of *Drosophila* SU(VAR)3-9 in Histone H3-K9 Methylation and Heterochromatic Gene Silencing.” *EMBO* 21(5):1121–31.
- Schultz, Jack. 1936. “Variegation in *Drosophila* and the Inert Chromosome Regions.” *Genetics* 22:27–33.
- Sinclair, Donald A. R., Vett K. Lloyd, and Thomas A. Grigliatti. 1989. “Characterization of Mutations That Enhance Position-Effect Variegation in *Drosophila Melanogaster*.” *Molecular Genetics and Genomics* 216:328–33.
- Slocum, Stephen L. and Thomas W. Kensler. 2011. “Nrf2: Control of Sensitivity to Carcinogens.” *Archives of Toxicology* 85:273–84.
- Sun, Qiang Kun, Qing Wei Meng, Qing Yu Xu, Pan Deng, Wen Chao Guo, and Guo Qing Li. 2017. “Leptinotarsa Cap ‘n’ Collar Isoform C/Kelch-like ECH Associated Protein 1 Signaling Is Critical for the Regulation of Ecdysteroidogenesis in the Larvae.” *Insect Biochemistry and Molecular Biology* 85:1–10.
- Sykiotis, Gerasimos P. and Dirk Bohmann. 2008. “Keap1/Nrf2 Signaling Regulates Oxidative Stress Tolerance and Lifespan in *Drosophila*.” *Developmental Cell* 14(1):76–85.
- Sykiotis, Gerasimos P. and Dirk Bohmann. 2010. “Stress-Activated Cap’n’collar Transcription Factors in Aging and Human Disease.” *Science Signaling* 3(112):re3. doi:10.1126/scisignal.3112re3.
- Taguchi, Keiko, Hozumi Motohashi, and Masayuki Yamamoto. 2011. “Molecular Mechanisms of the Keap1–Nrf2 Pathway in Stress Response and Cancer Evolution.” *Genes to Cells* 16:123–40.
- Tschiersch, Bettina, Annemarie Hofmann, Veiko Krauss, Rainer Dorn, Gunter Korgel, and Gunter Reuter. 1994. “The Protein Encoded by the *Drosophila* Position-Effect Variegation Suppressor Gene Su(Var)3-9 Combines Domains of Antagonistic Regulators of Homeotic Gene Complexes.” *EMBO Journal* 13(16):3822–31.
- Veraksa, A., N. McGinnis, X. Li, J. Mohler, and W. McGinnis. 2000. “Cap ‘n’ Collar B Cooperates with a Small Maf Subunit to Specify Pharyngeal Development and Suppress Deformed Homeotic Function in the *Drosophila* Head.” *Development* 127(18):4023–37.
- Wallrath, Lori L. and Sarah C. R. Elgin. 1995. “Position Effect Variegation in *Drosophila* Is Associated with an Altered Chromatin Structure.” *Genes and Development* 9:1263–77.
- Wang, Chao, Yeran Li, Weili Cai, Xiaomin Bao, Jack Girton, Jørgen Johansen, and Kristen M. Johansen. 2014. “Histone H3S10 Phosphorylation by the JIL-1 Kinase in Pericentric Heterochromatin and on the Fourth Chromosome Creates a Composite H3S10phK9me2 Epigenetic Mark.” *Chromosome* 123(3):273–80.
- Wu, Joy S. and Liqun Luo. 2006. “A Protocol for Mosaic Analysis with a Repressible Cell Marker (Marcm) in *Drosophila*.” *Nature Protocols* 1(6):2583–89.

Zeng, Weihua, Alexander R. Ball, and Kyoko Yokomori. 2010. "HP1: Heterochromatin Binding Proteins Working the Genome." *Epigenetics* 5(4):287–92.

Zhang, Donna D. 2006. "Mechanistic Studies of the Nrf2-Keap1 Signaling Pathway." *Drug Metabolism Reviews* 38(4):769–89.