Drosophila Keap1 xenobiotic response factor regulates nuclear architecture through interacting with B-type lamin

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Abstract

The Keap1-Nrf2 pathway is a central regulator that mediates transcriptional response to xenobiotic and oxidative stimuli. Recent studies revealed that Keap1 and Nrf2 can regulate transcription other than antioxidant and detoxifying genes, and the underlying mechanisms remain unclear. We have found that *Drosophila* Keap1 (dKeap1) and Nrf2 (CncC) proteins can control high-order chromatin structure including heterochromatin. Here, we describe evidence supporting that dKeap1 regulates heterochromatin through the interaction with lamin, the intermediate filament proteins that form nuclear lamina and organize chromatin architecture. dKeap1 forms complex with the *Drosophila* B-type lamin, lamin Dm0, in the nucleus. Ectopic expression of dKeap1 relocated lamin to the intra-nuclear area and caused a spreading of the heterochromatin marker H3K9me2 to euchromatin regions. Mis-regulated dKeap1 also disrupted the nuclear lamina morphology. Knock down of *dKeap1* partially rescued the lethality induced by lamin overexpression, suggesting that dKeap1 and lamin function in the same pathway during development. Taken together, these results support a model where dKeap1 regulates chromatin structure and transcription through interaction with lamin protein. This study reveals a novel epigenetic function of the Keap1 oxidative/xenobiotic response factor.

Introduction

The Keap1-Nrf2 pathway plays an essential role in cell protection through mediating transcriptional responses to environmental toxins (xenobiotics) and oxidative stimuli (Itoh et al., 1999, Slocum and Kensler, 2011). Mis-regulation of Keap1 and Nrf2 can lead to a variety of diseases, including cancer (Taguchi and Yamamoto, 2017), respiratory diseases (Carlson et al., 2020), neurodegenerations (Uruno et al., 2020), and cardiovascular diseases (Smith et al., 2016). Nrf2 (NF-E2-Related Factor 2) is a transcription factor that activates a series of antioxidant and detoxifying genes (Malhotra et al., 2010, Taguchi et al., 2011, Zhang, 2006). Under basal conditions, Keap1 (Kelch-like ECH-associated protein 1) binds to Nrf2 in the cytoplasm and targets Nrf2 for ubiquitination and degradation. Xenobiotic/oxidative compounds disrupt the Keap1-Nrf2 interaction, thereby releasing Nrf2 to enter the nucleus and activate target genes (Eggler et al., 2005, Itoh et al., 1999).

Recent studies have found that Keap1 and Nrf2 can also regulate developmental programs in several model systems (Deng, 2014, Pitoniak and Bohmann, 2015). Some of these developmental functions are mediated by reactive oxygen species (ROS) (Hochmuth et al., 2011, Zhou et al., 2021). Keap1 and Nrf2 can also directly target and activate some developmental genes. For example, Keap1 and Nrf2 proteins regulate adipogenesis genes in both mouse and *Drosophila* (Carlson et al., 2022, Huang et al., 2010, Kim et al., 2018, Pi et al., 2010). Mouse Nrf2 promotes cell proliferation by transcriptional activation of glucose metabolic enzymes (Mitsuishi et al., 2012) and promotes neuronal stem cell differentiation by activating genes that inhibit self-renewal (Khacho et al., 2016). CncC and dKeap1 promote *Drosophila* metamorphosis by activating ecdysone biosynthetic genes and response genes in specific tissues (Deng and Kerppola, 2013). The dKeap1-CncC signaling also plays a role in neuronal remodeling as CncC regulates dendrite pruning by activating gene expression of proteasomal subunits (Chew et al., 2021).

The mechanisms whereby Keap1/Nrf2 family proteins regulate developmental genes remain unclear. It is notable that in *Drosophila*, dKeap1 on one hand suppresses the CncC-activated transcription of detoxifying genes while on the other hand cooperates with CncC to activate developmental transcripts (Deng and Kerppola, 2014). This indicates that the dKeap1-CncC complex employs distinct molecular mechanisms to control detoxification and development. We previously found that dKeap1 and CncC function at both euchromatin and heterochromatin regions. dKeap1 and CncC activate ecdysone-response genes through binding to the highly-decondensed “puffs” on the polytene chromosome (Deng and Kerppola, 2013), while they are also required for gene silencing at the pericentromeric heterochromatin (Carlson et al., 2019). Given the roles of epigenetic mechanisms in transcriptional regulations of development, we hypothesize that Keap1-Nrf2 regulate developmental genes through controlling chromatin structure.

The nuclear lamina plays an important role in the organization and regulation of chromatin. In general, euchromatic regions of the chromatin tend to be centrally localized in the nucleoplasm while heterochromatic regions tend to be associated with the nuclear lamina (Foster and Bridger, 2005). The nuclear lamina is assembled by lamin intermediate filament proteins. In mammals and *Drosophila*, there are two types of lamin proteins: A-type lamin (lamin A/C in mammals; Lamin C in *Drosophila*) and B-type lamin (lamin B1/B2 in mammals; lamin Dm0 in *Drosophila*) (Adam and Goldman, 2012). Knockout of all three lamins in mice causes peripheral localization of euchromatin and central localization of heterochromatin; this defect is partially restored by adding back lamin B1 (Zheng et al., 2018). Lamins can interact with chromatin either directly through binding to histones (Goldberg et al., 1999, Hoskins et al., 2021) or indirectly through interactions with other proteins such as HP1, LBR, Emerin, BAF, MAN1, LAP2β, PRR14, and NET (Shevelyov and Ulianov, 2019, Towbin et al., 2013). Mutations in *lamin A* are associated with the Hutchinson-Gilford progeria syndrome, a genetic disease characterized by premature aging (Eriksson et al., 2003). The developmental roles of lamin proteins have been established in both mice and Drosophila model systems (Chen et al., 2013, Osouda et al., 2005, Vergnes et al., 2004). It is widely accepted that lamins should control development thought the regulation of chromatin architecture in the nucleus.

Since Keap1 and lamin proteins both regulate heterochromatin and development, we tested the hypothesis that Keap1 and lamin interact with each other and co-regulate chromatin architecture. Here, we demonstrate molecular and genetic interactions between dKeap1 and lamin Dm0 in *Drosophila*. We found that dKeap1 overexpression re-localized lamin Dm0 into the intra-nuclear area and resulted in the spreading of the heterochromatin marker H3K9me2. dKeap1 is also required for the maintenance of a normal unclear lamina morphology. We conclude that dKeap1 likely regulates heterochromatin structure and gene expression through its interaction with lamin.

Results And Discussion

1. dKeap1 and Lamin co-localize and form protein complexes in the nucleus

Lamin Dm0 (also named as “Lamin” in Flybase, stated as “Lamin” in this article) is the major type of lamin protein that forms the nuclear lamina in most of the *Drosophila* tissues. To explore the molecular function of dKeap1 in heterochromatin architecture, we tested whether dKeap1 associates with Lamin. We first visualized the subcellular localization of dKeap1 and Lamin in salivary gland cells via co-immunostaining (Fig. 1A). Although most of the dKeap1 immunosignals were in the nucleoplasm, co-localization of dKeap1 and Lamin signals were detected at the nuclear lamina, indicating that some dKeap1 proteins localize to the nuclear lamina.

We next visualized the potential formation of dKeap1-Lamin complexes in the *Drosophila* S2 cell line using the bimolecular fluorescence complementation (BiFC) assay (Hu *et al*, 2002). In the BiFC assay, the N-terminus and C-terminus of YFP (YN and YC) were fused to dKeap1 and Lamin, respectively (Fig. 1B). Fluorescence signals representing BiFC complexes were detected in nuclei of S2 cells that co-expressed YN-dKeap1 and YC-Lamin (Fig. 1B). As a negative control, no BiFC signal was detected in S2 cells that expressed BiFC fusions for dKeap1 and a cardiac transcription factor Tinman (Liu et al. , 2009). Therefore, dKeap1 and Lamin can form specific complexes in the nucleus.

2. Ectopic dKeap1 re-distributes Lamin and heterochromatin

dKeap1-Lamin BiFC complexes were detected mainly in the nucleoplasm of S2 cells (Fig. 1B). This is in contrast with the peripheral localization of endogenous Lamin in the nucleus (Fig. 1A). We hypothesized that the mis-localization of dKeap1-Lamin BiFC complex was caused by the overexpression of dKeap1 fusion proteins. To test this possibility, we overexpressed the UAS-controlled dKeap1 full length (FL) fusion protein (Fig. 2A) in salivary gland cells using *Sgs3-GAL4* (Cherbas et al. , 2003). Significant amounts of Lamin proteins were detected in the nucleoplasm, with a pattern partially overlapping with dKeap1 fusion proteins (Fig. 2B). The levels of Lamin proteins were not altered upon dKeap1 overexpression (Fig. 2C). Therefore, overexpression of dKeap1 proteins caused a redistribution of Lamin proteins from the original peripheral sites to the center area of the nucleus.

A similar Lamin redistribution phenotype was also observed in salivary gland cells that overexpressed YFP-dKeap1-ΔKelch, a dKeap1 truncation lacking the CncC-interacting Kelch domain (Fig. 2A,B). The signals of YFP-dKeap1-ΔKelch were mainly detected in the nucleus. As this truncation induced the same effects as YFP-dKeap1-FL did, we concluded that the dKeap1-CncC interaction is not involved in the relocation of lamina by ectopic dKeap1. Expressing dKeap1 N-terminal deletion (YFP-dKeap1-ΔNTD) or C-terminal deletion (YFP-dKeap1-ΔCTD) had no or only moderate effect on Lamin distribution. YFP-dKeap1-ΔCTD localized almost exclusively to the cytoplasm (Fig. 2B) (Carlson et al., 2022). YFP-dKeap1-ΔNTD localized to both the cytoplasm and nucleus, and the ratio of different portions varied in different cells (Fig. 2B). The nuclear accumulation of dKeap1-ΔNTD had no significant effect to the Lamin distribution, indicating that the N-terminal domain of dKeap1 is required for the relocation of Lamin. The expression levels of all the fusion proteins were comparable and none of them altered Lamin protein levels (Fig. 2C; Fig. 3B), indicating that ectopic dKeap1 proteins induced Lamin re-distribution rather than altering Lamin protein levels.

Intra-nuclear distributions of lamin proteins have been found in embryonic stem cells and some adult stem cells, presumably associated with a distinct global chromatin architecture in these cells (Dorland et al. , 2019, Meshorer and Misteli, 2006). Intra-nuclear lamins and abnormal nuclear lamina are also revealed in aging cells (Scaffidi and Misteli, 2006). Overexpression of dKeap1 fusion proteins cannot fully rescue the viability and fertility of the *dKeap1* null mutants (Carlson et al., 2022). We hypothesize that this developmental defect is associated with the dKeap1-induced relocation of Lamin and the consequent mis-organization of heterochromatin.

To test this hypothesis, we investigated the localization of heterochromatin marker histone H3K9me2 in salivary gland cells. In wildtype cells, H3K9me2 is found at the chromocenter, the pericentric heterochromatin region on the polytene chromosome (Fig. 2D) (Zhang et al. , 2006). Significant relocations of H3K9me2 immuno-signals to loci outside of the chromocenter were detected in nuclei with dKeap1 overexpression (Fig. 2D, Fig. S1A). dKeap1 overexpression had no effect on the level of H3K9me2 (Fig. 2E), suggesting that dKeap1 overexpression caused a spreading of H3K9me2 from the pericentric region to chromosome arms. The relocation of H3K9me2 is likely caused by ectopic dKeap1 and Lamin accumulation in the center area of the nucleus. On the other hand, *dKeap1* knockout reduced the level of H3K9me2 (Fig. 2E), consistent with our previous finding (Carlson et al., 2019). All these results support a positive role of dKeap1 in heterochromatin formation and/or maintenance.

3. Mis-regulated dKeap1 proteins disrupt nuclear lamina

Severely defected nuclear lamina morphologies were observed when the dKeap1 fusion proteins were expressed in a *dKeap1* null background. All the truncations, when expressed in the *dKeap1* null background, caused dramatic Lamin redistribution to the nucleoplasm (Fig. 3A). Interestingly, expression of dKeap1-FL, ΔNTD or ΔCTD altered the morphologies of nuclear laminas and the shapes of nuclei (Fig. 3A), regardless of whether the fusion proteins were primarily in the nucleus or cytoplasm. In around 30% of cells that expressed ΔCTD, nuclear shapes and organizations were the most severely affected. These cells showed partial nuclear fragmentation and potential breakdown of the nuclear envelope as indicated by the invading of ΔCTD fusion proteins from the cytoplasm into the nucleus (Fig. 2B; Fig. 3A). The re-distributed Lamin and defected nuclear lamina were also seen in other cell types such as the diploid follicle cells and polyploid nurse cells in ovaries (Fig. S1B).

The lack of native dKeap1 proteins in the *dKeap1* null background should account for the severe Lamin defects induced by ectopic dKeap1 fusions. However, no significant Lamin defect was detected in cells of the *dKeap1* null larvae (Fig. S1C). Intrinsic dKeap1 proteins localized to both the nucleoplasm and the nuclear lamina (Fig. 1A). However, none of the dKeap1 fusion proteins showed localization to the nuclear lamina (Fig. 2B), indicating that the YFP-dKeap1 fusions cannot function the same as intrinsic dKeap1 proteins in the nuclear lamina. In support of this, overexpression of YFP-dKeap1-FL largely but cannot fully rescue the viability and fertility of the *dKeap1* null mutant (Carlson et al., 2022). It is possible that the YFP tag interferes with the interaction of dKeap1 and Lamin. Taken together, we concluded that the disrupted Lamin morphology is a combinatory effect of both the ectopic expression dKeap1 fusion

proteins and the lack of endogenous dKeap1 proteins. Since that the dKeap1-ΔCTD induced the worst nuclear lamina disruption, the C-terminal domains of dKeap1 may play the most significant role in the maintenance of a normal nuclear lamina shape.

The lamin defects induced by mis-regulated dKeap1 is comparable but different to several other lamin phenotypes that have been reported. In *Drosophila*, reduction of a WAS family protein wash in salivary gland cells results in patternless strands of Lamin in the nucleoplasm (Verboon et al. , 2020). Overexpression of Lamin or an inner nuclear membrane protein Kugelkern result in “blebs”, “invagination” and “lobulation” of nuclear envelopes in different cell types (Brandt et al. , 2008, Polychronidou et al. , 2010, Uchino et al. , 2017). In mammalian cells, mutations in *Lamin A* or *Lamin B1* lead to “blebs”, “donuts”, and “honeycomb” defects of nuclear lamina (Jung et al. , 2013, van Tienen et al. , 2019). Compared to these phenotypes, the honeycomb-like lamina defect seen in our study shows much larger “holes” throughout the entire nucleus. We concluded that dKeap1 is directly involved in the maintenance of nuclear lamina morphology.

4. Molecular interaction between dKeap1 and Lamin

We examined the molecular interaction of dKeap1 and Lamin using the co-immunoprecipitation assay. Lamin immunoblotting signal was detected in the anti-GFP precipitation from embryos that expressed *YFP-dKeap1* (Fig. 4A), indicating that the overexpressed dKeap1 fusion proteins interacts with Lamin. The antiserum against dKeap1 also co-precipitated Lamin proteins from the lysate of wildtype embryos (Fig. 4B), suggesting that endogenous dKeap1 and Lamin form protein complexes *in vivo*.

To examine whether dKeap1 and Lamin directly interact with each other, the *in vitro* GST-pull down assay was conducted (Fig. 4C). GST-dKeap1 and GST-Lamin were expressed in and purified from *E. coli*. dKeap1 and Lamin proteins were generated by removal of the GST tag using protease. Both GST-dKeap1 and GST-Lamin were able to pull down Lamin and dKeap1, respectively (Fig. 4C). Therefore, dKeap1 and Lamin physically interact with each other.

The mechanism by which dKeap1 modulates chromatin structure in cooperation with Lamin remains to be elucidated. Lamin proteins can directly bind nucleosomes and can also regulate chromatin through interactions with other proteins (Shevelyov and Ulianov, 2019, Towbin et al., 2013). dKeap1 can bind to the euchromatin polytene chromosome arms using the C-terminal tail (Carlson et al., 2022). It is possible that dKeap1 interacts with heterochromatin through Lamin. It is also possible that Lamin controls chromatin structure through dKeap1 chromatin binding. We have found that CncC, the key interaction partner of dKeap1, also facilitates heterochromatin formation (Carlson et al., 2019). The molecular and biological functions of CncC in the dKeap1-Lamin complex and pathway remain to be explored.

5. dKeap1 functions downstream of Lamin in the genetic pathway

Given that dKeap1 molecularly interacts with Lamin and regulates Lamin localization, we hypothesized that dKeap1 and Lamin coregulate transcription. To determine if dKeap1 and Lamin regulate gene

expression in the same developmental pathway, we explored potential genetic interactions between *dKeap1* and *Lamin*. Lamin overexpression causes early lethality in *Drosophila* (Munoz-Alarcon et al. , 2007). In our experiments, larvae that overexpressed Lamin driven by *tub-GAL4* died at L1 or early L2 larval stage (Fig. 5A). Double mutants which contained overexpressed Lamin and a heterozygous *dKeap1* null allele survived to late L2 or early L3 stage (Fig. 5A), indicating that reduction of dKeap1 was able to partially rescue the lethality caused by Lamin overexpression. Excessive Lamin proteins could cause lethality via mis-regulation of developmental transcription. These results suggest that dKeap1 can act down-stream of Lamin in the regulation of gene expression during *Drosophila* development.

Mutations in *lamin Dm0* show reduced viability, abnormal tissue differentiation, and defects in fertility, ovary size, ventriculus, and locomotion (Lenz-Bohme et al. , 1997, Munoz-Alarcon et al., 2007, Osouda et al., 2005). A dKeap1 mutant with disrupted dKeap1 chromatin binding also shows reduced viability and fertility (Carlson et al., 2022). It would be interesting to determine the developmental genes and programs that are coregulated by dKeap1 and Lamin in the future.

Conclusions

In contrast to the well-established model in which Keap1-Nrf2 controls antioxidant/detoxifying transcription, the mechanisms whereby Keap1-Nrf2 regulates other genes remains unclear. Interestingly, dKeap1 functions both in gene activation as transcription activators and in gene silencing as a heterochromatin modifier (Carlson et al., 2022, Carlson et al., 2019). We hypothesized that dKeap1 conducts these opposite roles through interacting with different protein partners. In this study, we revealed that the dKeap1-Lamin complex likely controls heterochromatin architecture in the nucleus, which would indicate a novel epigenetic mechanism whereby Keap1 family proteins regulate transcription (Fig. 5B). Our model is supported by these observations: First, dKeap1 and Lamin molecularly interact with each other. Second, ectopic dKeap1 expression causes a relocation of Lamin to the center area of nuclei, which is likely mediated by the N-terminus of dKeap1. Third, dKeap1 is required for the maintenance of a normal nuclear lamina morphology, in which the C-terminus of dKeap1 likely plays an important role. Fourth, ectopic dKeap1 causes a spreading of the heterochromatin marker from the pericentric heterochromatin region to the euchromatic chromosome arms. Finally, the genetic interaction between *dKeap1* and *Lamin* suggests that they act in the same developmental pathway.

It is thought that epigenetic mechanisms can mediate transcriptional responses to oxidative/xenobiotic stimuli. Environmental epigenetic studies have revealed broad influences of environmental toxins on human epigenomes and disease (Feil and Fraga, 2012, Marsit, 2015). However, the molecular factors that bridge epigenetic alterations and environmental stimuli are less understood. Keap1 family proteins are the primary cellular sensors for xenobiotic stresses. The novel dKeap1-Lamin interaction revealed in this study luminates a hypothesis in which the dKeap1-Lamin pathway mediates epigenetic and transcriptional adaptations to environmental toxins. It remains to be explored whether the dKeap1-Lamin complex responds to xenobiotic stimuli, how dKeap1-Lamin controls nuclear architecture and the epigenome, and the full range biological functions of the dKeap1-Lamin pathway. These studies are also

expected to broaden our understanding of the essential but complicated roles of Keap1 and Nrf2 in human diseases.

Materials And Methods

Drosophila stocks

Fly stocks were maintained at 25°C according to standard protocol. Constructs of *UAS-YFP-dKeap1-FL*, *UAS-YFP-dKeap1-ΔNTD, UAS-YFP-dKeap1-ΔKelch,* or *UAS-YFP-dKeap1-ΔCTD* were generated in pUAST vector and injected into the w^{1118} background. *Sgs3-GAL4*, *tub-GAL4*, and *UAS-YFP-Lamin* (BL7376) were from the Bloomington Stock Center. $dKeap1^{EY5}$ was provided byDirk Bohmann (Sykiotis and Bohmann, 2008). $dKeap1^{EY5}$ and *tub-GAL4* are combined with the *TM6,Tb,Sb,Hu,e,Dfd-YFP (TM6)* balancer. Appropriate progenies were identified based on *Tb* marker in larvae and pupae or *Sb* marker in adults. Embryos with or without the *Dfd-YFP* marker were sorted under a Leica MZ10 F fluorescence stereomicroscope.

Immunofluorescence

Salivary glands isolated from L3 larvae and ovaries isolated from adults are fixed in 3.7% paraformaldehyde for 5 minutes. Tissues were then washed with PBST (PBS + 0.2% Triton X-100) and stained with anti-lamin Dm0 (1:500, ADL67.10, Developmental Studies Hybridoma Bank) or anti-GFP (1:200, Novus, NB600) at 4°C overnight. After washing with PBST, the tissues were stained with 1:2000 goat anti-mouse Alexa Fluor 594 and goat anti-rabbit Alexa Fluor 488 secondary antibodies (Invitrogen) at 25°C for 2 hours, washed with PBST, and then stained with Hoechst in PBS for 10 minutes. Slides were mounted in Vectashield (Vector Laboratories) and imaged using a Zeiss LSM 710 confocal microscope.

Co-immunoprecipitation

UAS-YFP-dKeap1/+; tub-GAL4/+ embryos were collected on apple juice plates. Approximately 2000 embryos were ground in IP buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 11 mM EDTA, 0.2% Triton X-100, 0.2% NP-40, 2 mM Na_3VO_4, 1 mM PMSF, and 1.5 µg/ml aprotinin). 10 µl of GFP antibody (Novus, NB600) or negative control antibody normal rabbit IgG (Cell Signaling) was added to the cell lysate and the samples were rotated at 4°C overnight. For each IP, 30 µl pre-blocked Protein G agarose beads (Cell Signaling) were added to the lysate-antibody solution and rotated for 2 hours at 4°C. Beads were spun down and the proteins were eluted using SDS-PAGE dye at 100°C for 10 minutes, followed by western blotting.

Western blotting

Proteins were separated using 4-12% Bis-Tris gels (Invitrogen) and transferred to nitrocellulose membranes (Bio-Rad). Membranes were blocked with 5% milk in TBST (TBS + 0.1% Tween-20) and then probed with primary antibodies against lamin Dm0 (1:400, Developmental Studies Hybridoma Bank

ADL67.10), dKeap1 (1:100) (Deng and Kerppola, 2013), tubulin (1:500, Developmental Studies Hybridoma Bank 12G10), H3K9me2 (1:200 , abcam ab1220), Histone H3 (1:1000, Proteintech 17168), GFP (1:500, Novus NB600), or GST (1:500, Genscript A00865), followed by 1:3000 HRP-conjugated Goat anti-rabbit or Goat anti-mouse secondary antibodies (Bio Rad). The membranes were developed by ECL reagents (GE Healthcare) and then exposed to X-ray film (AGFA).

GST pull down

Full length *dKeap1* and *Lamin* were cloned into pGEX plasmids (*Lamin* plasmid was provided by Kristen Johansen). *E. coli* BL21 competent cells were transformed with the plasmids, grown to an OD600 of ~1.0 and induced with 0.1 mM IPTG for 2 hours. To generate proteins without the GST tag, GST-Lamin and GST-dKeap1 proteins were treated with PreScission Protease (APExBIO). GST pull downs were conducted using Glutathione Sepharose 4B beads (Cytiva 17075601) according to the accompanying protocol. GST-tagged Lamin or dKeap1 proteins were bound to beads and incubated untagged dKeap1 or Lamin, respectively. The GST-protein complexes were eluted from the beads. The primary (E1) and secondary (E2) elutes from the beads were analyzed by western blotting. In negative controls, proteins were pulled down with beads only.

Bimolecular Fluorescence Complementation (BiFC) assay

Drosophila Schneider 2 (S2) cells were cultured according to standard protocols (Thermo Fisher Scientific) and transfected with *pMT-GAL4* (*Drosophila* Genomics Resource Center) and *pUAST* plasmids containing YN and YC fusions using the calcium phosphate transfection kit (Invitrogen). 10 mg of each construct were used. After 24 hours, the cells were spun down, resuspended in PBS, and imaged using a Zeiss LSM 710 confocal microscope.

Genetic Assays

To generate *Lamin* overexpression flies in combination with or without *dKeap1* knockdown, *UAS-GFP-Lamin* were crossed with either *tub-GAL4/TM6,Tb,Sb,Hu,e,Dfd-YFP* or $dKeap1^{EY5}$*,tub-GAL4/TM6,Tb,Sb,Hu,e,Dfd-YFP*. L1 larvae were cultured on apple juice plates and appropriate genotypes (*UAS-GFP-Lamin/+; EY5, tub-GAL4/+* and *UAS-GFP-Lamin/+; tub-GAL4/+*) were sorted using a Leica MZ10 F fluorescence stereomicroscope based on the ubiquitin *GFP-Lamin* fluorescence and the absence of the *Dfd-YFP* marker. Larvae were counted daily to assess the number of larvae that survived to specific stages. T-tests were used for statistical analysis.

Declarations

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Competing interest statement

The authors declare that they have no competing interest.

Availability of data and material

The data used to support the findings of this study are included within the article.

Author contributions

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

Ethics approval

All of the material is owned by the authors and/or no permissions are required.

The manuscript in part or in full has not been submitted or published on another peer-reviewing journal. The manuscript has been preprinted on bioRxiv (https://doi.org/10.1101/2022.04.27.489742).

Consent to participate

This study did not involve human subjects.

Consent to publish

all the authors mentioned in the manuscript have agreed for authorship, read and approved the manuscript, and given consent for submission and subsequent publication of the manuscript.

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Figures

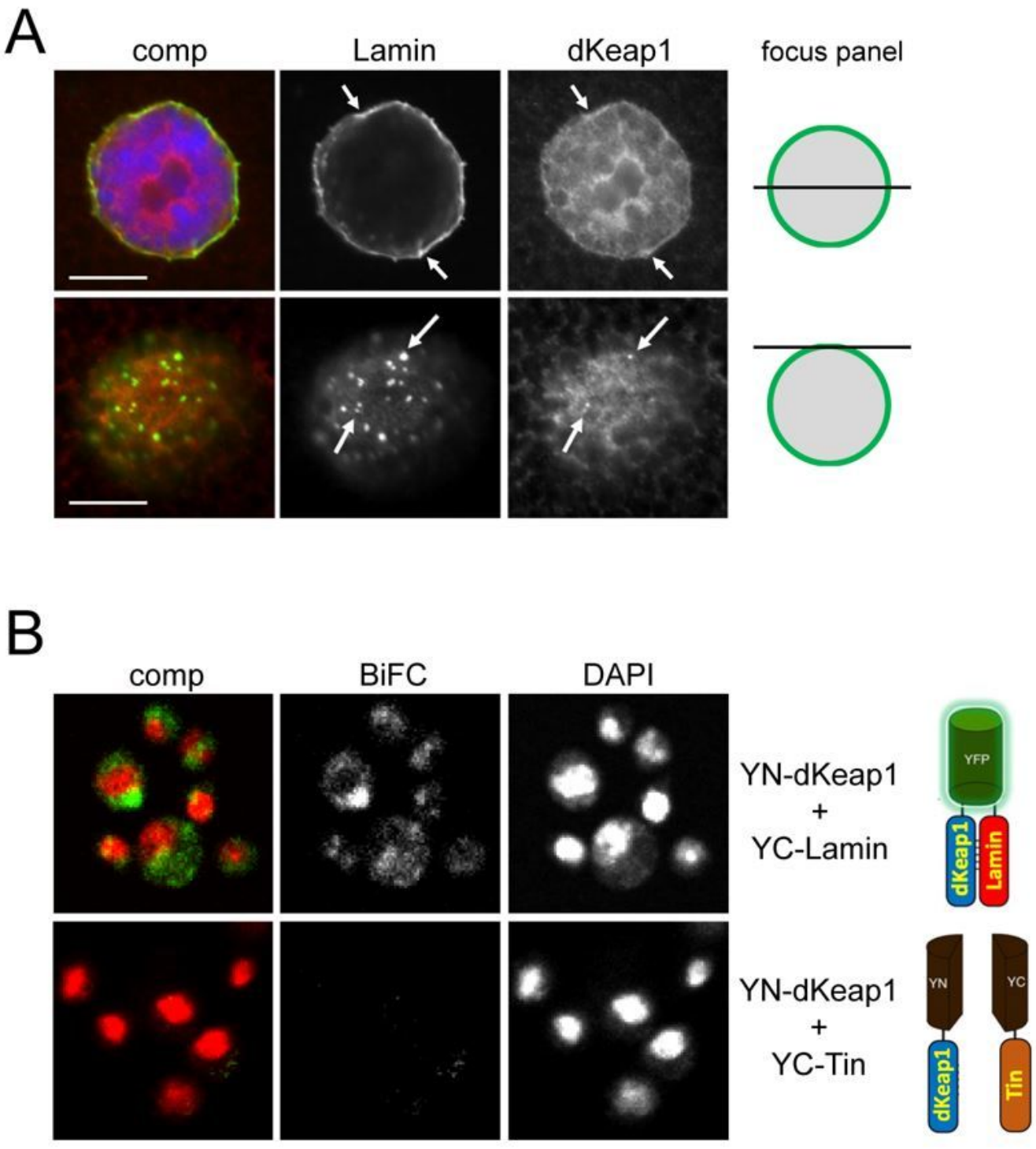

Figure 1

dKeap1 and Lamin form a complex in the nucleus

A. Subcellular localization of dKeap1 and Lamin. Wildtype (Oregon R) salivary gland cells were immunostained with anti-Lamin (green) and anti-dKeap1 (red). DNA was stained with Hoechst (blue). The same nucleus was visualized in cross-section (top) and top surface (bottom). Selective loci where dKeap1 colocalized with Lamin are indicated by arrows. Scale bars: 10 μm.

B. dKeap1-Lamin BiFC assay. S2 cells were transfected with *YN-dKeap1* and *YC-Lamin* or *YN-dKeap1* and *YC-Tin* (negative control). YFP florescence (green) represents the formation of dKeap1-Lamin BiFC complexes. DNA was labeled by DAPI and pseudo-colored as red. The diagrams on the right depict BiFC assay.

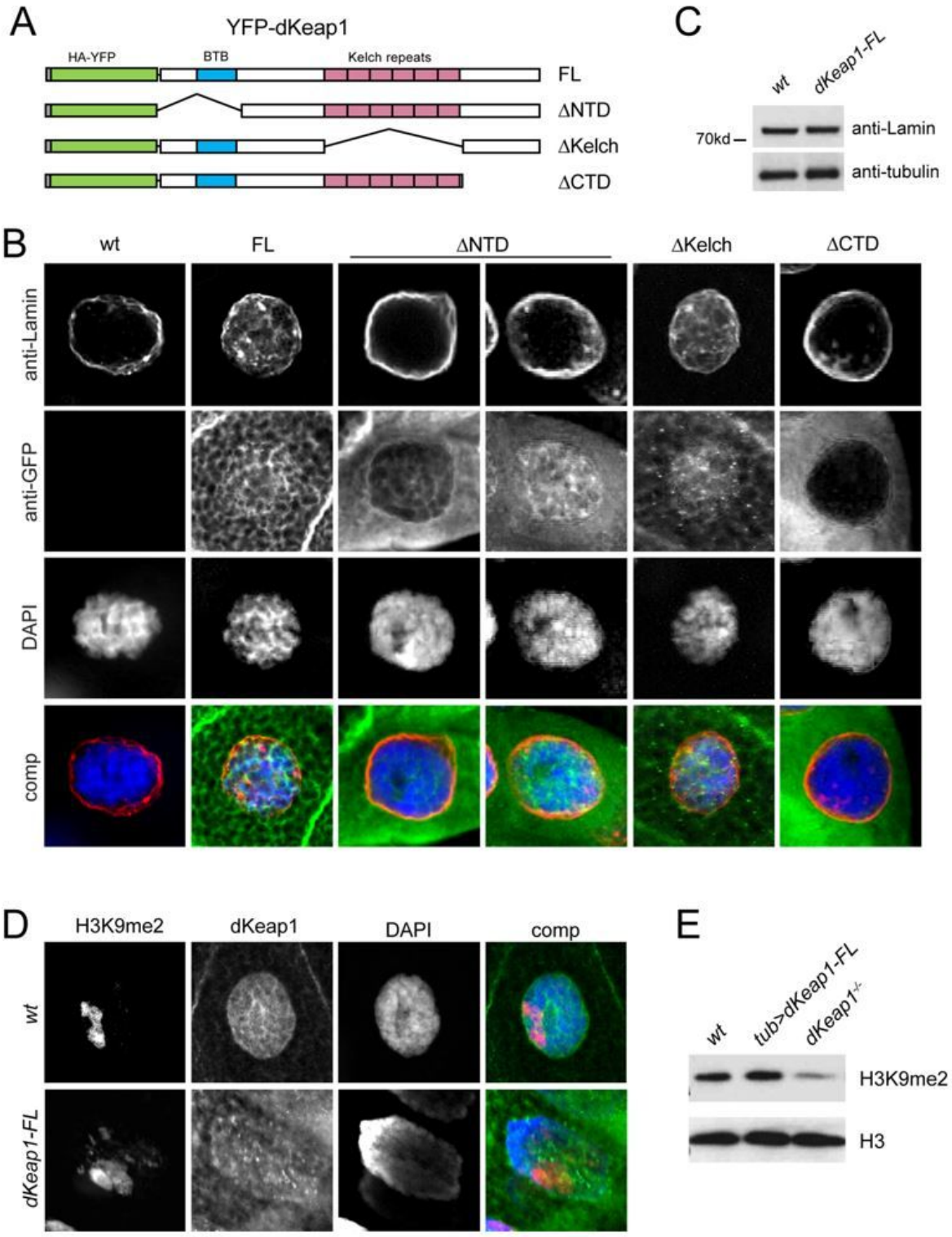

Figure 2

Ectopic dKeap1 relocalizes Lamin and heterochromatin

A. Diagram of dKeap1 fusion proteins. dKeap1 full length (FL) and truncated proteins including ΔNTD (missing the N-terminus and BTB domain), ΔKelch (missing the Kelch repeats), and ΔCTD (missing the C-terminal domain) were tagged by HA and YFP.

B. Effects of dKeap1 overexpression on Lamin. UAS-controlled dKeap1 fusion proteins described in A were expressed in the salivary glands via *Sgs3-GAL4*. Resulting salivary glands were immunostained with anti-GFP (green) and anti-Lamin (red). DNA was stained with Hoechst (blue).

C. Lamin protein levels in dKeap1 overexpression flies. Lamin levels in wildtype (*wt*) L3 larvae and L3 larvae that overexpressed *YFP-dKeap1-FL* using *tub-GAL4* were examined via western blotting. Anti-tubulin was used as a loading control.

D. H3K9me2 distributions in salivary gland nuclei. Salivary glands from wildtype larvae or larvae that expressed *YFP-dKeap1-FL* using *tub-GAL4* were immunostained with anti-H3K9me2 (Red) and anti-GFP (green). DNA was stained with DAPI.

E. Levels of H3K9me2 in dKeap1 overexpression or knock out larvae. H3K9me2 levels were detected in early L1 larvae of wildtype (*wt*), YFP-dKeap1-FL overexpression (driven by *tub-GAL4*), and $dKeap1^{EY5/EY5}$ null mutant (lethal at early L1 stage) using western blotting. Levels of histone H3 were used as the loading control.

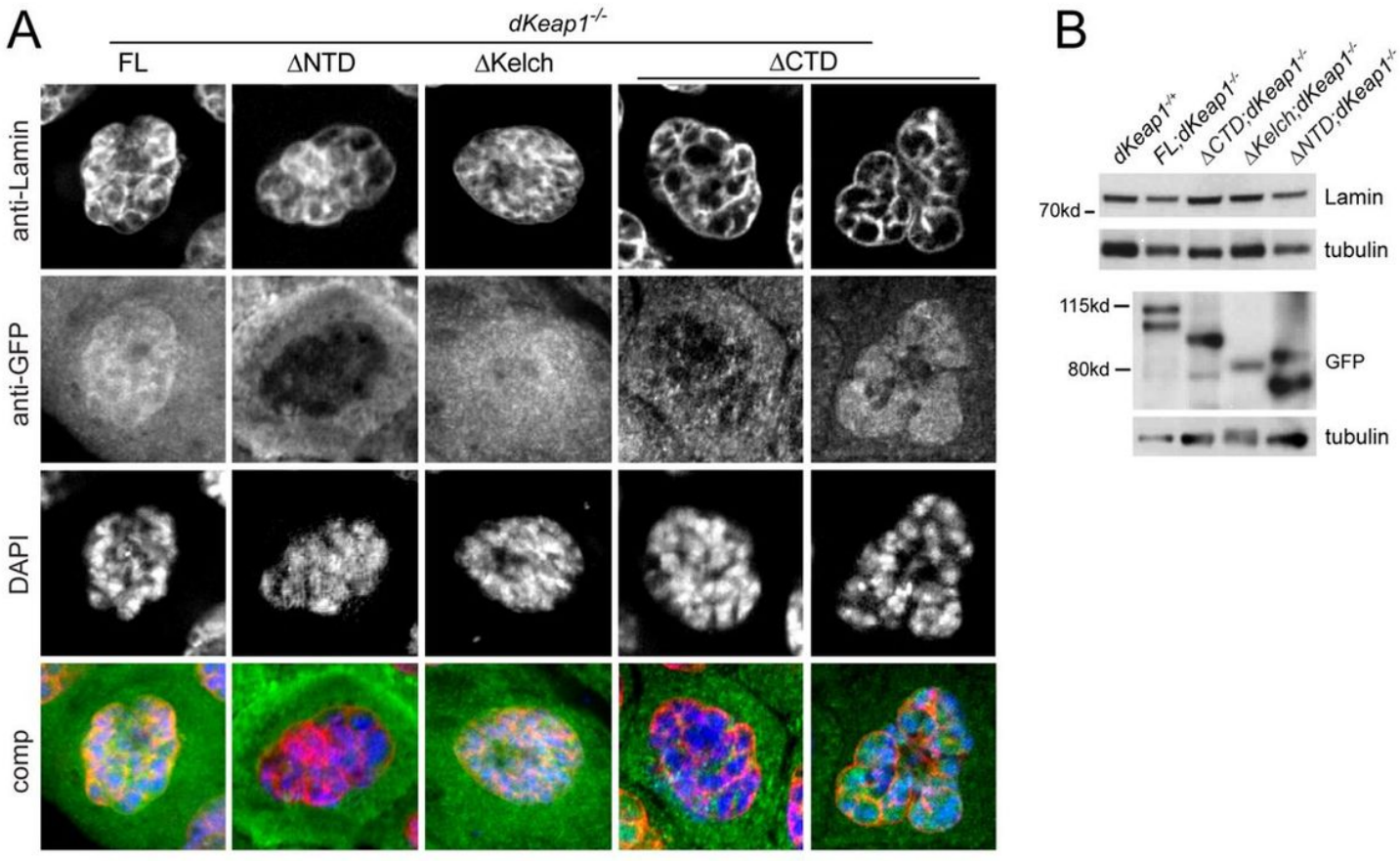

Figure 3

Mis-regulated dKeap1 disrupts nuclear lamina morphology

A. Effects of dKeap1 overexpression on Lamin in a dKeap1 null background. UAS-controlled dKeap1 fusion proteins described above were expressed in the *dKeap1*$^{EY5/EY5}$ null mutant using *tub-GAL4*. Resulting salivary glands were immunostained with anti-GFP (green) and anti-Lamin (red). DNA was stained with Hoechst (blue).

B. Protein levels of Lamin and ectopic dKeap1. Protein levels of Lamin and YFP-dKeap1 fusions in L3 larvae with genotypes described above were examined via western blotting using anti-Lamin and anti-GFP, respectively. Anti-tubulin was used as a loading control.

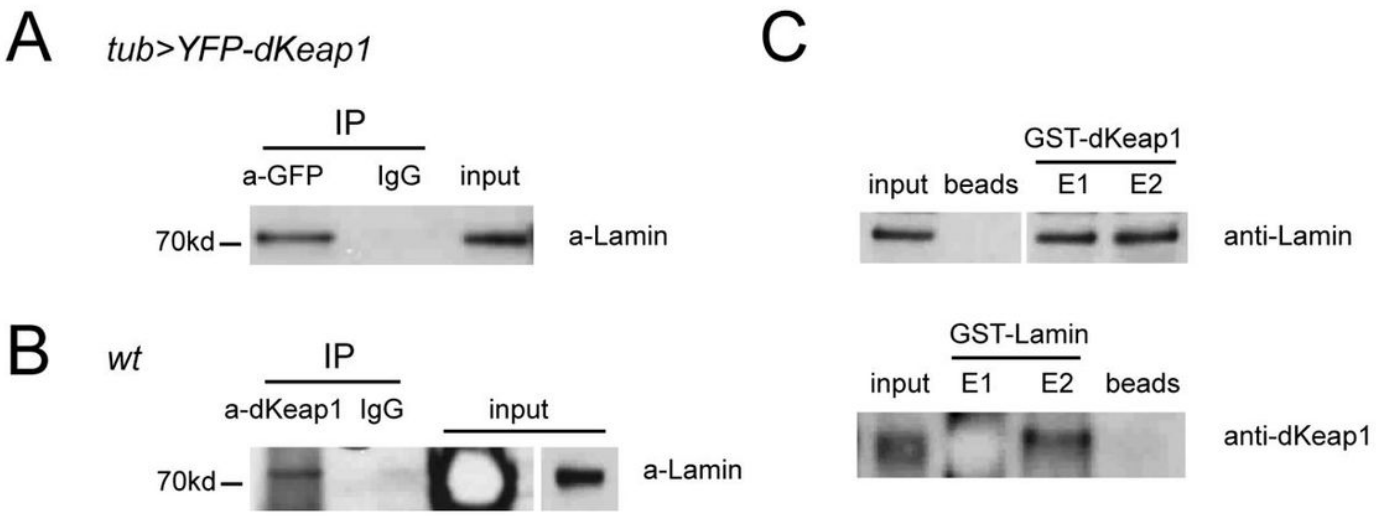

Figure 4

Molecular interactions between dKeap1 and Lamin

A. Coimmunoprecipitation of ectopic dKeap1 and Lamin. YFP-*dKeap1* expressed by *tub-GAL4* in embryos were precipitated with anti-GFP or IgG control. Lamin proteins were detected in the input and precipitations by western blotting.

B. Coimmunoprecipitation of endogenous dKeap1 and Lamin. Wildtype embryos were precipitated with anti-dKeap1 serum or IgG control. Lamin proteins were detected in the input and precipitations by western blotting. A short exposure of the Lamin signal in the input was shown at right.

C. GST pull down of dKeap1 and Lamin. GST-dKeap1 or GST-Lamin proteins were mixed with Lamin or dKeap1 proteins, respectively, and pulled down using Glutathione Sepharose 4B beads. In negative controls, Lamin or dKeap1 proteins were pulled down with beads only. The primary (E1) or secondary (E2) elutes from the beads were probed with anti-dKeap1 or anti-Lamin via western blotting.

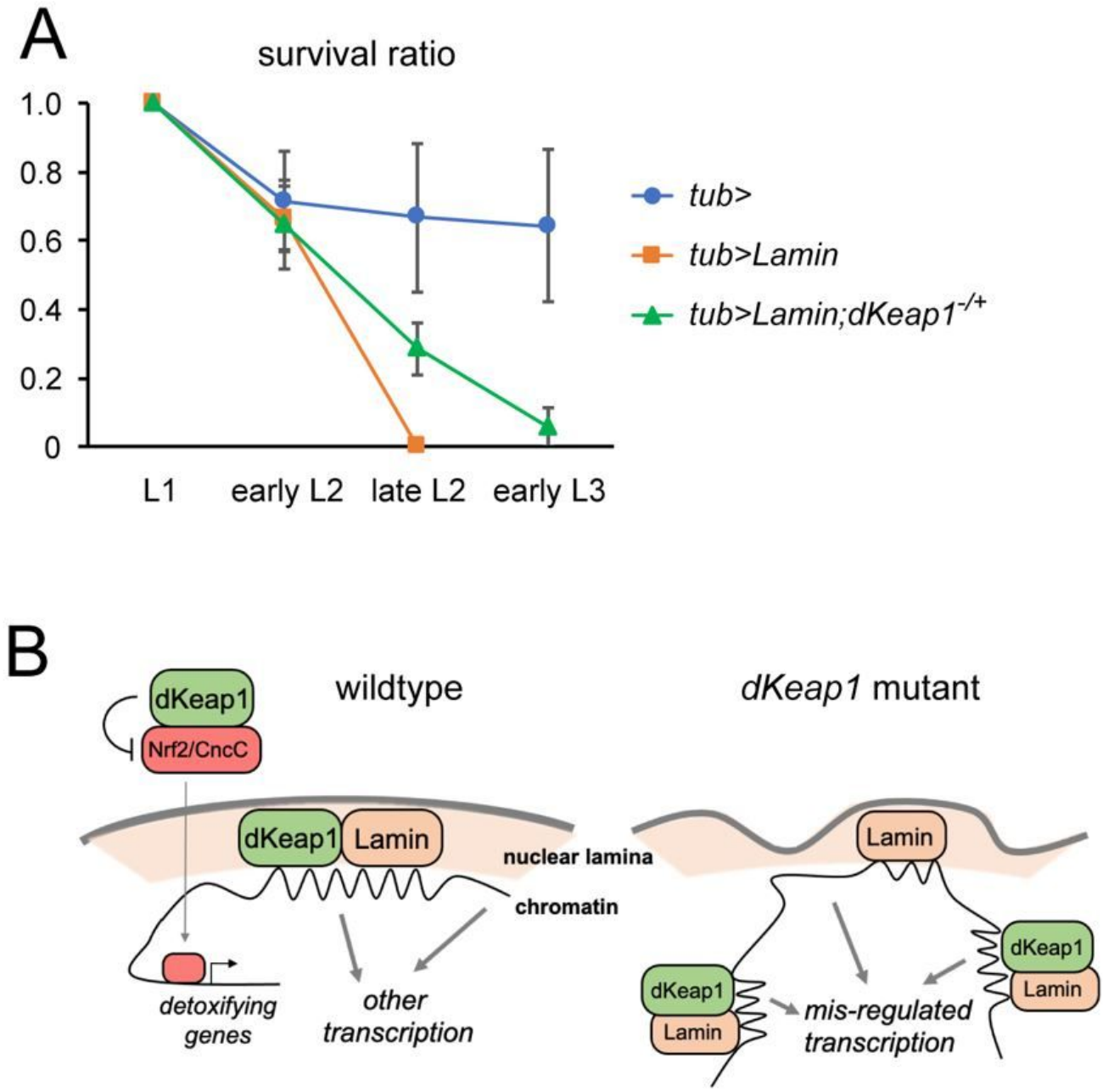

Figure 5

dKeap1 and Lamin function in the same genetic pathway

A. Genetic interaction between dKeap1 and Lamin mutants. Survival ratios of control flies (*tub-GAL4/+*), Lamin overexpression flies (U*AS-YFP-Lamin/+; tub-GAL4/+*), and the double mutant combining Lamin overexpression and dKeap1 knock down (*UAS-YFP-Lamin/+; dKeap1^{EY5},tub-GAL4/+*) were measured at larval stages listed below. Error bars represent the standard deviation based on two independent experiments. T-tests were used to compare the survival ratios of *Lamin* overexpression flies (*tub>Lamin*) and the double mutant flies (*tub>Lamin;dKeap1$^{-/+}$*) ($p<0.05$).

B. Model of dKeap1-Lamin interaction and function. Left: the classic function of Keap1 proteins in oxidative/xenobiotic responses is inhibiting Nrf2/CncC in the cytoplasm and preventing the activate of detoxifying genes by Nrf2/CncC. The novel dKeap1-Lamin interaction mediates the maintenance of normal lamina morphology and the regulation of chromatin architecture, especially heterochromatin. This would indicate a potential epigenetic mechanism whereby Keap1 family proteins regulate transcription. Right: Mis-regulated dKeap1 disrupts nuclear lamina, relocates Lamin, resulting in mis-organized chromatin architecture and mis-regulated transcription.

Supplementary Files

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