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LETTER

Regulation of position effect variegation at pericentric heterochromatin by *Drosophila* Keap1-Nrf2 xenobiotic response factors

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Summary

The Keap1-Nrf2 signaling pathway plays a central role in the regulation of transcriptional responses to oxidative species and xenobiotic stimuli. The complete range of molecular mechanisms and biological functions of Keap1 and Nrf2 remain to be fully elucidated. To determine the potential roles of Keap1 and Nrf2 in chromatin architecture, we examined the effects of their *Drosophila* homologs (dKeap1 and CncC) on position effect variegation (PEV), which is a transcriptional reporter for heterochromatin formation and spreading. Loss of function mutations in *cncC*, *dKeap1*, and *cncC/dKeap1* double mutants all suppressed the variegation of w^{m4} and Sb^{V} PEV alleles, indicating that reduction of CncC or dKeap1 causes a decrease of heterochromatic silencing at pericentric region. Depletion of CncC or dKeap1 in embryos reduced the level of the H3K9me2 heterochromatin marker, but had no effect on the transcription of the genes encoding Su(var)3-9 and HP1. These results support a potential role of dKeap1 and CncC in the establishment and/or maintenance of pericentric heterochromatin. Our study provides preliminary evidence for a novel epigenetic function of Keap1-Nrf2 oxidative/xenobiotic response factors in chromatin remodeling.

KEYWORDS

dKeap1-CncC, heterochromatin, Keap1-Nrf2, oxidative and xenobiotic responses, position effect variegation

1 | INTRODUCTION

Epigenetic modifications are thought to be important for mediating long-term developmental adaptations to continuous 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 regulations 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, & 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; Kobayashi et al., 2004; McMahon, Thomas, Itoh, Yamamoto, & Hayes, 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 (Eggler, Liu, Pezzuto, van Breemen, & Mesecar, 2005). Misregulations of Keap1 and Nrf2 are associated with many diseases, including cancer, neurodegeneration, and cardiovascular dysfunctions (Sykiotis & Bohmann, 2010; Taguchi, Motohashi, & 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 & Kerppola, 2013). In mice, Nrf2 binds to and activates genes involved in adipogenesis and lipid metabolism in specific tissues (Huang, Tabbi-Anneni, Gunda, & Wang, 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 pathogeneses.

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 & Kerppola, 2014). Both dKeap1 and CncC occupy ecdysone-response early puffs on polytene chromosomes and activate ecdysone-induced genes located at these puffs (Deng & Kerppola, 2013). The specific localization of dKeap1 and CncC at the polytene chromosome puffs, which represent highly decondensed chromatin regions, indicates 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.

2 | 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 & 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 & Reuter, 2013). The loss-of-function alleles of *cncC* and *dKeap1* are homozygous lethal at early larval stage (Sykiotis & Bohmann, 2008; Veraksa, McGinnis, Li, Mohler, & McGinnis, 2000). We performed the PEV assay by introducing heterozygous mutations of $cncC^{k6}$ and $dKeap1^{036}$ into the w^{m4} background. Surprisingly, both $cnc^{k6/+}$ and $dKeap1^{036/+}$ significantly suppressed the variegation of w^{m4}, represented by the observation of more flies containing a higher percentage of red ommatidia (Figure 1). 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 & 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 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 1).

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 cnc^{K22} and $dKeap1^{EY5}$ alleles have red eyes and cannot be used for w^{m4} PEV assay. Instead, we tested the effects of the cnc^{K22} and $dKeap1^{EY5}$ alleles on the PEV of the Sb^{V} locus (Figure 2). 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 hence the restoration of wild-type size of some scutellar bristles (Sinclair, Lloyd, & Grigliatti, 1989). We genetically combined Sb^{V} with cnc^{K22}, cnc^{K6}, $dKeap1^{EY5}$, or $dKeap1^{036}$ heterozygous mutations as well as relevant *cncC/dKeap1* double mutants. All of the mutants had an increased number of short bristles in the scutellar region, indicating significant suppression of Sb^{V} PEV (Figure 2). These results further support

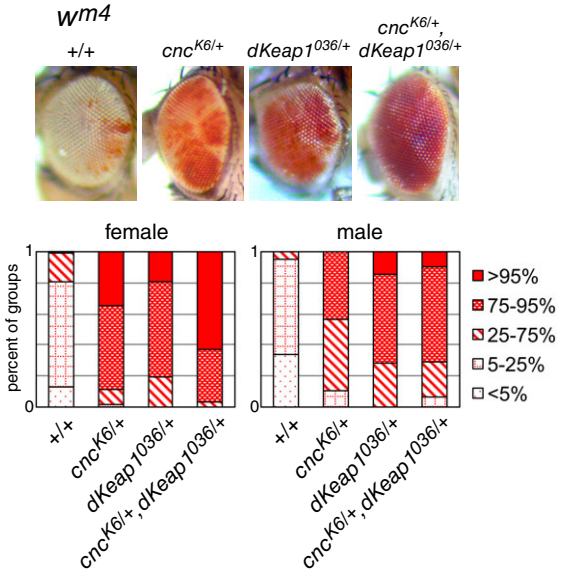

FIGURE 1 Regulation of w^{m4} position effect variegation (PEV) by *cncC* and *dKeap1* loss-of-function mutations. *Images*: Examples of the degree of PEV in the eyes of wild-type (*wt*), $cnc^{K6/+}$, $dKeap1^{036/+}$, and $cnc^{K6/+}$,$dKeap1^{036/+}$ flies in the w^{m4} background. *Histograms*: Distribution of the percentage of five groups categorized by the portion of red ommatidia in flies with genotypes labeled below columns. For each genotype, at least 100 flies were counted. Females and males were counted separately. Introduction of *cncC* and *dKeap1* loss-of-function alleles increased the number of flies with more red ommatidia, suggesting that reducing CncC or dKeap1 suppresses the variegation at the w^{m4} locus

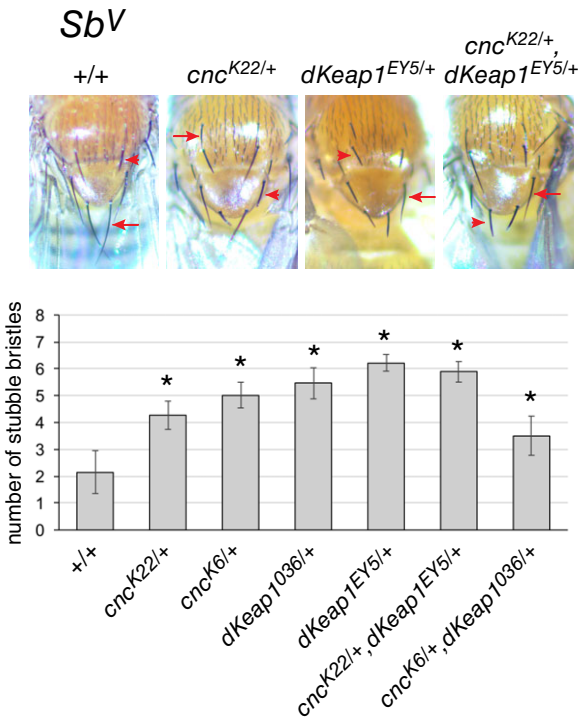

FIGURE 2 Regulation of Sb^V position effect variegation (PEV) by *cncC* and *dKeap1* loss-of-function mutations. *Images*: Examples of the degree of PEV in the scutellar regions of wild-type (*wt*), $cnc^{K22/+}$, $dKeap1^{EY5/+}$, and $cnc^{K22/+}$, $dKeap1^{EY5/+}$ flies in the Sb^V background. Examples of normal and stubble bristles are labeled by arrows and arrow heads, respectively. *Histograms*: Average numbers of stubble bristles among the eight scutellar bristles were counted in flies with genotypes listed below columns. For each genotype, the *SD* was calculated based on 200 flies that were counted. The mean values that differ from wild type at 95% confidence level ($p < 0.05$) are indicated by an asterisk. Introduction of *cncC* and *dKeap1* loss-of-function alleles increased the number of stubble bristles, suggesting that reducing CncC or dKeap1 suppresses the variegation at the Sb^V locus

the Su(var) effects of *cncC* and *dKeap1* loss-of-function alleles 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 & Elgin, 1995). Introduction of $cnc^{K6/+}$ or $dKeap1^{036/+}$ caused no significant effect on *39C-5* PEV (Supporting Information Figure S1). However, moderate suppression of *39C-5* PEV was observed in the females of the $cnc^{K6/+}$,$dKeap1^{036/+}$ double mutant (Supporting Information Figure S1). 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 3a). 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 *Su(var)205* gene) are the key factors that promote heterochromatin formation in *Drosophila* (James & Elgin, 1986; Schotta et al., 2002; Tschiersch et al., 1994). Given that CncC targets numerous genes (Misra, Horner, Lam, & Thummel, 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 3b). No significant changes in *Su(var)3-9* or *Su(var)205* transcripts were detected in any of the *cncC* or *dKeap1* mutants (Figure 3b). 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 (Figures 1 and 2), 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 and the transcription of antioxidant/detoxifying genes (Taguchi et al., 2011). Studies in *Drosophila* have confirmed that dKeap1 counteracts CncC in the regulations of lifespan and xenobiotic responses (Deng & Kerppola, 2014; Sykiotis & Bohmann, 2008). On the other hand, we have revealed that dKeap1 can bind chromatin and activate ecdysone biosynthetic and response genes in cooperation with CncC (Deng & Kerppola, 2013), and that dKeap1-CncC form complexes on chromatin and coactivate *Jheh* and *dKeap1* genes (Deng & Kerppola, 2014). The discovery that dKeap1 and CncC coregulate 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 4).

The current finding that sufficient dKeap1 and CncC proteins are required for the maintenance of heterochromatic silencing and 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 4). 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.,

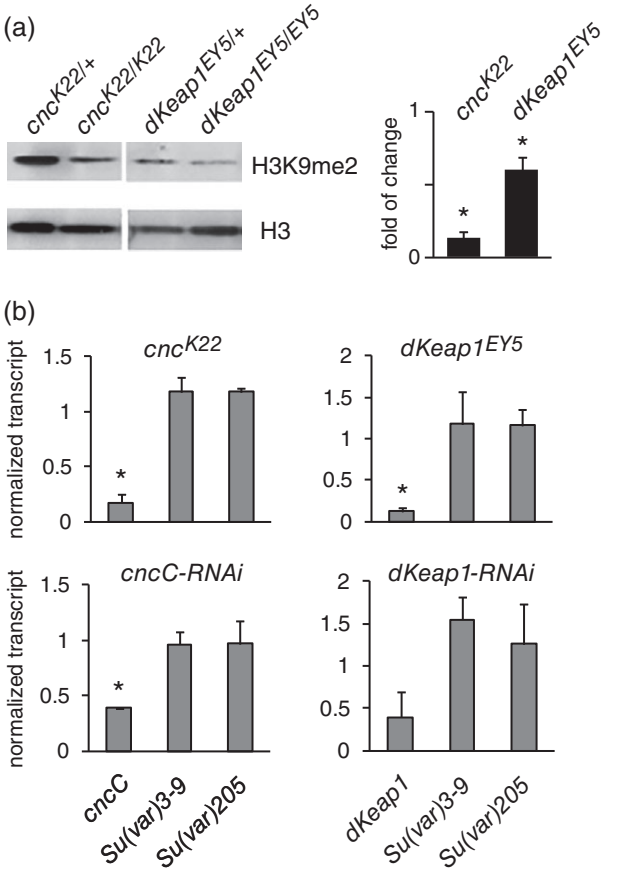

FIGURE 3 Regulation of heterochromatin level by CncC and dKeap1. (a) Effects of cncC and dKeap1 null mutations on H3K9me2 heterochromatin marker. Histone H3K9me2 and histone H3 (loading control) were detected in protein extractions from embryos with the genotypes labeled above using immunoblotting. The 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 *SD* from three experiments using different biological replicates (*$p < 0.05$). (b) Effects of CncC and dKeap1 depletions on transcripts. Quantitative reverse transcription polymerase chain reaction (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 small hairpin RNA 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 *SD* 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

2005; Piacentini, Fanti, Berloco, Perrini, & Pimpinelli, 2003; Piacentini et al., 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

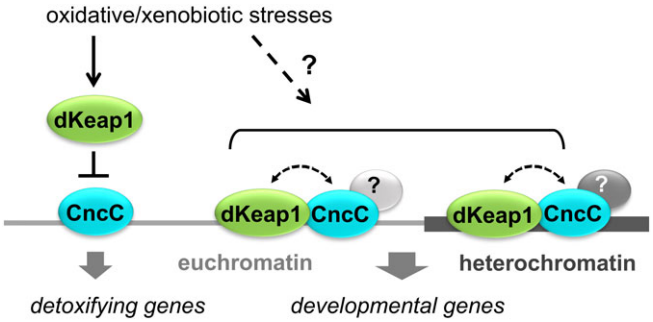

FIGURE 4 Model for multiple dKeap1-CncC functions on chromatin. The dKeap1-CncC complex regulates different sets of genes using distinct mechanisms: dKeap1-CncC interactions in the cytoplasm or nucleoplasm suppress CncC activity and the activation of antioxidant/detoxifying genes (left). In addition, 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)

elucidate whether dKeap1-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 epigenome, as well as the full range of roles of Keap1 and Nrf2 in human diseases.

3 | MATERIALS AND METHODS

3.1 | *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 & 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 are 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.

3.2 | PEV assays

PEV assays were performed as previously described (Deng et al., 2010; Gandhi, Bag, Sengupta, Pal-Bhadra, & Bhadra, 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 five different classes was counted based on the percentage of red color in the compound eyes. For Sb^{V} assays, the number of stubble bristles among eight major scutellar bristles in each fly was 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 (AmScope, Irvine, CA, USA).

3.3 | Immunoblotting

Since that null mutations of *cncC* and *dKeap1* are homozygous lethal at early first instar larval stage, late (12–16 hr) embryos were used for protein extraction and immunoblotting assay (Deng & 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 (Leica, Wetzlar, Germany). Embryos (~15 in each sample) were homogenized in 50 μL ice-cold Buffer (20 mM Tris-HCl pH 8.0, 0.2% NP-40, 0.2% Triton X-100, 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), 2 mM $NaVO_3$, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1.5 μg/mL aprotinin) and centrifuged at 10,000*g* for 3 min to remove precipitations. The supernatant samples were resolved using the NuPAGE 4–12% Bis-Tris gel (Invitrogen, Carlsbad, CA, USA). The proteins were transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA, USA) and probed using H3K9me2 mouse monoclonal antibody (1:400; Abcam, Cambridge, MA, USA) or H3 rabbit polyclonal antibody (1:3,000; Proteintech, Rosemont, IL, USA), followed by horseradish peroxidase-conjugated secondary antibodies (1:3,000, Bio-Rad). Intensities of western bands were measured using ImageJ software. The levels of H3K9me2 were calculated by normalizing the intensities of H3K9me2 bands with those of H3 bands. Three replicates of this experiment were performed using independent biological samples. Statistical tests were conducted using two-way Analysis of Variance (ANOVA) test.

3.4 | Transcript quantitation

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

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AUTHOR CONTRIBUTIONS

H.D. designed the project and planned the experiments. J.C., T.S., and C.S. conducted the experiments. J.C. and H.D. interpreted the data and wrote the paper.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interests.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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