Visualization of the *Drosophila* dKeap1-CncC interaction on chromatin illuminates cooperative, xenobiotic-specific gene activation

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**ABSTRACT**

Interactions among transcription factors control their physiological functions by regulating their binding specificities and transcriptional activities. We implement a strategy to visualize directly the genomic loci that are bound by multi-protein complexes in single cells in *Drosophila*. This method is based on bimolecular fluorescence complementation (BiFC) analysis of protein interactions on polytenic chromosomes. *Drosophila* Keap1 (dKeap1)-CncC complexes localized to the nucleus and bound chromatin loci that were not bound preferentially by dKeap1 or CncC when they were expressed separately. dKeap1 and CncC binding at these loci was enhanced by phenobarbital, but not by tert-butylhydroquinone (tBHQ) or paraquat. Endogenous dKeap1 and CncC activated transcription of the *Jheh* (*Jheh1*, *Jheh2*, *Jheh3*) and *dKeap1* genes at these loci, whereas CncC alone activated other xenobiotic response genes. Ectopic dKeap1 expression increased CncC binding at the *Jheh* and *dKeap1* gene loci and activated their transcription, whereas dKeap1 inhibited CncC binding at other xenobiotic response gene loci and suppressed their transcription. The combinatorial chromatin-binding specificities and transcriptional activities of dKeap1-CncC complexes meditated the selective activation of different sets of genes by different xenobiotic compounds, in part through feed-forward activation of *dKeap1* transcription.

**KEY WORDS:** Imaging, Bimolecular fluorescence complementation (BiFC), DNA-binding specificity, Combinatorial regulation, Transcription complex, Transcriptional response, *Drosophila* polytene chromosome, Chromatin, Xenobiotic compounds

**INTRODUCTION**

The combinatorial regulation of transcription is essential for the selectivity of eukaryotic gene expression. Chromatin immunoprecipitation studies have revealed that many transcription factors bind overlapping genomic regions (Negre et al., 2011; ENCODE Project Consortium, 2012). In tissues and in heterogeneous cell populations it is difficult to determine whether proteins that bind to the same region contact each other or whether they bind independently to adjacent recognition sequences in different cells.

Bimolecular fluorescence complementation (BiFC) analysis enables the visualization of protein interactions in living cells and tissues (Hu et al., 2002; Plaza et al., 2008; Gohi et al., 2010; Bai and Kerppola, 2011; Hudry et al., 2011; Papadopoulos et al., 2012; Boube et al., 2014). In the BiFC assay, the interaction between two proteins is detected based on the fluorescence produced by the association of two fragments of a fluorescent protein that are fused to two interaction partners. BiFC analysis has also been used to image interactions between epigenetic regulatory proteins and chromatin (Vincenz and Kerppola, 2008). However, it has not been possible to determine the genes that are bound by multi-protein complexes using BiFC analysis because of the difficulty of mapping the genomic loci that are bound by BiFC complexes in diploid cells. To overcome this limitation, we adapted BiFC analysis to study multi-protein complex binding on *Drosophila* polytene chromosomes.

Transcriptional responses to synthetic chemicals (xenobiotic compounds) are regulated by xenobiotic response regulators (Jennings et al., 2013). Cap-n-collar isofom C (CncC) and Nrf2 (also known as Nfe2l2) are homologous *Drosophila* and mammalian bZIP family transcription factors that can bind to and activate genes that express proteins that can protect cells from the deleterious effects of many xenobiotic compounds (Moi et al., 1994; Venugopal and Jaiswal, 1996; McGinnis et al., 1998; Sykiotis and Bohmann, 2008; Malhotra et al., 2010; Misra et al., 2011). Nrf2-deficient mice are more sensitive to chemical carcinogens than wild-type mice, presumably owing to the loss of cytoprotective functions of Nrf2 (Slocum and Kensler, 2011). Depletion of CncC in adult flies reduces the activation of many genes by xenobiotic compounds (Misra et al., 2011). *Drosophila* Keap1 (dKeap1) and mammalian Keap1 are homologous Kelch family proteins that can inhibit the activation of some xenobiotic response genes by CncC and Nrf2, respectively (Itoh et al., 1999; Wakabayashi et al., 2003; Sykiotis and Bohmann, 2008; Misra et al., 2011). Keap1-Nrf2 interaction is essential for Keap1 inhibition of transcriptional activation by Nrf2 (Itoh et al., 1999; McMahon et al., 2006). Depletion of dKeap1 in adult flies increases the transcription of some xenobiotic response genes that are activated by CncC (Misra et al., 2011). The conservation of many dKeap1-CncC and Keap1-Nrf2 functions between insects and mammals (Sykiotis and Bohmann, 2008; Misra et al., 2011) suggested that their interactions might also have conserved effects on their functions.

Both dKeap1 and CncC in *Drosophila* and Keap1 and Nrf2 in mouse also regulate development. Keap1 deletion in mouse causes hyperkeratosis in the esophagus and forestomach, and Nrf2 deletion causes defects in adipogenesis and in hematopoiesis (Wakabayashi et al., 2003; Pi et al., 2010; Tsai et al., 2013). Loss-of-function mutations in dKeap1 and in cncC cause larval lethality (Veraksa et al., 2000; Sykiotis and Bohmann, 2008). dKeap1 and CncC regulate *Drosophila* pupation and metamorphosis by binding to and activating ecdysone biosynthesis genes and ecdysone response genes in different tissues (Deng and Kerppola, 2013). The progression of metamorphosis, which is promoted by ecdysone, can be counteracted by juvenile hormone (Dubrovsy, 2005; Riddiford et al., 2010). Juvenile hormone

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epoxide hydrolases can catalyze the degradation of juvenile hormone in vitro (Touhara and Prestwich, 1993; Khlebodarova et al., 1996), and their expression is correlated with specific developmental stages in insects (Seino et al., 2010). The mechanisms that coordinate the balance between ecdysone and juvenile hormone levels during different stages of development have not been identified.

Keap1 is predominantly cytoplasmic in cultured mammalian cells (Itoh et al., 1999; Watari et al., 2007). The interaction between Keap1 and Nrf2 has been proposed to inhibit Nrf2 transcriptional activity by confining Nrf2 to the cytoplasm, by facilitating Nrf2 export from the nucleus, and by inducing Nrf2 ubiquitylation and degradation (Itoh et al., 1999; McMahon et al., 2003; Kobayashi et al., 2004; Sun et al., 2007; Misra et al., 2011). The modification of selected cysteine residues in Keap1 by reactive oxygen species and by xenobiotic compounds has been proposed to block the effects of Keap1 on Nrf2 localization or ubiquitylation (Eggl er et al., 2005; Kobayashi et al., 2009). This is thought to result in the accumulation of Nrf2 in the nucleus and the activation of xenobiotic response genes (Taguchi et al., 2011).

Nrf2 is required for the activation of different genes in response to different xenobiotic compounds in different mammalian cell lines and tissues (Thimmulappa et al., 2002; Kwak et al., 2003; Lee et al., 2003; Hu et al., 2006; Nair et al., 2006; Yates et al., 2009). The mechanisms that determine which genes are activated in response to different xenobiotic compounds have not been elucidated. We hypothesized that dKeap1 interacts with CncC on chromatin, and that xenobiotic compounds modulate their chromatin-binding specificities. To test this hypothesis, we used BiFC analysis to visualize interactions between dKeap1 and CncC on polytene chromosomes. Mapping the genetic loci bound by dKeap1-CncC complexes enabled the identification of genes that are selectively regulated by dKeap1-CncC complexes. Elucidating the effects of interactions between dKeap1 and CncC family proteins on their chromatin-binding specificities and on their transcriptional activities provided a new model for a mechanism that can mediate selective responses to different xenobiotic compounds and developmental signals.

**RESULTS**

**Nuclear localization of dKeap1-CncC complexes in *Drosophila* tissues**

The subcellular localization of protein complexes can provide insights into their functions. dKeap1 is localized to both the nucleus and the cytoplasm of cells in several different tissues (Fig. 1A; supplementary material Fig. S1A) (Deng and Kerppola, 2013). CncC is predominantly nuclear in all tissues that have been examined. To investigate interactions between dKeap1 and CncC in living tissues, we visualized dKeap1-CncC complexes in *Drosophila* larvae using BiFC analysis (Hu et al., 2002). dKeap1-CncC BiFC complexes were localized almost exclusively to the nuclei in living salivary gland cells (Fig. 1A). BiFC complexes formed by two different combinations of fusion proteins localized to the nucleoplasm and to subnuclear foci. The difference in subnuclear localization could be caused by the difference in the expression levels of these fusion proteins (supplementary material Fig. S2E). The nuclear localization of dKeap1-CncC complexes is likely to reflect the localization of endogenous dKeap1-CncC complexes, since endogenous dKeap1 and CncC were also localized to the nuclei in several cell types (supplementary material Fig. S1A) (Deng and Kerppola, 2013). dKeap1-CncC BiFC complexes were also localized to nuclei in diploid imaginal disc cells and in polyploid prothoracic gland cells (supplementary material Fig. S1B).

We investigated whether dKeap1 or CncC formed homodimers, and if their homodimerization affected their localization. BiFC complexes formed by dKeap1 homodimers were localized predominantly to the cytoplasm, whereas BiFC complexes formed by CncC homodimers were exclusively nuclear (Fig. 1A; supplementary material Fig. S1C). The non-overlapping distributions of dKeap1 homodimers and dKeap1-CncC complexes suggested that dKeap1-CncC complexes did not contain dKeap1 homodimers. This is in contrast to the hypothesis that mammalian Keap1 interacts with Nrf2 as a homodimer (Tong et al., 2006; Ogura et al., 2010).

To evaluate the specificity of BiFC complex formation by dKeap1 and CncC, we examined BiFC complex formation by dKeap1 and CncB, which lacks the N-terminal sequences that are homologous to those that mediate Nrf2 interaction with Keap1 (supplementary material Fig. S1D) (Veraksa et al., 2000). No fluorescence was detected in cells that expressed dKeap1 and CncB fused to fluorescent protein fragments (Fig. 1A). CncB-YC was expressed at a level that was intermediate between those of CncC-YN and CncC-YC, was localized to the nucleus and bound to chromatin (Fig. 1A; supplementary material Fig. S2C-E). BiFC complex formation by dKeap1 and CncC therefore required the N-terminal region of CncC.

**Chromatin binding by dKeap1-CncC complexes**

To determine whether dKeap1 and CncC form complexes at specific chromatin loci, we adapted BiFC analysis to visualize dKeap1-CncC complexes on polytene chromosome spreads. Surprisingly, dKeap1-CncC BiFC complexes bound loci distinct from those that were bound by the individual dKeap1 and CncC proteins (Fig. 1B; supplementary material Fig. S1E). None of the loci that were bound by the BiFC complexes (e.g. 55F, 95E and 94E) was bound by both endogenous dKeap1 and CncC. Most of these loci were bound by low or undetectable levels of the dKeap1 and CncC fusion proteins when they were expressed separately (Fig. 1B, Fig. 2A, C; supplementary material Fig. S2A). Conversely, none of the loci that were bound by both endogenous dKeap1 and CncC had detectable BiFC fluorescence. In particular, no BiFC signal was detected at the ecdysone-inducible early puffs (e.g. 2B, 74EF and 75B). These puffs were bound predominantly both by endogenous dKeap1 and CncC as well as by independently expressed dKeap1 and CncC fusion proteins (Fig. 1B, Fig. 2A, C; supplementary material Fig. S2A) (Deng and Kerppola, 2013). The BiFC complexes bound to fewer loci than either dKeap1 or CncC that were expressed separately, suggesting that dKeap1-CncC complexes bound chromatin with a higher specificity than either dKeap1 or CncC individually (Fig. 2C).

Neither dKeap1-dKeap1 nor CncC-CncC homodimer BiFC complexes were detected on polytene chromosomes, although the overall fluorescence intensities of these BiFC complexes were comparable to that of dKeap1-CncC BiFC complexes. All of the dKeap1 and CncC BiFC fusion proteins (dKeap1-YC, dKeap1-YN, CncC-YC and CncC-YN) bound to many of the same loci on chromatin (Fig. 2A; supplementary material Fig. S2A). These results suggest that BiFC complex binding on chromatin did not result from non-specific association between BiFC fusion proteins on chromatin.

**Effects of ectopic dKeap1 and CncC co-expression on their chromatin-binding specificities**

The difference between the loci that were bound by dKeap1-CncC BiFC complexes versus dKeap1 and CncC expressed separately
Fig. 1. Subcellular localization and locus-specific polytene chromosome binding by dKeap1-CncC BiFC complexes. (A) Subcellular localization of dKeap1-CncC, dKeap1-dKeap1 and CncC-CncC BiFC complexes and of the individual dKeap1 and CncC fusion proteins in Drosophila live salivary gland cells. The BiFC fusion proteins indicated in each image were expressed in salivary glands. The intrinsic BiFC and YFP fluorescence (green) were superimposed on Hoechst fluorescence (blue). CncB fusion protein localization was determined by immunostaining with anti-GFP antibodies (yellow). The fluorescence intensities of different cells varied over a wide range. The exposure times were adjusted to produce images of similar brightness. The subcellular distributions are representative of the majority of cells in each population. The diagram on the right depicts BiFC analysis. (B) Visualization of loci that are bound by dKeap1-CncC BiFC complexes and by endogenous dKeap1 and CncC proteins on polytene chromosomes. (Top row) Intrinsic BiFC fluorescence was visualized on polytene chromosome spreads from larvae that expressed dKeap1-YC and CncC-YN. (Bottom row) Endogenous dKeap1 and CncC were visualized on polytene chromosomes from wild-type (w^1118) larvae by immunostaining. The BiFC (green), immunofluorescence (yellow) and Hoechst (blue) signals were superimposed. Selected loci that were bound by the BiFC complexes are underlined and loci that were predominantly bound by endogenous dKeap1 and CncC proteins are indicated without underlining. The diagram (top left) depicts BiFC analysis of complexes on a polytene chromosome. The binding patterns are representative of at least eight spreads from four separate experiments. Scale bars: 10 µm.
Fig. 2. Effects of dKeap1 and CncC co-expression on their binding specificities on polytene chromosomes. (A) Loci that were bound by dKeap1, CncC and CncB when they were co-expressed and when they were expressed separately. The proteins indicated at the top of each image were expressed in salivary glands. The loci that were bound by the fusion proteins were visualized by immunostaining (shown in yellow) using the antibodies indicated. Loci that were bound predominantly by co-expressed dKeap1 and CncC are underlined, whereas loci that were bound predominantly by dKeap1 and CncC that were expressed separately are indicated without underlining. The loci that were bound by different combinations of dKeap1 and CncC fusion proteins and polytene chromosome spreads that are stained with additional antibodies are shown in supplementary material Fig. S2A-C. (B) Quantitative analysis of dKeap1 and CncC binding specificities when they were co-expressed versus expressed separately. (Left) The images show sections of polytene chromosomes that were stained using the antibodies indicated. The fusion proteins that were expressed in the larvae are indicated to the right of the images. Horizontal pairs of images separated by a vertical bar were from larvae that expressed the dKeap1 and CncC fusion protein separately; the other pairs of images were from larvae that co-expressed both the dKeap1 and CncC fusion proteins. The top pair of images is from wild-type (w1118) larvae. The 55F (arrow) and 56E (arrowhead) loci are indicated. (Right) The bar graph shows the average ratio (with s.d.) of the fluorescence intensities (55F/56E) from at least six spreads alongside the corresponding images. (C) Selected loci that were bound by endogenous dKeap1 and CncC, by dKeap1 and CncC fusion proteins expressed separately or in combination, and by dKeap1-CncC BiFC complexes. The intensities of the colored blocks indicate the relative levels of binding by each protein and complex at each locus. Only loci that had higher fluorescence intensities than the average intensity of all bands in the spread are indicated by the colored blocks. Thus, the absence of a colored block does not signify a lack of binding. (D) Loci that were bound by dKeap1 and CncC fusion proteins expressed separately or in combination. The proteins as indicated at the top of each image were expressed in salivary glands. Polytene chromosome spreads were stained using the antibodies indicated. Selected loci are labeled as in A. The binding patterns in A and D are representative of at least ten separate images from three separate experiments. Scale bars: 10 µm.
could reflect selective BiFC complex formation at a subset of the loci that were bound by dKeap1 and CncC, or it could reflect a change in the selectivity of chromatin binding by dKeap1 and CncC when they were co-expressed. To distinguish between these possibilities, we compared the loci that were bound by the dKeap1 and CncC fusion proteins when they were expressed separately versus in combination, dKeap1 and CncC fusion proteins that were expressed separately bound most of the loci that were bound by endogenous dKeap1 and CncC (Fig. 2A; supplementary material Fig. S2A; e.g. 2B, 74EF and 75B). When the same fusion proteins were co-expressed, they bound preferentially to the loci that were bound by dKeap1-CncC BiFC complexes (Fig. 2F, 2G; supplementary material Fig. S2A; e.g. 55F and 89E). The same change in binding specificities was detected by immunostaining with antibodies directed against dKeap1, CncC, as well as GFP (Fig. 2A,C; supplementary material Fig. S2A,B). Co-expression of dKeap1 and CncB fusion proteins, which did not form BiFC complexes, did not enhance their binding to the loci that were bound by dKeap1-CncC BiFC complexes (Fig. 2A; supplementary material Fig. S2C). Taken together, these results indicate that the difference between the loci that were bound by dKeap1-CncC BiFC complexes and those that were bound by dKeap1 and CncC independently reflects the effects of dKeap1-CncC interaction on their binding specificities.

To quantify the effect of dKeap1 and CncC co-expression on their binding specificities, we measured their relative levels of binding at neighboring loci when they were expressed separately and in combination. dKeap1 and CncC co-expression resulted in a reciprocal shift in their binding at the 55F and 56E loci compared with binding by endogenous dKeap1 and CncC as well as by ectopic dKeap1 expressed separately (Fig. 2B). Ectopic CncC expressed separately bound to many loci and exhibited little selectivity between the 55F and 56E loci. dKeap1 co-expression with CncC increased the selectivity of CncC binding between these loci and throughout the genome, suggesting that the dKeap1-CncC interaction enhanced the binding specificity of ectopic CncC.

We investigated whether BiFC complex formation or the protein fusions altered the binding specificities of dKeap1 and CncC. The same shift in binding was observed when dKeap1 and CncC fused to intact fluorescent proteins were co-expressed (Fig. 2D). BiFC complex formation was therefore not required for the effect that dKeap1 and CncC co-expression had on their binding specificities. Co-expression of dKeap1 and CncC fusion proteins containing either N- or C-terminal fusions caused the same changes in their binding specificities (Fig. 2A,D; supplementary material Fig. S2A). The positions of the protein fusions were therefore not important for the effects that dKeap1 and CncC co-expression had on their binding specificities.

We investigated the potential effects of differences in the levels of ectopic CncC and dKeap1 expression on their binding specificities by comparing the loci that were bound by CncC and dKeap1 fusion proteins that were expressed at different levels (supplementary material Fig. S2E). Co-expression of different dKeap1 and CncC fusion proteins caused the same changes in their binding specificities regardless of their expression levels (supplementary material Fig. S2A). It is therefore unlikely that changes in the levels of dKeap1 and CncC expression when they were expressed separately in combination caused the changes in their binding specificities.

All of the dKeap1 and CncC fusion proteins were expressed at higher levels than endogenous dKeap1 and CncC (supplementary material Fig. S2E). It is likely that the higher levels of ectopic dKeap1 and CncC co-expression caused the changes in their binding specificities as compared with the binding specificities of endogenous dKeap1 and CncC. We hypothesized that ectopic dKeap1 and CncC co-expression mimicked the effects of specific inducers of dKeap1 and CncC activity.

**Selective regulation of dKeap1 and CncC binding and of gene transcription by phenobarbital**

To test the hypothesis that xenobiotic inducers of dKeap1 and CncC activity had effects similar to that of ectopic dKeap1 and CncC co-expression, we examined the effects of compounds that can activate xenobiotic response genes on dKeap1 and CncC binding on polytene chromosomes. CncC and Nrf2 are required for the induction of many xenobiotic response genes by phenobarbital and paraquat in adult *Drosophila* and by tBHQ in mammalian cells (Lee et al., 2003; Sykiotis and Bohmann, 2008; Misra et al., 2011). Paraquat can cause oxidative stress and is thought to activate Nrf2 through the oxidation of cysteine residues on Keap1 (Suntres, 2002). tBHQ can activate Nrf2 by increasing mitochondrial reactive oxygen species and the tBHQ metabolite tert-butylbenzoquinone can directly modify Keap1 (Imhoff and Hansen, 2010; Abiko et al., 2011). The mechanisms by which phenobarbital regulates Keap1-Nrf2 as well as dKeap1-CncC were not known.

Phenobarbital feeding induced endogenous dKeap1 binding at the 55F and 89E loci (Fig. 3A). The increase in dKeap1 binding at these loci was selective, as no dKeap1 binding was detected at loci containing other xenobiotic response genes (Fig. 3B; 47D, 51D, 55C, 87B; Sykiotis and Bohmann, 2008; Misra et al., 2011). There was no detectable change in dKeap1 binding at other loci that were bound by dKeap1 in untreated larvae (Fig. 3A; 13C, 21B, 50C, 63F, 74EF). Phenobarbital feeding also increased the levels of ectopically expressed CncC binding at the 55F and 89E loci (supplementary material Fig. S3C). No endogenous CncC binding was detected at these loci, potentially because of the higher level of background staining of polytene chromosomes by anti-CncC antibodies.

Among the compounds tested, only phenobarbital induced dKeap1 and CncC binding at the 55F and 89E loci (Fig. 3C; supplementary material Fig. S3A). dKeap1 binding at other loci including 98E increased in larvae that were fed tBHQ, indicating that the lack of dKeap1 binding at the 55F and 89E loci in these larvae was not due to a failure of tBHQ feeding to influence dKeap1 binding (supplementary material Fig. S3B).

We examined the effects of phenobarbital, tBHQ and paraquat feeding on the transcription of genes at the loci that were bound by dKeap1-CncC BiFC complexes. *Juvenile hormone epoxide hydrolase 1 (Jheh1)* and *Jheh2*, as well as the gene encoding endogenous dKeap, which are located at 55F and 89E, respectively, were induced in larvae that were fed phenobarbital (Fig. 3D,E). The level of *dKeap1* transcripts increased more rapidly than those of *Jheh* or other xenobiotic response genes, indicating that dKeap1 was probably activated directly in response to phenobarbital (Fig. 3E). The slower induction of other xenobiotic response gene transcripts indicates that their activation could be mediated in part by the *de novo* synthesis of other proteins, including dKeap1.

Phenobarbital feeding also activated the transcription of a subset of previously identified xenobiotic response genes, including *Cyp6a2, Cyp6a8, Cyp12d1* and *GstD2*, but did not activate other xenobiotic response genes (*GstD1* and *GstE1*) (Fig. 3D; supplementary material Fig. S3D). Phenobarbital activated most of the genes that were tested both in salivary glands and in whole larvae, suggesting that salivary glands were an appropriate model for the investigation of their regulation (Fig. 3D, Fig. 4A,B).
Fig. 3. Effects of xenobiotic compounds on dKeap1 binding at selected loci on polytene chromosomes and on the transcription of xenobiotic response genes. (A) Effects of phenobarbital feeding on the loci that were bound by dKeap1. Polytene chromosome spreads were prepared from larvae (w1118) that were fed either phenobarbital (+PB) or control food (−PB) for 24 h, and were stained using antibodies against dKeap1. The immunostaining (yellow) and Hoechst (blue) signals were superimposed. Magnified views of the regions encompassing the 55F and 89E loci (arrows or arrowheads) are shown to the left of the spreads. The effects of phenobarbital feeding on CncC binding are shown in supplementary material Fig. S3C. (B) Effect of phenobarbital feeding on dKeap1 binding at loci encompassing known xenobiotic response genes. dKeap1 binding was examined at the indicated loci in larvae that were fed phenobarbital (+PB). Selected genes at each locus are indicated. (C) Effects of phenobarbital, tBHQ and paraquat feeding of larvae on dKeap1 binding at the 55F locus. Polytene chromosomes from third instar larvae that were fed phenobarbital, tBHQ, paraquat or control food for 24 h were stained using anti-dKeap1 antibodies. Segments of polytene chromosomes containing the 55F (arrows) and 56E (arrowheads) loci are shown. The effects of these compounds on dKeap1 binding at additional loci are shown in supplementary material Fig. S3A,B. (D) Effects of phenobarbital, tBHQ and paraquat feeding on the transcription of Jheh, dKeap1 and other xenobiotic response genes. Transcript levels were measured in four third instar larvae that were fed phenobarbital, tBHQ, paraquat or control food for 24 h. All transcript levels were normalized to the levels of Rp49 (Rpl32) transcripts. **P<0.05, ***P<0.01 (ANOVA) for treated versus control groups from two separate experiments. Error bars indicate s.d. (E) Temporal changes in dKeap1 and Jheh transcript levels following phenobarbital, tBHQ and paraquat feeding. Transcript levels were plotted relative to the levels of the same transcripts in larvae that were fed control food. All transcript levels were normalized to the levels of Rp49 transcripts and represent the mean and s.d. from two separate experiments. The changes in the levels of additional transcripts are shown in supplementary material Fig. S3D. (F) Levels of endogenous dKeap1 and CncC proteins in larvae that were fed different xenobiotic compounds. Extracts of larvae that were fed control food or food containing phenobarbital, paraquat or tBHQ were analyzed by immunoblotting using the antibodies indicated. The bands corresponding to endogenous dKeap1 and CncC are indicated by arrows (asterisks indicate cross-reactive bands). Marker sizes (kDa) are indicated. Scale bars: 10 µm.
Among the xenobiotic compounds tested, phenobarbital had the largest effects on Jheh1, Jheh2 and dKeap1 transcription as well as on dKeap1 binding at the 55F and 89E loci (Fig. 3D,C; supplementary material Fig. S3A). tBHQ had smaller effects on these transcripts and paraquat had no detectable effect, consistent with the undetectable levels of dKeap1 binding at the 55F and 89E loci in larvae that were fed tBHQ or paraquat. By contrast, tBHQ and paraquat had larger effects on dKeap1 binding as well as on transcription of several other xenobiotic response genes. Different xenobiotic compounds induced different combinations of the genes tested, suggesting that they activated these genes by distinct mechanisms.

We examined the effects of feeding different xenobiotic compounds on the levels of dKeap1 and CncC proteins. The level of dKeap1 increased in larvae that were fed phenobarbital, but not in those that were fed tBHQ or paraquat (Fig. 3F). By contrast, tBHQ and paraquat had larger effects on dKeap1 binding as well as on transcription of several other xenobiotic response genes. Different xenobiotic compounds induced different combinations of the genes tested, suggesting that they activated these genes by distinct mechanisms.

Roles of dKeap1 and CncC in phenobarbital induction of Jheh and dKeap1 gene transcription
The observations that phenobarbital induced dKeap1 and CncC binding at the loci encompassing the Jheh and dKeap1 genes and activated their transcription suggested that dKeap1 and CncC could mediate the activation of Jheh and dKeap1 transcription by phenobarbital. We investigated the effects of endogenous dKeap1 and CncC depletion by shRNA expression on transcription of the Jheh, dKeap1 and other xenobiotic response genes in larvae that were fed phenobarbital. Phenobarbital feeding increased transcription of dKeap1, Jheh1, Jheh2, Jheh3 and many other xenobiotic response genes in salivary glands (Fig. 4). dKeap1 depletion reduced Jheh1, Jheh2 and Jheh3 transcription in the salivary glands of phenobarbital-fed larvae (Fig. 4A). By contrast, dKeap1 depletion had no significant effect on the transcription of other xenobiotic response genes tested, with the exception of Cyp6a2. dKeap1 was therefore required for phenobarbital activation of most of the other xenobiotic response genes examined. CncC depletion reduced the levels of Jheh1, Jheh2, Jheh3 and dKeap1 as well as most other xenobiotic response gene transcripts in phenobarbital-fed larvae (Fig. 4B). Thus, both endogenous dKeap1 and CncC activated Jheh and dKeap1 gene transcription in response to phenobarbital, whereas CncC, but not dKeap1, activated the transcription of most of the other xenobiotic response genes examined in phenobarbital-fed larvae.

dKeap1 has opposite effects on CncC binding as well as on transcription at the Jheh and dKeap1 versus other xenobiotic response genes
We investigated the combined effects of dKeap1 and CncC on chromatin binding and on transcriptional activation at genes that were bound by dKeap1-CncC complexes and at other xenobiotic response genes. Ectopically expressed CncC bound many loci, including those encompassing the Jheh and dKeap1 genes as well those encompassing other xenobiotic response genes (Fig. 5A). Co-expression of ectopic dKeap1 with CncC enhanced CncC
binding at the former loci, but suppressed CncC binding at the latter loci.

Ectopic CncC expression activated the transcription of \textit{Jheh} and \textit{dKeap1} as well as other xenobiotic response genes (Fig. 5B). Co-expression of \textit{dKeap1} with CncC further increased \textit{Jheh} and endogenous \textit{dKeap1} gene transcription. By contrast, \textit{dKeap1} co-expression inhibited CncC activation of the other xenobiotic response genes examined. The same synergistic and antagonistic effects on transcription of these genes were observed when different \textit{dKeap1} and CncC fusion proteins containing either N- or C-terminal fusions were co-expressed (Fig. 5B; supplementary material Fig. S4). The different fusions did not therefore alter the effects of \textit{dKeap1} and CncC on transcription of the genes examined. Thus, ectopic \textit{dKeap1} co-expression with CncC had opposite effects on CncC binding as well as on transcriptional activation at the \textit{Jheh} and \textit{dKeap1} genes versus the other xenobiotic response genes.

We examined the combined effects of phenobarbital feeding and \textit{dKeap1}-CncC co-expression on the transcription of these genes. Phenobarbital feeding of larvae that co-expressed \textit{dKeap1} and CncC in salivary glands did not further increase \textit{Jheh} or \textit{dKeap1} transcription (Fig. 5C). By contrast, phenobarbital feeding did increase the transcription of most of the other xenobiotic response genes examined in the salivary glands of these larvae. Ectopic \textit{dKeap1} and CncC bound the 55F and 89E loci and activated \textit{Jheh} and \textit{dKeap1} transcription at much higher levels than endogenous \textit{dKeap1} and CncC did in response to phenobarbital feeding (Fig. 5A, Figs 2 and 3). The distinct effects of ectopic \textit{dKeap1} and CncC co-expression on phenobarbital activation of \textit{Jheh} and \textit{dKeap1} versus other xenobiotic response genes are consistent with the hypothesis that \textit{dKeap1} and CncC regulate the transcription of these two groups of genes through distinct mechanisms.

**Effects of \textit{Jheh} gene activation by \textit{dKeap1} and CncC co-expression on juvenile hormone-dependent functions**

To test the hypothesis that \textit{dKeap1} and CncC regulate development by altering juvenile hormone levels through the activation of \textit{Jheh} gene transcription, we examined the effects of \textit{dKeap1} and CncC co-expression on the timing of ommatidial development in pupae. When juvenile hormone was depleted by ablation of the corpus allatum premature ecdysone receptor expression was observed in the

![Fig. 5. Effects of ectopic \textit{dKeap1} and CncC expression on transcription and on phenobarbital activation of genes bound by \textit{dKeap1}-CncC complexes and of other xenobiotic response genes. (A) CncC binding at selected loci when it was expressed separately versus co-expression with \textit{dKeap1}. Sections of polytene chromosomes from larvae that expressed the fusion proteins indicated above each panel were stained using anti-CncC antibodies. Loci that encompass \textit{Jheh} or \textit{dKeap1} genes as well as other xenobiotic response genes are indicated. (B) Effects of ectopic \textit{dKeap1} and CncC expression separately and in combination on the transcription of different genes. Transcript levels were measured in ten pairs of salivary glands from larvae that expressed YFP or the \textit{dKeap1} and CncC fusion proteins indicated under the control of the Sgs3-GAL4 driver. The effects of separate and combined expression of \textit{dKeap1} and CncC BiFC fusion proteins on transcription are shown in supplementary material Fig. S4. (C) Effects of ectopic \textit{dKeap1} and CncC co-expression on phenobarbital activation of different genes. Transcript levels were measured in ten pairs of salivary glands of third instar larvae that were fed either phenobarbital (red bars) or control food (black bars) for 1 h. Control larvae that expressed YFP and larvae that co-expressed \textit{dKeap1} and CncC fusion proteins were reared and analyzed in parallel. All transcript levels were normalized to the levels of \textit{Rp49} transcripts. **P<0.05, ***P<0.01 (ANOVA) for transgene versus control groups from two separate experiments. Error bars indicate s.d. Scale bars: 10 µm.
eye disc (supplementary material Fig. S5A,B) (Riddiford et al., 2010; Riddiford, 2012). Conditional dKeap1 and CncC co-expression activated Jheh1 and Jheh2 transcription in third instar larvae and in pupae, but did not cause premature ec Dysone receptor expression (supplementary material Fig. S5A,C,D). Ectopic dKeap1 and CncC expression also had no detectable effect on transcription of the Kruppel homolog 1 (Kr-h1) gene, which is activated by juvenile hormone (supplementary material Fig. S5B-D) (Minakuchi et al., 2008). Ectopic dKeap1 and CncC co-expression in third instar larvae caused pupal lethality that was not suppressed by feeding the larvae with the juvenile hormone mimic methoprene. Thus, whereas dKeap1 and CncC co-expression induced Jheh gene transcription and arrested pupal development, it is likely that factors other than juvenile hormone depletion contributed to the pupal lethality.

**DISCUSSION**

BiFC imaging of transcription factor interactions on polytene chromosomes provides a unique strategy for visualization of the genome-wide loci that are bound by combinatorial transcription regulatory complexes. The discoveries that dKeap1-CncC complexes are localized to nuclei and are bound to specific loci on polytene chromosomes challenge previous models for the effects of dKeap1 and CncC family protein interactions on their functions. The effects of dKeap1-CncC interaction on their chromatin-binding specificities and transcriptional activities have identified a combinatorial mechanism for the regulation of their functions that mediated selective gene activation by phenobarbital (Fig. 6).

**Chromatin-binding specificity of dKeap1-CncC complexes**

The distinct loci bound by dKeap1-CncC BiFC complexes compared with the loci bound by dKeap1 and CncC expressed separately suggest that the interaction between dKeap1 and CncC altered their binding specificities. When dKeap1 and CncC were expressed separately, both proteins bound to the ec Dysone response gene loci (Deng and Kerppola, 2013). However, they did not form BiFC complexes or enhance binding by each other at these loci. Three distinct modes of dKeap1 and CncC binding on chromatin were observed: (1) binding to the same loci without cooperative complex formation, as represented by the ec Dysone response genes; (2) cooperative complex formation, as represented by the Jheh and dKeap1 genes; and (3) independent binding to separate loci. These results indicate that multiple mechanisms, including cooperative DNA binding, influence the binding specificities and combinatorial regulation of transcription by dKeap1 and CncC.

Phenobarbital feeding induced endogenous dKeap1 and ectopic CncC binding at the Jheh and dKeap1 loci, suggesting that phenobarbital induced dKeap1-CncC complex binding at these loci. The molecular mechanisms for the selective induction of dKeap1 and CncC binding at these loci remain to be fully characterized. The feed-forward loop produced by dKeap1-CncC complex activation of dKeap1 gene transcription and the resulting increase in the level of dKeap1 protein is likely to be an integral component of this mechanism (Fig. 6).

**Selective binding and transcriptional activation by dKeap1-CncC**

The selectivity of dKeap1-CncC BiFC complex formation at the Jheh and dKeap1 loci correlated with the selectivity of dKeap1 activation of Jheh and dKeap1 transcription in concert with CncC. The opposite effects of ectopic dKeap1 co-expression on ectopic CncC binding and on transcriptional activation at the Jheh and dKeap1 genes versus other xenobiotic response genes are consistent with distinct mechanisms for the regulation of dKeap1 and CncC functions at different genes. Both endogenous dKeap1 and CncC activated Jheh gene transcription in phenobarbital-fed larvae. By contrast, CncC, but not dKeap1, mediated phenobarbital activation of the other xenobiotic response genes examined. Thus, dKeap1 and CncC synergistically activated the Jheh and dKeap1 genes, whereas dKeap1 had an antagonistic effect on CncC activation of other xenobiotic response genes (Fig. 6). Phenobarbital feeding of larvae that expressed ectopic dKeap1 and CncC did not further activate Jheh or dKeap1 transcription, whereas it activated other xenobiotic response genes. These results are consistent with the hypothesis that phenobarbital activates Jheh and dKeap1 transcription by enhancing dKeap1-CncC complex formation at these loci, whereas phenobarbital activates other xenobiotic response genes by a distinct mechanism.

**Feed-forward amplification of dKeap1 transcription by dKeap1-CncC**

The increased levels of dKeap1 transcripts, dKeap1 protein and dKeap1 binding at the dKeap1 locus in phenobarbital-fed larvae suggested that dKeap1 expression was regulated by a feed-forward loop (Fig. 6). The increase in dKeap1 transcription preceded the increases in Jheh gene transcription, suggesting that the auto-activation of dKeap1 transcription amplified the activation of other dKeap1-CncC target genes. The feed-forward activation of dKeap1 transcription is likely to also modulate the activities of other xenobiotic response genes. Feed-forward loops regulate many developmental transitions and gene networks that maintain cellular states. Some inducers of Nrf2 activity can protect cells and organisms from subsequent exposure to toxic compounds and carcinogens (Liby et al., 2007; Kensler et al., 2013). In particular, phenobarbital pretreatment increases mouse resistance to parathion and reduces the incidence of chemically induced bladder cancer in rats (Vitarius et al., 1995). Feed-forward regulatory loops that activate dKeap1-CncC as well as Keap1-Nrf2 transcriptional networks could mediate such sensitization by xenobiotic agents.

**Selective effects of different xenobiotic compounds on dKeap1 and CncC binding and on transcription**

dKeap1 and CncC binding and transcription at the dKeap1 and Jheh genes were selectively enhanced by phenobarbital, but not by tBHQ.
or paraquat. Different xenobiotic compounds also have distinct effects on the Nr2-dependent transcription factors in different mammalian cell lines and tissues (Thimmulappa et al., 2002; Kwak et al., 2003; Lee et al., 2003; Hu et al., 2006; Nair et al., 2006; Yates et al., 2009). The mechanisms that mediate the selective regulation of different genes in response to different xenobiotic compounds could involve differences in the formation and binding specificities of dKeap1-CncC, as well as of Keap1-Nr2, complexes. The multiple mechanisms that can control the transcriptional activities of dKeap1 and CncC family protein complexes potentially provide many ways to coordinate transcriptional responses with different xenobiotic compounds and developmental signals.

**Visualization of combinatorial transcription complex binding specificity by BiFC imaging**

The results of our experiments have established that genetic loci that are selectively bound by transcription factor complexes can be identified by mapping BiFC complex binding on polytene chromosomes. dKeap1 and CncC fusions formed BiFC complexes only at loci where dKeap1 and CncC bound cooperatively to chromatin, indicating that adventitious collisions between the fluorescent protein fragments did not facilitate BiFC complex formation. Thus, BiFC analysis can be used to identify differences between the binding specificities of protein complexes and those of their constituent subunits.

Ectopic overexpression of dKeap1 and CncC fusion proteins affected the loci that were bound by dKeap1 and CncC. In this case, ectopic dKeap1 expression mimicked the effect of feed-forward amplification of dKeap1 expression in response to selected xenobiotic compounds. In other situations, it might be necessary to control the levels of ectopic fusion proteins by using inducible expression vectors or endogenous promoters (Hudry et al., 2011). BiFC analysis has also been used to visualize Hox family transcription factor binding at a tandem array of integrated binding sites on polytene chromosomes (Papadopoulos et al., 2012). Our results demonstrate that at least some BiFC complexes bind to native loci that are bound and regulated by the endogenous proteins in response to specific stimuli.

Many biochemical, genetic, cell biological and computational approaches have been used to study combinatorial mechanisms of transcriptional regulation. Studies using different experimental approaches in different model systems have focused on different mechanisms of combinatorial transcriptional regulation (Arnosti et al., 1996; Burns and Kerppola, 2012; Takaya et al., 2012). BiFC analysis of protein interactions on polytene chromosomes combines various aspects of these approaches by providing information about molecular interactions in genetically modified animals at high spatial resolution. This strategy provides a unique and complementary approach for the investigation of regulatory processes on chromatin.

**MATERIALS AND METHODS**

**Drosophila stocks**

Plasmids encoding dKeap1, CncC and CncB fused to intact fluorescent proteins (nYFP, here designated YFP) and fluorescent protein fragments (YN and YC) were constructed using the pUAST vector (Brand and Perrimon, 1993) and microinjected in the w1118 background. Additional information about the plasmid expression vectors is available in the supplementary materials and methods. Lines that were not developed during this study were generously provided by Dirk Bohmann (Sykiotis and Bohmann, 2008). Salivary gland expression was obtained using the Sgs3-GAL4 driver line (Cherbas et al., 2003). All studies were conducted with larvae maintained at 25°C with the exception of larvae expressing BiFC fusion proteins, which were maintained at 21°C to enhance the polyplody of salivary gland cells, and larvae carrying the UAS-dKeap1-RNAi transgene, which were maintained at 29°C to improve the efficiency of dKeap1 depletion.

**BiFC analysis of protein interactions on polytene chromosomes**

Salivary glands that expressed BiFC fusion proteins were isolated from early wandering third instar larvae. Polyteny chromosome spreads were prepared using an acid-free squash technique to avoid quenching of the BiFC fluorescence (Johansen et al., 2009). One pair of dissected salivary glands was incubated in freshly prepared 2% paraformaldehyde in Brower’s Fixation Buffer (0.15 M PIPES, 3 mM MgSO4, 1.5 mM EGTA, 1.5% NP40, pH 6.9) for 3 min, in PBS+0.2% Triton X-100 for 3 min, and in 50% glycerol for 5 min. The salivary glands were then squashed in 10 µl 50% glycerol and stained with Hoechst 33258 before mounting in 80% glycerol, 10 mM Tris pH 9.0. Live tissues were mounted in PBS and imaged within 5 min after dissection. Images were acquired on an Olympus IX81 inverted fluorescence microscope with a Hamamatsu ORCA-ER digital CCD camera. BiFC signal was visualized using 504 nm excitation and 542 nm emission wavelengths. The signals corresponding to each combination of excitation and emission wavelengths were pseudocolored and merged in RGB color space. Detailed experimental procedures related to BiFC analysis of protein interactions on polytene chromosomes are available in the supplementary materials and methods.

**Polytene chromosome immunostaining**

Polyteny chromosome spreads were prepared from salivary glands of early wandering third instar larvae using conventional squash and immunostaining protocols (Johansen et al., 2009). Whole salivary glands isolated from early wandering third instar larvae were immunostained as described (Phalle Bde, 2004). Antibodies used for immunostaining were: anti-GFP (Fitzgerald Industries, 20R-GR011; 1:200), anti-dKeap1 (1:100) and anti-CncC (1:100) (Deng and Kerppola, 2013), Alexa Fluor 594-conjugated goat anti-rabbit secondary antibody (Invitrogen, A-11012; 1:1000). The immunostained samples were mounted in VectaShield (Vector Laboratories). Additional information about the antisera and experimental procedures for polytene chromosome squash, immunostaining and imaging are available in the supplementary materials and methods.

**Xenobiotic compound feeding to larvae**

Larvae were collected at third instar and were transferred to plates with Formula 24 Drosophila diet (Carolina Biological Supply) containing 0.1% phenobarbital (Sigma), 0.1% tBHQ (Sigma), 1% paraquat (Sigma) or water (control). Fewer than 20 larvae were placed on each plate to avoid overcrowding. After the indicated times, mRNA or protein was isolated and analyzed by RT-qPCR or immunoblotting. The sequences of the primers used for RT-qPCR are listed in supplementary material Table S1. Detailed experimental procedures for immunoblotting, transcript quantification and statistical analyses of the data are available in the supplementary materials and methods.

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**Competing interests**

The authors declare no competing financial interests.

**Author contributions**

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

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