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Drosophila Keap1 xenobiotic response factor regulates developmental transcription through binding to chromatin

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Abstract

The Keap1-Nrf2 complex is a central regulator that mediates transcriptional responses to xenobiotic stimuli and is highly related with multiple human diseases. The molecular mechanisms and biological functions of Keap1 and Nrf2 are not fully understood. The *Drosophila* Keap1 homolog (dKeap1) is conserved with mammalian Keap1 except that dKeap1 contains a 156 aa C-terminal tail (CTD). A dKeap1 truncation with the CTD removed (dKeap1-ΔCTD) shows abolished nuclear localization and chromatin-binding. Expression of dKeap1-ΔCTD in the *dKeap1* null background significantly rescues this mutant to the adult stage, but the files showed partial lethality, sterility and defects in adipose tissue. In the rescued flies, expression levels of ecdysone-response genes, ecdysone-synthetic genes and adipogenesis genes were down-regulated in specific tissues, indicating that the chromatin-binding of dKeap1 mediates the activation of these developmental genes. As the same time, dKeap1-ΔCTD can still suppress the basal expression of detoxifying genes and mediate the activation of these genes in response to xenobiotic stimuli, suggesting that the chromatin-binding of dKeap1 is not required for the regulation of detoxifying genes. These results support a model in which dKeap1 on one hand functions as an inhibitor for the Nrf2-mediated transcription in the xenobiotic response pathway and on the other hand functions as a chromatin-binding transcription activator in the developmental pathway. Our study reveals a novel mechanism whereby Keap1-Nrf2 xenobiotic response signaling regulates development using a mechanism independent of redox signaling.

Graphical Abstract

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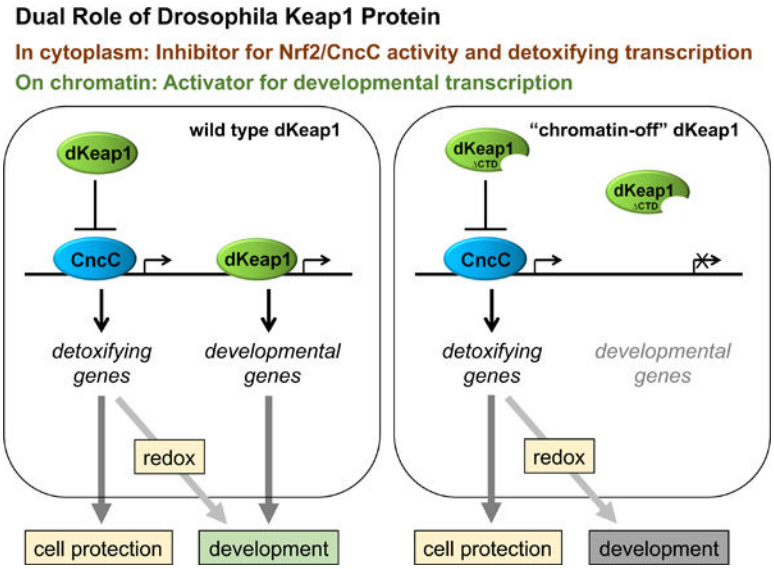
Author contributions

H.D. designed the project and planned the experiments. J.C., L.P., and I.C. conducted the experiments. H.D. and J.C. interpreted the data and wrote the paper.

Competing interest statement

The authors declare that they have no competing interest.



Keywords

Keap1-Nrf2; dKeap1-CncC; oxidative and xenobiotic responses; chromatin binding; transcription activator; development; ecdysone; oogenesis; adipogenesis

1. Introduction

The molecular mechanisms whereby environmental toxins influence development remain to be fully elucidated. Environmental toxins can induce multiple protective pathways that mediate transcriptional responses to xenobiotic compounds in the cell (Jennings et al., 2013). It is hypothesized that factors that can both respond to xenobiotic stimuli and regulate developmental transcription could serve as a mechanism to mediate long-term developmental responses to constant toxin exposure.

The Keap1-Nrf2 complex is an essential pathway that protects cells from both reactive oxygen species and xenobiotics (Kensler et al., 2007). Nrf2 (NF-E2-Related Factor 2) is a bZIP family transcription factor that can bind to antioxidant response elements (AREs) 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 interacts with Nrf2 in the cytoplasm and induces Nrf2 ubiquitination and degradation (Itoh et al., 1999; McMahon et al., 2006). Selective modifications of cysteine residues on Keap1 by oxidative/xenobiotic compounds disrupt Keap1-Nrf2 interactions, resulting in the nuclear accumulation of Nrf2 and the activation of detoxifying genes (Eggler et al., 2005). Mis-regulations of Keap1 and Nrf2 are related to many diseases including cancer, neurodegeneration, cardiovascular dysfunctions, and respiratory diseases (Carlson, 2020; Sykiotis and Bohmann, 2010; Taguchi et al., 2011).

Recent studies in mice and *Drosophila* have revealed that Keap1 and Nrf2 family proteins can also target and regulate developmental genes in a tissue-specific manner. *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 (Huang et al., 2010; Pi et al., 2010). Nrf2 can also promote cell proliferation by activating genes that encode glucose metabolic enzymes (Mitsuishi et al., 2012). Nrf2 can regulate neuronal stem cell fate by activating genes that inhibit self-renewal or promote differentiation (Khacho et al., 2016). It is thought that the multiple developmental functions of Keap1 and Nrf2 account for their complex roles in physiology and pathogenesis (Slocum and Kensler, 2011; Taguchi et al., 2011).

In contrast to the well-understood ARE-dependent xenobiotic response pathway, it remains unclear how the Keap1-Nrf2 complex regulates developmental genes. Our previous studies in *Drosophila* indicated that the mechanism by which dKeap1-CncC regulates developmental genes is different from the classic mechanism whereby Keap1-Nrf2 controls detoxifying genes (Deng, 2014). Notably, dKeap1 can bind to specific chromatic loci and function as a transcriptional coactivator with CncC (Deng and Kerppola, 2014). Both dKeap1 and CncC bind to and activate ecdysone-biosynthetic genes in prothoracic glands and ecdysone-response genes in salivary glands (Deng and Kerppola, 2013). Therefore, the developmental function of the dKeap1-CncC pathway is likely associated with the chromatin-binding of dKeap1. dKeap1 and CncC can also co-regulate heterochromatin silencing, providing additional evidence to support the model that chromatin-bound dKeap1 cooperates with CncC when controlling chromatin structure and developmental genes (Carlson et al., 2019).

To better understand the molecular mechanism underlying the developmental function of dKeap1, we mapped the domain that mediates chromatin-binding of dKeap1. We found that removal of the C-terminal domain (CTD) abolished dKeap1 nuclear localization and chromatin-binding, while retaining its function in the inhibitions of CncC and xenobiotic response transcription. Expressing a dKeap1 truncation without CTD (dKeap1-ΔCTD) in the *dKeap1* null background reduced the expression of some developmental genes and resulted in developmental defects in oogenesis and adipogenesis. This *dKeap1-ΔCTD* hypomorphic mutant allows for the dissection of specific developmental functions of dKeap1 that are independent of the redox signaling.

2. Materials and Methods

2.1. Drosophila stocks

Plasmids encoding YFP-dKeap1 and YFP-dKeap1-ΔCTD were constructed using the pUAST vector (Brand and Perrimon, 1993) and microinjected in the w^{1118} background. $dKeap1^{EY5}$, *UAS-cncC-RNAi*, and *tubGS5-GAL4* strains were provided by Dirk Bohmann (Sykiotis and Bohmann, 2008). *tub-GAL4* and *Sgs3-GAL4* (Cherbas et al., 2003) were from the Bloomington Stock Center. All fly stocks were maintained using standard protocols. $dKeap1^{EY5}$ and *tub-GAL4* are combined with *TM6,Tb,Sb,Hu,e,Dfd-YFP (TM6)* balancers. Rescue experiments were conducted by crossing *UAS-YFP-dKeap1-ΔCTD,*$dKeap1^{EY5}$*/TM6* or *UAS-YFP-dKeap1,*$dKeap1^{EY5}$*/TM6* with *tub-GAL4,*$dKeap1^{EY5}$*/TM6*. Appropriate progenies were identified based on *Tb* marker in larvae and pupae or

Sb marker in adults. Embryos with or without *Dfd-YFP* marker were sorted under a Leica MZ10 F fluorescence stereomicroscope. Conditional *cncC* RNAi knockdown driven by *tubGS5-GAL4* was induced by feeding adults with RU486 (Sigma) as described (Sykiotis and Bohmann, 2008).

2.2. Western blotting

Three third instar larvae were homogenized in 200 μl ice-cold IP Buffer (20 mM Tris-HCl pH 8.0, 0.2% NP-40, 0.2% Triton X-100, 150 mM NaCl, 5 mM EDTA, 1 mM EGTA, 2 mM $NaVO_3$, 1 mM PMSF, and 1.5 μg/ml aprotinin). Samples were resolved using the NuPAGE 4–12% Bis-Tris gel (Invitrogen). The proteins were transferred to nitrocellulose membrane (Bio-Rad) and probed using anti-GFP rabbit polyclonal antibody (1:1000, Novus Biologicals, NB600), anti-dKeap1 rabbit polyclonal antibody (1/200) or anti-tubulin mouse monoclonal antibody (1:3000, Developmental Studies Hybridoma Bank, 12G10), followed by HRP-conjugated secondary antibodies (1:3000, Bio-Rad).

2.3. Transcript quantitation

Around 50 embryos, 10 pairs of salivary glands dissected from early wandering third instar larvae, fat tissues dissected from 5 third instar larvae or 5 pairs of ovaries dissected from adults (aged for 5 days) were used for mRNA extraction using RNeasy kit (Qiagen). Isolated mRNA was treated with RQ1 RNase-Free DNase (Promega) and reverse transcribed using iScript™ cDNA Synthesis kit (Bio-Rad). Real-time qPCR was performed using SYBR green qPCR master mix (Apex) in an Eppendorf realplex Mastercycler. The relative transcript levels were calculated by assuming that they were proportional to 2^{-Cp}, and normalized with the levels of *Rp49* transcripts. Primer sequences were designed using Universal ProbeLibrary software (Roche) and are listed in Table S1. Statistical analyses for the significance of the differences in the relative transcript levels were evaluated using two-way ANOVA.

2.4. Immunostaining of tissues and polytene chromosome spreads

Salivary glands and guts isolated from L3 larvae and ovaries isolated from adults were immunostained as described (Phalle Bde, 2004). Polytene chromosome spreads were prepared using conventional squash and immunostaining protocols (Johansen et al., 2009). Antibodies used for immunostaining were: anti-GFP (1:200, Novus Biologicals, NB600), anti-dKeap1 (1:100) and anti-CncC (1:100) (Deng and Kerppola, 2013), anti-Lamin Dm0 (1/200, Developmental Studies Hybridoma Bank, ADL67.10), Alexa Fluor 594-conjugated goat anti-rabbit secondary antibody (1:1000, Invitrogen). The stained samples were mounted in mounting solution (80% glycerol, 10 mM Tris pH9.0 and 2% n-Propyl Gallate) and examined under a Nikon Eclipse 80 fluorescence microscope with SPOT Insight 4 MP color digital camera.

2.5. Xenobiotic compound feeding and resistance assay

5 larvae of each genotype were collected at the early L3 stage and transferred to plates with Formula 24 *Drosophila* diet (Carolina Biological Supply) containing 0.1% tBHQ (Sigma) or water (control). After 24 hours, mRNA was isolated and analyzed by RT-qPCR. Xenobiotic

resistance of adults to Paraquat (Sigma) was measured as described (Wang et al., 2005). Newly eclosed flies were collected and aged for 4 days. After being starved for 3 hours in an empty vial, flies were fed with a semi-lethal dose of Paraquat (50 mM) dissolved in 5% sucrose. Survivors were scored after 16 hours. T-tests were used for statistical analysis.

2.6. Viability and fertility quantification

To quantify the viability, dKeap1-ΔCTD or FL rescuing flies were generated using the crosses described above. Parental flies were removed after 4 days and the vials were incubated at 21°C. Different genotypes of pupae and adults generated in each vial were counted. Appropriate rescued animals and control animals were identified based on the *Tb* marker in pupae and *Hu* marker in adults. To quantify the female fertility, 6 virgin females with selected genotypes were placed in a vial containing 6 wildtype males (Oregon R). To quantify the male fertility, 6 young males with selected genotypes were placed in a vial containing 6 wildtype virgins. Parental flies were removed after 4 days and the number of F1 pupae were counted. Chi-squared statistical analysis was used to evaluate the significance of differences.

2.7. Fat tissue quantification

The relative fat tissue levels in L3 larvae were measured by a buoyancy-based method as described (Hazegh and Reis, 2016). L3 larvae cultured at 21°C were placed into a 50-ml conical tube with 11.5 ml PBS and 9 ml 20% sucrose. The tube was inverted two times and then swirled to create a vortex. After allowing the larvae to settle, the number of floating larvae was recorded. 20% sucrose was added in 1 ml increments (to increase the density of the solution) and the number of floating larvae were recorded at each density until all of the larvae were floating.

2.8. Nile red staining and quantification

Fat tissues were removed from L3 wandering larvae and fixed in PFA for 20 minutes, then stained with 1 μg/mL Nile red (Sigma) in PBS for 1 hour. Tissues were washed 3 times for 10 minutes in PBS and then mounted in mounting solution and imaged using a Zeiss LSM 710 confocal microscope. The diameters of the 100 largest lipid droplets were measured using Image J. The cell area was also measured for 15 cells of each genotype. T-tests were used to assess statistical significance in lipid droplet size.

2.9. Ovary size quantification

Female adults were aged for 4 days by culturing with wildtype males at 25°C. Ovaries were dissected, suspended in PBS, and imaged alongside a ruler by an Olympus SZ61 stereo microscope with Amscope MU900 microscope digital camera. The areas of the ovaries were then measured using image J. T-tests were used for statistical analysis.

3. Results

3.1. CTD is required for nuclear localization and chromatin binding of dKeap1

Unlike mammalian Keap1 proteins, which primarily localize to the cytoplasm, the majority of *Drosophila* dKeap1 proteins are in the nuclei (Deng and Kerppola, 2013). Immunostaining of dKeap1 on polytene chromosomes reveals hundreds of loci that are bound by dKeap1 (Deng and Kerppola, 2013). Comparing the amino acid sequences of dKeap1 and mouse/human Keap1 revealed a high conservation overall except that dKeap1 contains extra 155 amino acids at the C-terminus following the Kelch repeats (Fig. 1A) (Sykiotis and Bohmann, 2008). Motif predictions based on amino acid sequence revealed a potential non-classic nuclear localization sequence (NLS) in the C-terminus of dKeap1 but no NLS was predicted in mammalian Keap1 proteins (Kosugi et al., 2009; Nguyen Ba et al., 2009). We therefore hypothesize that the C-terminal domain (CTD) of dKeap1 mediates the nuclear localization and chromatin binding of dKeap1.

To test this hypothesis, we generated a CTD truncation (dKeap1-ΔCTD) by deleting 156 amino acids from the C-terminus of dKeap1 (Fig. 1A). This truncated dKeap1, as well as the full length dKeap1 protein (dKeap1-FL), were fused with a YFP tag and expressed in *Drosophila* tissues using the UAS-GAL4 system. The expression levels of these two transgenes and fusion proteins are comparable (Fig. 1B-C). YFP-dKeap1-ΔCTD signal was found mainly in the cytoplasm of the salivary gland cell (Fig. 1D). On the contrary, YFP-dKeap1 and endogenous dKeap1 localized to both the cytoplasm and the nucleus, primarily in the nucleoplasm (Fig. 1D). The mis-localization of dKeap1-ΔCTD proteins was detected in both wildtype and *dKeap1* null backgrounds (Fig. 1D, S1A) and also was revealed in other cell types including polyploid gut cells and diploid imaginal disc cells (Fig. S1B-C).

We next examined the abilities of these fusion proteins to bind to the polytene chromosome. Anti-GFP immunostaining revealed the binding of YFP-dKeap1 at many interband loci (Fig. 1E) (Deng and Kerppola, 2014). No detectable signal of YFP-dKeap1-ΔCTD was identified on polytene chromosomes (Fig. 1E). Taken together, the CTD is required for dKeap1 to enter the nucleus and bind to chromatin.

3.2. Removal of the dKeap1-CTD reduces viability and ecdysone-response transcripts

dKeap1 can directly bind to and activate ecdysone-synthetic and response genes (Deng and Kerppola, 2013). It also binds to many other genomic loci that are not occupied by CncC. We speculated that the binding of dKeap1 to many loci mediates a broad role of dKeap1 in the transcriptional regulation of development. To test this model, we specifically depleted dKeap1-chromatin binding by expressing dKeap1-ΔCTD in a *dKeap1* null background. The $dKeap1^{EY5/EY5}$ null mutant survives only to the early first instar stage. Ubiquitous expression of dKeap1-FL in the *dKeap1* null mutant (*UAS-YFP-dKeap1/+; tub-GAL5,*$dKeap1^{EY5/EY5}$) rescued the viability almost fully (~93%) at the pupal stage and largely (~83%) at the adult stage (Fig. 2A). The partial lethality of these flies is likely due to overexpression of the YFP-dKeap1 fusion protein. Expression of dKeap1-ΔCTD in the same *dKeap1* null background (*UAS-YFP-dKeap1-ΔCTD/+; tub-GAL5,*$dKeap1^{EY5/EY5}$) partially rescued the viability. Compared with control flies (*UAS-YFP-dKeap1-ΔCTD/+;* $dKeap1^{EY5/}$

TM6), the viability of the rescued animals was 89% at the pupal stage and 62% at the adult stage (Fig. 2A). The *dKeap1* null mutants that were rescued by YFP-dKeap1-ΔCTD (ΔCTD flies) showed significantly lower viability than those rescued by YFP-dKeap1 (FL flies). These results suggest that the CTD of dKeap1 is required for the survival of *Drosophila* at pupal and adult stages.

Drosophila development is regulated by the dKeap1-CncC pathway through ecdysone signaling. Both dKeap1 and CncC are required for the activation of early puff genes, the genes that are directly targeted and activated by ecdysone receptors (Deng and Kerppola, 2013). To determine if dKeap1-CTD mediates the activation of ecdysone-response genes, we examined the transcription of these genes in the salivary glands of both ΔCTD and FL flies. Transcription levels of most of the early puff genes were down-regulated in the ΔCTD larvae compared to those of FL larvae (Fig. 2B). Therefore, the chromatin-bound dKeap1 proteins may facilitate the activation of ecdysone-response genes during development. Compared with control flies, the timing of pupation and eclosion of ΔCTD flies was delayed by approximately one and two days, respectively (Fig. S2A). These results are consistent with our previous finding that dKeap1 regulates the timing of metamorphosis by activating both ecdysone biosynthetic and response transcripts (Deng and Kerppola, 2013). Expression of the dKeap1-FL had no effect on the length of metamorphosis but delayed pupation for approximately one day (Fig. S2A), probably due to the suppression of CncC by the overexpressed dKeap1.

3.3. Regulation of adipogenesis by dKeap1 and dKeap1-CTD

We observed significant reduction of fat tissue in both FL and ΔCTD L3 larvae. In support of this, these larvae showed higher density than control larvae (*tub-GAL4/+*) due to the loss of low-density fats (Fig. 2C). The reduction of fat tissue was caused by reduced cell numbers since the overall cell sizes did not change in FL and ΔCTD fat tissues (Fig. S2B). The average sizes of lipid droplets in adipose cells from FL and ΔCTD larvae were smaller than those from wildtype larvae (Fig. 2D, S2C), indicating a disrupted lipid storage in both FL and ΔCTD cells. The lipid droplets in ΔCTD adipose cells were even smaller than those in FL cells (Fig. 2D, S2C), suggesting that the chromatin-binding of dKeap1 is required for a normal lipid storage in adipose cells.

To determine if dKeap1 regulates adipogenesis at the transcriptional level, we examined the expression of adipogenesis genes including *adp*, *SREBP*, and *FASN1* in fat tissues isolated from ΔCTD, FL or wildtype L3 larvae (Fig. 2E). All these genes were down-regulated in FL fat tissue and *adp* expression was further reduced in ΔCTD fat tissue. These results suggest that overexpression of dKeap1 suppresses the transcription of several adipogenesis genes, and the CTD of dKeap1 is required for the activation of the *adp* gene. Taken together, dKeap1 likely binds to and activates some adipogenesis genes such as *adp*.

3.4. dKeap1-ΔCTD can still suppress CncC and mediate xenobiotic response

The Keap1-Nrf2 complex is a master regulator for redox homeostasis. Both knockdown and over-expression of CncC cause lethality of *Drosophila* at embryonic and early first instar larval (L1) stages, theoretically due to the mis-regulation of oxidative and xenobiotic

response genes. In support of this, knockout of dKeap1 (*dKeap1*$^{EY5/EY5}$) and RNAi-knockdown of dKeap1 cause dramatic upregulation of xenobiotic response genes including *gstD2*, *gstE1* and *Cyp12d1* (Fig. 3A) (Deng and Kerppola, 2013). Expressing dKeap1-FL in the *dKeap1*$^{EY5/EY5}$ null mutant suppressed the expression of these genes to basal levels (Fig. 3B), suggesting that overexpressed dKeap1 proteins can inhibit xenobiotic response genes. Expressing dKeap1-ΔCTD in the *dKeap1* null mutant also restored the expression of these genes to basal levels (Fig. 3B), indicating that dKeap1-ΔCTD is also able to suppress xenobiotic response genes. Feeding FL and ΔCTD L3 larvae with an Nrf2 inducer (tBHQ) activated the transcription of these xenobiotic response genes to levels comparable to the gene activation seen in wildtype control larvae (Fig. 3B), indicating that both YFP-dKeap1 and YFP-dKeap1-ΔCTD fusion proteins can normally mediate transcriptional response to xenobiotic stimuli. In a xenobiotic resistance assay, large portions of FL and ΔCTD adults were able to survive after being challenged with a semi-lethal dose of another Nrf2 inducer Paraquat (Fig. S3A). The survival ratio of ΔCTD flies was slightly lower than wildtype and FL flies, which was likely associated with developmental defects of ΔCTD flies. Taken together, a dKeap1 protein without CTD can still function in the xenobiotic response pathway.

Keap1 family proteins suppress the nuclear accumulation of CncC/Nrf2 by interacting with CncC/Nrf2 and inducing their proteasomal degradation (McMahon et al., 2006). We directly tested if CncC protein levels were altered in ΔCTD flies. In our western blotting experiment, the basal CncC protein level was not detectable in FL or ΔCTD flies (Fig. 3C). As a control, CncC level was upregulated in larvae that expressed a dKeap1 truncation deleting the CncC-interacting Kelch domain in the *dKeap1* null background (Fig. 3C). Taken together, dKeap1-ΔCTD can efficiently suppress CncC protein level and transcriptional activity. The lethality of ΔCTD flies is not caused by the up-regulation of CncC activity and xenobiotic response transcripts.

3.5. dKeap1-CTD is required for normal oogenesis of Drosophila

Partial sterility was detected in ΔCTD female flies that survived to the adult stage. The F1 offspring produced by ΔCTD females were only ~40% of those produced by FL females (Fig. 4A), suggesting that the CTD of dKeap1 is required for the normal oogenesis of *Drosophila*. The morphology of egg chambers in ΔCTD ovaries showed no difference compared to those of FL and wildtype flies (Fig. S4A). However, the average size of ΔCTD ovaries was larger than those of wildtype and FL ovaries (Fig. S4B). It is possible that both the enlarged ovary and reduced fertility were the consequence of defects in oogenesis which accumulated premature eggs in ΔCTD flies. Both FL and ΔCTD males showed significant reduction of fertility at comparable levels (Fig. S4E), suggesting that dKeap1 overexpression may cause partial male sterility but dKeap1-CTD plays no role in the regulation of spermatogenesis.

3.6. dKeap1 and CncC regulates ecdysone synthetic genes in ovaries

dKeap1 and CncC bind to and activate ecdysone-biosynthetic genes in the prothoracic gland, the organ that produces the steroid hormone ecdysone during the larval stage (Deng and Kerppola, 2013). Ecdysone signaling also plays multiple roles in the regulation of

Drosophila oogenesis (Belles and Piulachs, 2015). We hypothesized that dKeap1-CncC controls oogenesis through ecdysone signaling, and speculated that the oogenesis defect in ΔCTD flies was caused by the reduction of dKeap1-targeted ecdysone-biosynthetic genes. Immunostaining of *Drosophila* ovaries revealed dKeap1 signal in egg chamber follicle cells, primarily in nuclei (Fig. 4B). Transcripts of most of the ecdysone-biosynthetic genes were detected in ovaries (Figure 4C). We next examined and compared the expression levels of these genes in the ovaries from ΔCTD and FL flies as well as wildtype control flies. Expressing dKeap1-FL in *dKeap1* null ovaries had no significant effect on most of these genes except the slight up-regulation of *dib* and *shd* (Fig. 4C). Expressing dKeap1-ΔCTD in *dKeap1* null ovaries significantly down-regulated *nvd*, *phm*, and *sad* transcripts (Fig. 4C). This downregulation was not caused by a decrease of CncC as the transcription level of *cncC* was not affected. Therefore, the chromatin-bound dKeap1 facilitates the activation of several ecdysone-biosynthetic genes.

Immunostaining of *Drosophila* ovaries also revealed CncC in nuclei of egg chamber follicle cells (Fig. S2C). Conditional knockdown of CncC by RNAi at the adult stage down-regulated most of the ecdysone-biosynthetic genes in ovaries (Fig. S2D), suggesting that CncC is also required for ecdysone synthesis during oogenesis. We conclude that dKeap1 and CncC likely regulate oogenesis by cooperatively binding to and activating ecdysone-biosynthetic genes in follicle cells.

4. Discussion

In contrast to the well-known function of the Keap1-Nrf2 pathway in the regulation of oxidative and xenobiotic responses, the developmental roles of Keap1 and Nrf2 remain to be fully elucidated. One mechanism by which Keap1-Nrf2 can control development is through the redox signaling, since the regulatory role of ROS in several developmental programs has been revealed (Bigarella et al., 2014; Oswald et al., 2018; Owusu-Ansah and Banerjee, 2009). For example, dKeap1 and CncC regulate intestinal stem cell proliferation through the redox pathway (Hochmuth et al., 2011). Interestingly, recent studies in both mammalian and *Drosophila* systems have identified that Nrf2 and Keap1 can directly bind to and activate some developmental genes (Burns, 2020; Deng and Kerppola, 2013; Huang et al., 2010), indicating another mechanism underlying the developmental roles of Keap1 and Nrf2. However, genetic manipulations of Keap1 and/or Nrf2 result in redox imbalances, hindering the identification of the redox-independent functions of Keap1 and Nrf2. For example, knockouts of Keap1 proteins in both *Drosophila* and mice cause lethality, likely due to the upregulation of CncC/Nrf2 and consequent reductive stress (Sykiotis and Bohmann, 2008; Wakabayashi et al., 2003). Our study identified a dKeap1 truncation that lost chromatin binding but retained the ability to inhibit CncC. The dKeap1-ΔCTD truncation significantly rescues the *dKeap1* null mutant, indicating that dKeap1-ΔCTD can restore the CncC-induced redox imbalance. This “chromatin-off” *dKeap1* hypomorphic mutant provides a powerful experimental system for the identification of dKeap1-functions independent of the redox pathway (Fig. 4D). This mutant also allows a genomic transcription assay to fully identify the genes that are directly targeted by dKeap1.

Using this “chromatin-off” *dKeap1* mutant, we found that the chromatin-binding dKeap1 activates the transcription of ecdysone-biosynthetic genes in ovary cells. *Drosophila* ecdysone signaling regulates multiple developmental programs during oogenesis, including egg chamber follicle formation, border cell migration, germline stem cell development and stem cell niche formation (Ables and Drummond-Barbosa, 2010; Belles and Piulachs, 2015; Carney and Bender, 2000; Domanitskaya et al., 2014; Konig et al., 2011; Morris and Spradling, 2012; Naora and Montell, 2005). The dKeap1-CncC pathway therefore could control developmental programs, including oogenesis, through the ecdysone pathway. However, the role of dKeap1-CncC in oogenesis may also be mediated by other developmental pathways. In support of this hypothesis, we found that dKeap1 can bind to and activate many other genes in salivary glands and a loss-of-function *dKeap1* mutation reduced the expression of many developmental genes in embryos (data not shown). We also found a novel function of dKeap1 in the transcriptional regulation of adipogenesis. Interestingly, *adp* and *FASN1* have opposite effects on the size of lipid droplets: Loss of function mutation of *adp* results in enlarged lipid droplets (Hader et al., 2003) while knockdown of *FASN1* causes smaller lipid droplets (Ugrankar et al., 2019), indicating more complex relationships between adipogenesis genes and dKeap1 in lipogenesis. It has been reported that tissue-specific overexpression of a CncC N-terminal truncation significantly reduced fat tissue (Karim et al., 2015), and mouse Nrf2 binds to and activates adipogenesis and lipogenic genes (Huang et al., 2010; Pi et al., 2010). The functions of the dKeap1-CncC complex in adipogenesis remain to be fully investigated in the future. The viability of the *dKeap1* mutant could be affected by the disrupted adipogenesis, which could reduce fat storage and influence other pathways such as insulin signaling. It is also likely that the lethality of the *dKeap1* mutant is the consequence of a combination of developmental defects associated with the mis-regulation of dKeap1-targeting genes. Taken together, these results highlight the value of the “chromatin-off” *dKeap1* mutant in exploring novel dKeap1-regulated developmental genes and programs.

In the conventional redox-regulation pathway, Keap1 suppresses the Nrf2-mediated activation of antioxidant and detoxifying genes by interacting with Nrf2 in the cytoplasm and inducing its proteasomal degradation (Taguchi et al., 2011). Our previous studies in *Drosophila* suggest a novel pathway in which dKeap1 directly binds to and activates developmental genes (Deng, 2014; Deng and Kerppola, 2013). However, as most of the dKeap1 proteins are in the nucleoplasm and cytoplasm, it is possible that the down-regulation of developmental genes upon dKeap1 depletion is mediated by other Keap1-interacting factors such as Nrf2/CncC, E3 complex, actin, p62, and PGAM5 in the cytoplasm or nucleoplasm (Itoh et al., 1999; Komatsu et al., 2010; Lo and Hannink, 2006; McMahon et al., 2006). This study verifies that the C-terminal tail of dKeap1 mediates its binding on chromatin. Specific removal of the CTD abolishes the chromatin binding of dKeap1, selectively downregulating developmental genes but not detoxifying genes. These results support the model that dKeap1 proteins mediate the activation of developmental genes by functioning as a chromatin-binding transcription activator (Fig. 4D).

It is possible that the lack of dKeap1-ΔCTD binding on chromatin could be a consequence of reduced dKeap1-ΔCTD in the nucleoplasm. However, this is unlikely as a low level of dKeap1-ΔCTD was still detected in the nucleoplasm (Fig. 1D) and dKeap1 chromatin

binding is insensitive to the nuclear level of dKeap1. Overexpressed YFP-dKeap1 proteins strongly accumulate in the nucleoplasm but bind to the polytene chromosome at comparable levels to endogenous dKeap1 proteins (data not shown). In support of this, dKeap1 overexpression does not enhance the transcription of most dKeap1-target genes (Fig. 4C) (Deng and Kerppola, 2014). Additionally, nuclear Keap1 proteins are required for shuttling Nrf2 proteins back to the cytoplasm (Sun et al., 2011). Given that dKeap1-ΔCTD functions normally in the suppression of CncC activity (Fig. 3B-C), dKeap1-ΔCTD proteins that function in CncC-shuttling should be at least at the same levels as endogenous dKeap1 proteins in the nucleus. Therefore, the lack of chromatin-binding of dKeap1-ΔCTD is likely the result of the loss of CTD rather than the result of reduction of overall protein level in the nucleus.

Our results verify that the CTD of dKeap1 contains a functional NLS, which mediates the nuclear localization of dKeap1. The function of abundant dKeap1 proteins in the nucleoplasm remains unclear, and we hypothesize that a large portion of nuclear dKeap1 proteins function independently of CncC-suppression and oxidative/xenobiotic responses. The mechanism whereby CTD mediates dKeap1 chromatin binding remains to be investigated. dKeap1 contains no classic DNA-binding motif. A coil domain (698–718) is predicted in the CTD by InterPro (Blum et al., 2021), suggesting that dKeap1 could dimerize with a chromatin-binding protein. It is also proposed that some NLS can serve as a DNA-binding motif (Cokol et al., 2000), indicating a possibility that dKeap1-CTD directly binds to DNA through the NLS sequence.

The novel chromatin-binding and transcriptional activation function of dKeap1 is likely conserved in mammalian cells. It was recently found that Keap1 binds to cytokine promoters in mouse embryo fibroblasts and activates transcripts in response to virus infection (Burns, 2020). The full chromatin-binding map of mammalian Keap1 remains to be determined. It is believed that Keap1 binds to a limited number of genomic loci, which differs from the global binding of dKeap1 throughout the genome (Deng and Kerppola, 2013, 2014). The lack of the C-terminal tail in mammalian Keap1 could account for this difference. The chromatin binding of Keap1 is likely mediated by other factors. For example, the activation of cytokine genes in viral-infected mouse embryo fibroblasts requires the interactions of NFB p50 and G9a-GLP with Keap1 (Burns, 2020). It remains to be determined if Keap1 family proteins in *Drosophila* and mammalian systems share at least some conserved mechanisms in developmental regulations. Overall, our studies are expected to reveal novel developmental functions of Keap1/Nrf2 family proteins, which will provide more insight into the complicated roles of these factors in human diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Drosophila Keap1 binds to chromatin using the C-terminal domain
- dKeap1 that loses chromatin-binding functions normally in the xenobiotic response pathway
- Chromatin-bound dKeap1 facilitates the activation of developmental genes
- dKeap1 and CncC activates ecdysone-synthetic genes during oogenesis
- dKeap1 regulates adipogenesis genes in the fat tissue

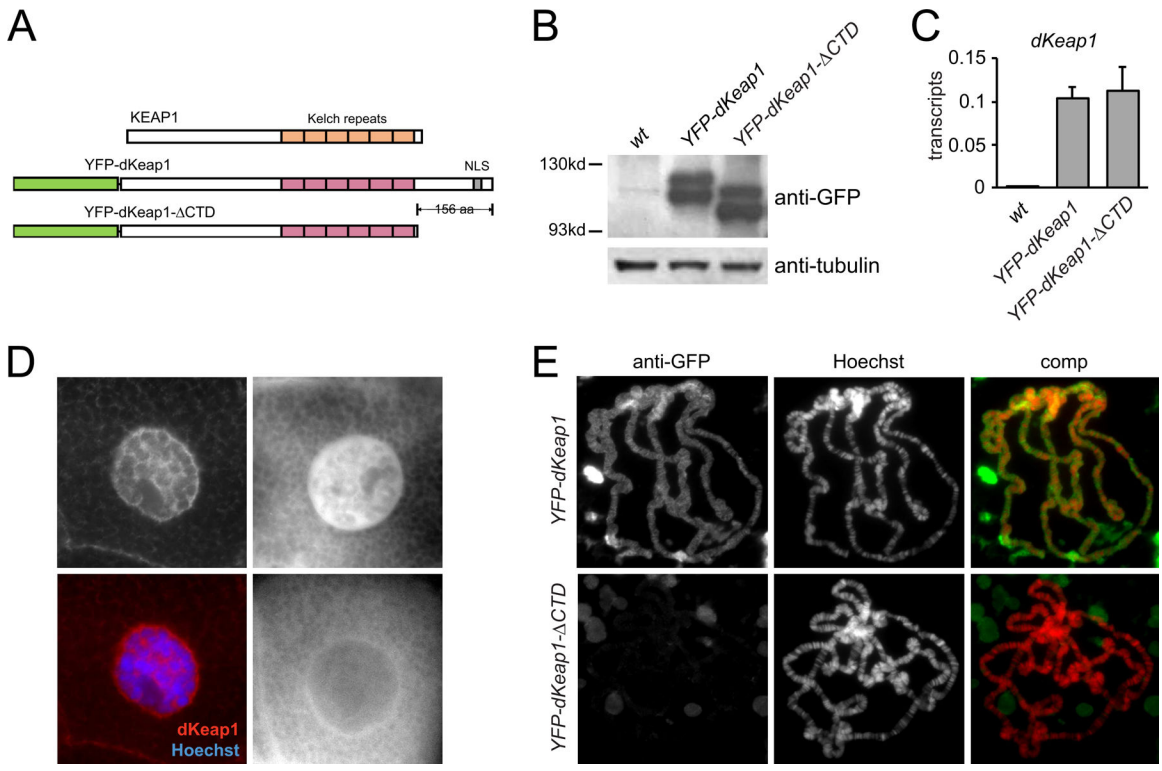

Fig.1. Removal of CTD abolishes dKeap1 nuclear localization and chromatin binding

(A) Comparison of human KEAP1, dKeap1, and dKeap1-ΔCTD. dKeap1 is conserved with KEAP1 except the extra 156 amino acids in the C-terminus, which contains a predicted monopartite NLS at amino acids 709–717 or a bipartite NLS at amino acids 709–741.

(B-C) Expression levels of dKeap1 fusion proteins. UAS-controlled *YFP-dKeap1* or *YFP-dKeap1-ΔCTD* were expressed using *tub-GAL4*. Protein extracts from L3 larvae that express fusion proteins indicated and from wildtype (*wt*) control larvae (*tub-GAL4/+*) were detected by western blotting using anti-GFP for fusion proteins or anti-tubulin as loading control (B). Transcription levels of *dKeap1* and *dKeap1* transgenes were measured by RT-qPCR in the same sets of L3 larvae (C).

(D) Subcellular localization of dKeap1 and dKeap1-ΔCTD. Left: Endogenous dKeap1 was detected by anti-dKeap1 immunostaining in a salivary gland cell from wildtype (*wt*) control larvae (*Sgs3-GAL4/+*). DNA was stained with Hoechst (blue). Right: Live imaging of YFP-dKeap1 or YFP-dKeap1-ΔCTD in salivary gland cells from L3 larvae expressing these fusion proteins using *Sgs3-GAL4*.

(E) Chromatin binding of dKeap1 and dKeap1-ΔCTD. Polytene chromosomes from salivary glands that express YFP-dKeap1 or YFP-dKeap1-ΔCTD using *Sgs3-GAL4* were spread and stained with anti-GFP (green) and Hoechst (red). YFP-dKeap1 is globally localized to interband regions while YFP-dKeap1-ΔCTD signal is not detected on the polytene chromosome. Scale bars: 10 μm.

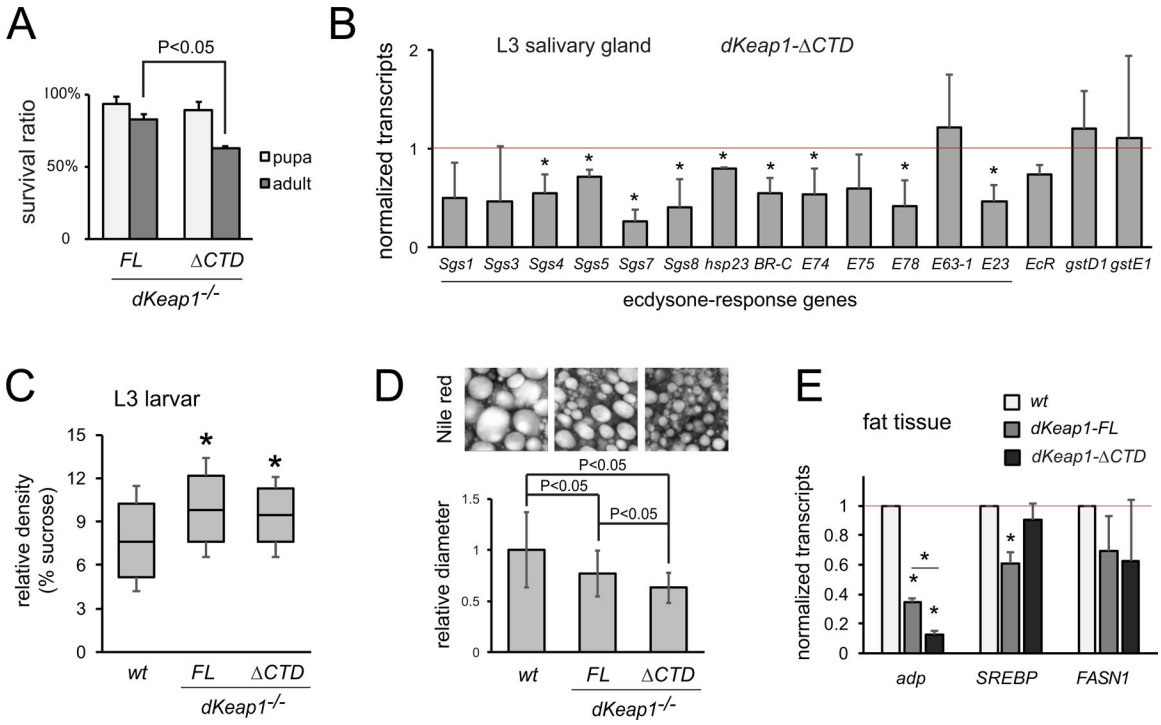

Fig. 2. Removal of dKeap1-CTD affects viability and developmental transcripts

(A) Viability of dKeap1 null flies rescued by YFP-dKeap1 (FL) or YFP-dKeap1-ΔCTD (ΔCTD). dKeap1 fusion proteins were expressed in the *dKeap1*$^{EY5/EY5}$ null mutation background using *tub-GAL4*. Survival ratios of the rescued flies were calculated by normalizing the numbers of pupae or adults to those of control flies (*dKeap1*^{EY5}*/TM6*) cultured in the same vials. For each genotype, standard deviation was calculated based on two independent experiments in each of which more than 100 flies were counted.

(B) Effect of dKeap1-ΔCTD on ecdysone-response transcripts. Transcription levels of the genes labeled below were measured by RT-qPCR in the salivary glands that express YFP-dKeap1 or YFP-dKeap1-ΔCTD in *dKeap1*$^{EY5/EY5}$ using *tub-GAL4*. All transcript levels were normalized with the levels of *Rp49* transcripts. To facilitate comparison, the transcript levels in ΔCTD cells were normalized to the levels of the transcripts in FL cells. Standard deviations were calculated based on three independent experiments (*, $p<0.05$).

(C) Effects of dKeap1-FL and ΔCTD on the amount of fat tissue. L3 larvae expressing YFP-dKeap1 or YFP-dKeap1-ΔCTD in *dKeap1*$^{EY5/EY5}$ using *tub-GAL4* as well as wildtype (*wt*) L3 larvae (*tub-GAL4/+*) were measured using a buoyancy-based assay. The relative density of larvae was represented by the concentration of sucrose solution that allowed the larvae to float. Expression of dKeap1-FL and dKeap1-ΔCTD increased larval density, suggesting a reduction of the low-density fat tissues in these larvae (*, $p<0.05$).

(D) Effects of dKeap1-FL and ΔCTD on lipid droplets in adipose cells. Fat tissues from L3 larvae as described in Fig. 2C were stained with the fluorescent lipophilic dye Nile red (upper panel, also see Fig S2C). Diameters of lipid droplets were measured and normalized to the average diameter of lipid droplets in wildtype cells. Smaller lipid droplets were observed in FL cells and even smaller lipid droplets were observed in ΔCTD adipose cells. Scale bars: 10 μm.

(E) Effects of dKeap1-FL and ΔCTD on the transcription of adipogenesis genes.
Transcription levels of the genes labeled below were measured by RT-qPCR in fat tissues isolated from L3 larvae expressing YFP-dKeap1 or YFP-dKeap1-ΔCTD in *dKeap1*$^{EY5/EY5}$ using *tub-GAL4* or control (*wt*) larvae (*tub-GAL4*/*TM6*). Transcript levels were normalized with the levels of *Rp49* transcripts. To facilitate comparison, the transcript levels were normalized to the levels in *wt* fat tissue. Standard deviations were calculated based on two independent experiments (*, $p<0.05$).

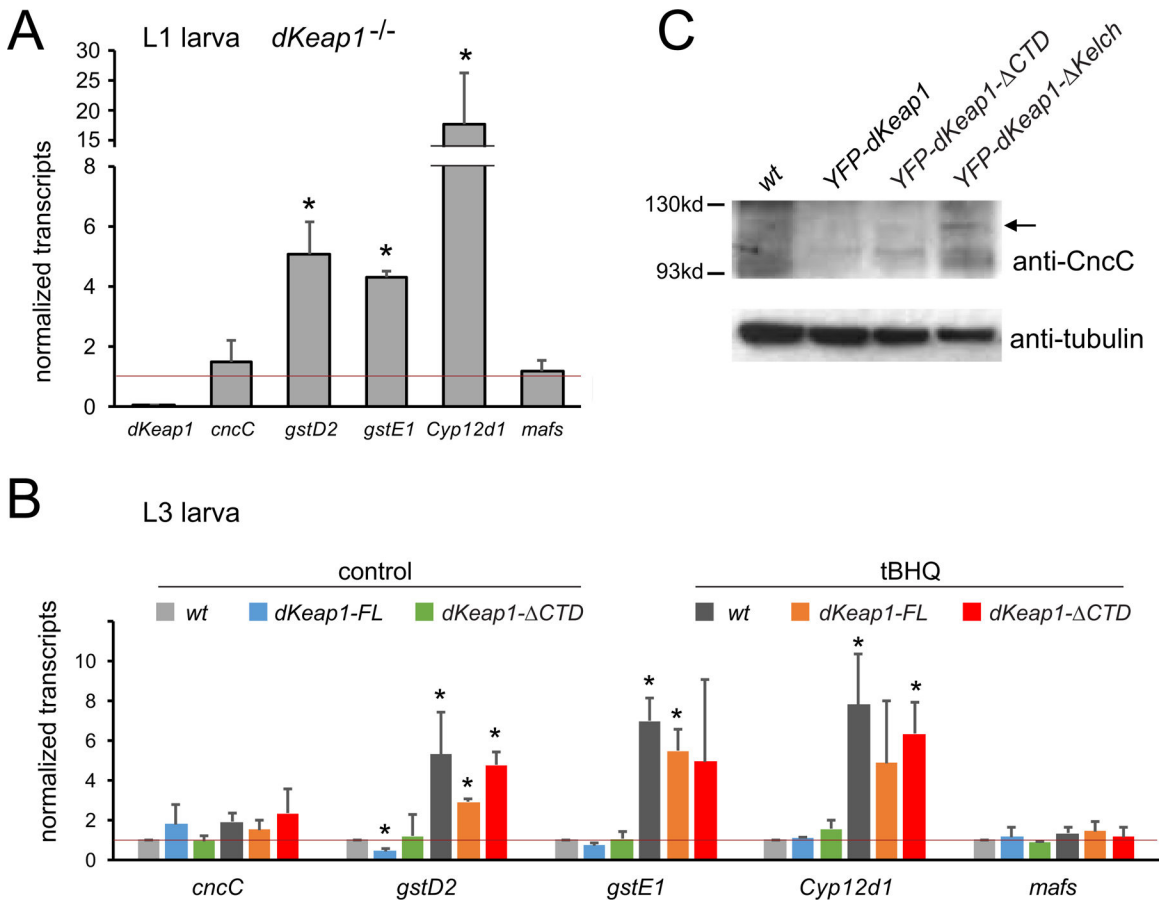

Fig. 3. Regulation of xenobiotic response transcripts by dKeap1 and dKeap1-ΔCTD

(A) Effect of dKeap1 knockout on the transcription of xenobiotic response genes. Transcription levels of the genes labeled below were measured by RT-qPCR in early L1 larvae of *dKeap1*$^{EY5/EY5}$ and control (*dKeap1*^{EY5}/*TM6*). All transcript levels were normalized with the levels of *Rp49* transcripts. To facilitate comparison, the transcript levels in *dKeap1*$^{EY5/EY5}$ were normalized to those in controls. Standard deviations were calculated based on two independent experiments (*, $p<0.05$). Knockout of dKeap1, verified by the abolished dKeap1 transcription, has no effect on *cncC* and *mafs* but significantly enhances selected xenobiotic response genes.

(B) Effect of dKeap1-FL and ΔCTD on the transcription of xenobiotic response genes. Transcription levels of the genes labeled below were measured by RT-qPCR in L3 larvae expressing YFP-dKeap1 or YFP-dKeap1-ΔCTD in *dKeap1*$^{EY5/EY5}$ using *tub-GAL4* or wildtype (*wt*) larvae (*tub-GAL4*/*TM6*) that were fed with tBHQ or vehicle (control). All transcript levels were normalized with the levels of *Rp49* transcripts. To facilitate comparison, the transcript levels were normalized to the levels of the transcripts in vehicle-feeding wildtype larvae. Standard deviations were calculated based on two independent experiments (*, $p<0.05$). Expressing either dKeap1-FL or ΔCTD can suppress transcription of xenobiotic response genes (compare with Fig. 3A) and has no effect on the activation of these genes by tBHQ.

(C) Effect of different dKeap1 fusion proteins on the protein levels of CncC. CncC levels were detected by western blotting in L3 larvae expressing the dKeap1 fusion proteins labeling above in *dKeap1*$^{EY5/EY5}$ using *tub-GAL4* or wildtype (*wt*) larvae (*tub-GAL4/TM6*). Endogenous CncC is undetectable in wt as well as FL or ΔCTD-rescued larvae. Upregulation of CncC (arrow) is detected in the *dKeap1*$^{EY5/EY5}$ null mutant that expresses a dKeap1 truncation lacking the CncC-interacting Kelch domain.

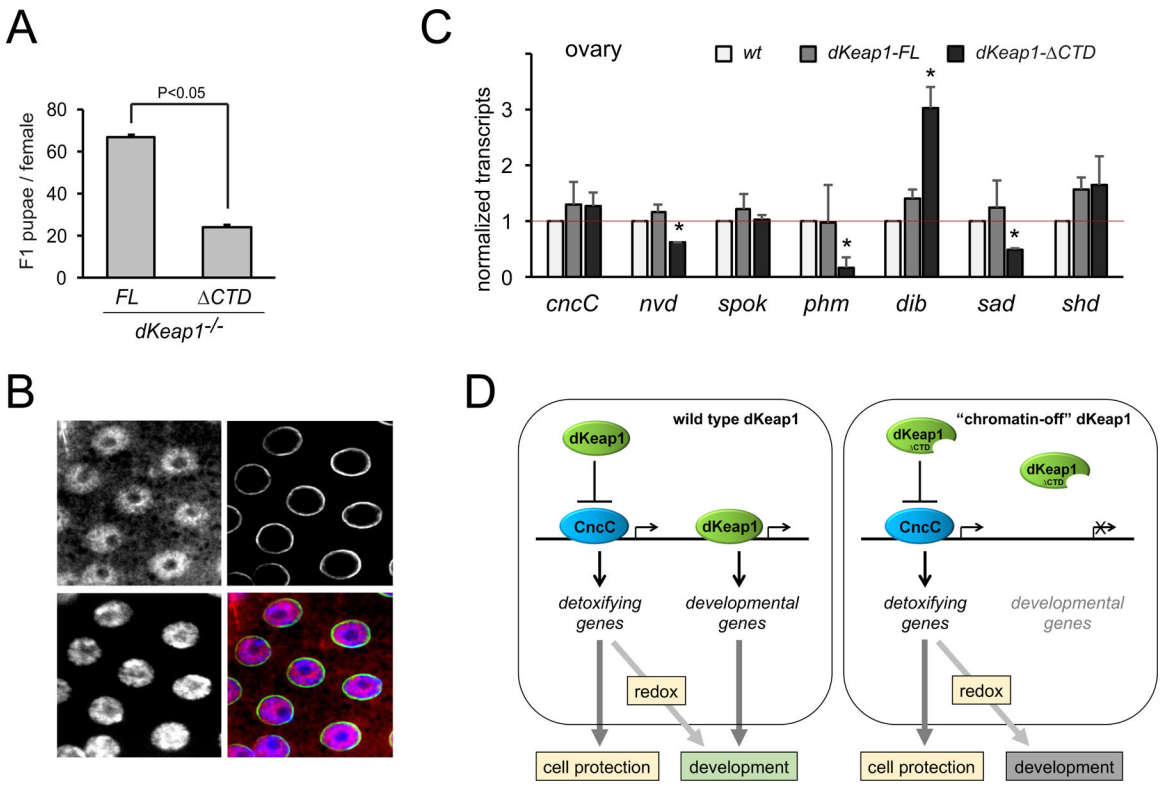

Fig. 4. Removal of dKeap1-CTD affects fertility and ecdysone synthetic transcripts in the ovary

(A) Female fertility of YFP-dKeap1 (FL) or YFP-dKeap1-ΔCTD (ΔCTD)-rescued flies. dKeap1 fusion proteins were expressed in *dKeap1*$^{EY5/EY5}$ using *tub-GAL4*. Numbers of F1 pupae were normalized to the numbers of FL or ΔCTD females that produced the progeny. Standard deviation was calculated based on two independent experiments.

(B) Localization of dKeap1 in ovarian follicle cells. Ovaries from wildtype flies (*w*^{1118}) were stained with antibodies against dKeap1 (red), Lamin Dm0 (green) and Hoechst (blue). Endogenous dKeap1 proteins were revealed in follicle cells with major localization in the nucleus. Scale bar: 2 μm.

(C) Effects of dKeap1-FL and ΔCTD on the transcriptions of ecdysone-synthetic genes in the ovary. Transcription levels of the genes labeled below were measured by RT-qPCR in ovaries isolated from flies expressing YFP-dKeap1 or YFP-dKeap1-ΔCTD in *dKeap1*$^{EY5/EY5}$ using *tub-GAL4* or wildtype (*wt*) larvae (*tub-GAL4/TM6*). Transcript levels were normalized with the levels of *Rp49* transcripts. To facilitate comparison, the transcript levels were normalized to the levels of the transcripts in *wt* ovaries. Standard deviations were calculated based on two independent experiments (*, $p<0.05$).

(D) Model of dKeap1 dual functions in both the detoxification pathway and the developmental pathway. In the conventional oxidative/xenobiotic response pathway, dKeap1 suppresses CncC and the transcription of detoxifying genes. In the developmental pathway, dKeap1 binds to chromatin and activates developmental genes. The binding of dKeap1 is mediated by the C-terminal domain of dKeap1.