Drosophila ELYS regulates Dorsal dynamics during development

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Running Title: ELYS is a Critical Player in Drosophila Development
Abstract:

Embryonic large molecule derived from yolk sac (ELYS) is a constituent protein of nuclear pores. It initiates assembly of nuclear pore complexes (NPCs) into functional nuclear pores toward the end of mitosis. Using cellular, molecular and genetic tools, including fluorescence and electron microscopy, quantitative PCR and RNAi mediated depletion, here; we report that ELYS ortholog (dElys) plays critical roles during Drosophila development. dElys localized to the nuclear rim in interphase cells, but during mitosis, it was absent from kinetochores and enveloped chromatin. We observed that RNAi-mediated dElys depletion leads to aberrant development and, at the cellular level, to defects in the nuclear pore and nuclear lamina assembly. Further genetic analyses indicated that dElys depletion reactivates the Dorsal (NF-κB) pathway during late larval stages. Re-activated Dorsal caused untimely expression of the Dorsal target genes in the post-embryonic stages. We also demonstrate that activated Dorsal triggers apoptosis during later developmental stages by up-regulating the pro-apoptotic genes reaper and hid. The apoptosis induced by Reaper and Hid was probably the underlying cause for developmental abnormalities observed upon dElys depletion. Moreover, we noted that dElys has conserved structural features, but contains a non-canonical AT-hook like motif through which it strongly binds to DNA. Together, our results uncover a novel epistatic interaction that regulates Dorsal dynamics by dElys during development.

Keywords: ELYS, NPCs, AT-hook motif, Drosophila, Development, Dorsal (NF-κB)

Introduction:

ELYS (Embryonic Large molecule derived from Yolk Sac) was characterized in the mouse as a putative transcription factor important for hematopoiesis (1). ELYS was characterized to possess nuclear localization signal (NLS), nuclear export signal (NES), N-terminal β-propeller region, central helical and, C-terminally disordered region (1,2). AT-hook motifs present in ELYS allow DNA binding, and the trans-activation domains (acidic region) can induce transcription (1). The β propeller-like domain present in ELYS mediates interaction with other nucleoporins and facilitates the nuclear pore complex (NPC) assembly (3). NPC is a multi-protein assembly of nucleoporins (Nups) with their sizes varying from ~60 MDa in yeast to ~125 MDa metazoans. Nucleoporins assemble into sub-complexes and are present on the cytoplasmic, nuclear membrane and nucleoplasmic faces of nuclear pores (4,5). In metazoans, at the onset of mitosis, sub-complexes of NPCs dissociate from each other and get redistributed inside the cell. ELYS was reported to be an integral member of the Nup107 complex and essential for post-mitotic NPC assembly (6-10). In interphase, ELYS is present at the nuclear envelope and nucleoplasm, but in mitosis, it associates with chromatin, kinetochores, and spindles (8). Importantly, the conserved ELYS domain is required for its NPC and kinetochore localization (11), where ELYS helps in microtubule
polymerization (12). ELYS tethers Nup107 complex to kinetochores and initiates their inclusion into post-mitotic nuclear pores (9).

ELYS is critically required for embryonic development as ELYS null mice die during embryonic stage E3.5 to E5.5, well before the onset of embryonic hematopoiesis (1,13). However, conditional inactivation of ELYS locus in adult mice showed reduced effects, and mice behaved normally (14). ELYS, also known as Mel-28, is required for the maintenance of the nuclear morphology and embryonic development in C. elegans (7,10). ELYS orthologue in zebrafish, flotte lotte (flo), is critically required for early embryonic and pharyngeal skeleton development (15). Additionally, flo deletion disrupted NPC formation and defective nuclear import induced replication stress in intestinal progenitor cells (16).

In addition to the NPC assembly, the depletion of ELYS leads to phosphorylation-dependent mislocalization of Lamin B Receptor (LBR) from the nuclear periphery (17). ELYS recruits Protein phosphatase-1 (PP1) to the kinetochores, and a reduction in phosphorylation levels of various molecules drives nuclear envelope assembly (18). Thus, ELYS being a nucleoporin and putative transcription factor is a critical molecule that may have a significant role to play in the early developmental events of an organism. However, no directed study has been performed on ELYS to obtain mechanistic insight of important roles played by ELYS in cellular homeostasis and early developmental events.

We chose Drosophila melanogaster (fruit flies) to address the importance of ELYS in early developmental processes. CG14215, ELYS-like molecule in Drosophila (hereafter as dElys) is known to be present at the nuclear periphery and in association with the promoter region of genes undergoing active transcription (19,20). Here we report the genetic, molecular, and cellular characterization of dElys. dElys binds with DNA through its non-canonical AT-hook like motif. RNAi mediated depletion of dElys induces loss of nucleoporins and lamins, thus affecting the nuclear pore and nuclear lamina assembly. Importantly, upon dElys depletion, Dorsal (NF-κB) accumulates inside the nucleus in later larval stages, without any immune challenge, and induces apoptosis. Through our results, we strongly imply that dElys is a key developmental molecule required in cellular processes integral to normal cellular homeostasis. In addition to maintaining the nuclear architecture, dElys contributes to the development by regulation of the Dorsal dynamics into post-embryonic tissues.

Results:

ELYS is highly conserved in Drosophila:

ELYS is a DNA binding nucleoporin that coordinates NPC assembly at the end of mitosis. Drosophila ELYS (dElys, CG14215) is known to localize at the nuclear periphery and found to be present at the promoter region of genes undergoing active transcription (19,20). However, any mechanistic details on the dElys function are not available. For molecular and cellular characterization of dElys, we started with primary sequence alignment and report that an overall ~20-21% identity exists among
dElys and mouse and human ELYS (Fig. 1A). Using bioinformatics tools, we have predicted the presence and conserved arrangement of structural features like the N-terminal β-propeller domain (aa 1-488), central helical domain (aa 489-977) and a C-terminal unstructured region (Fig. 1A). Homology-based 3D structure predictions of the N-terminal β-propeller domain of dElys identified a highly conserved seven-bladed β-propeller structure (Fig. S1A) appreciably similar to the reported structure of the N-terminal domain of mouse ELYS (3,21). Evolutionary divergence analysis indicates that dElys is present in a clade quite distinct and distant from vertebrate ELYS (Fig. S1B).

We raised polyclonal antibodies against the C-terminal antigenic fragment (aa 1796-2111) of dElys. Immunoblotting with anti-dElys antibody detected a band of ~215 kDa in control head complex lysates and a band of decreased intensity in lysates prepared from tissues where dElys was ubiquitously knocked down (ubiquitous knockdown was achieved by driving dElys RNAi with Act5C-GAL4 driver, to be denoted as ubiquitous here on) (Fig. 1B). dElys antibodies detected a conserved and strong nuclear rim staining pattern in syncytial embryos overlapping with mAb414 antibodies recognizing FG-nucleoporins in nuclear pores (Fig. 1C). dElys antibodies also detected a similar overlapping pattern at the nuclear rim of salivary gland nuclei from GFP-Nup107 expressing organisms (Fig. S1C). dElys antibodies also detected a similar overlapping pattern at the nuclear rim of salivary gland nuclei from GFP-Nup107 expressing organisms (Fig. S1C). Moreover, the EYFP-dElys expressing salivary glands also had a nuclear rim localization pattern identical to endogenous dElys (Fig. S1D). In salivary gland nuclei, dElys antibodies detect a pattern co-localizing with nuclear lamina-associated Lamin-B receptor at the nuclear periphery (Fig. S1E). We probed if dElys also exhibits cell-cycle dependent subcellular localization changes as seen in other organisms. Metaphase-arrested EYFP-dElys expressing Drosophila S2 cells were co-stained with centromere identifier (CID, Drosophila CENP-A homolog) (22) to mark the kinetochores. dElys localized on mitotic chromosomes but not on kinetochores rather in the close vicinity of CID puncta (Fig. 1D). It is important to note that ELYS recruits the Nup107 complex to the kinetochores in cell culture, Xenopus egg extracts, and C. elegans embryos (6-8,12). However, the Nup107 complex was reported to be absent from kinetochores in Drosophila (23).

ELYS is also known as AT-hook containing transcription factor 1 (AHCTF1). Using canonical mouse ELYS AT-hook motif (Mm_Elys_AT-hook), we have predicted three AT-hook like motifs in dElys. We noticed a lack of glycine and proline amino acids in the conserved -RGRP- core (overscored) present in several characterized AT-hook proteins (Fig. S2A) (24). We thus asked if such a divergent non-canonical AT-hook can bind DNA. A carboxy-terminal fragment (aa 1858-1962) of dElys bearing all three predicted AT-hook like motifs, dElys_AT-1, 2, and 3, was purified and tested in DNA binding assay. Together with this, we mutated the arginines in each predicted AT-hook like motifs individually (mAT-1, mAT-2, and mAT-3, “m” denotes arginines of AT-hook mutated to alanines) or combined mutations of all three AT-hooks (mAT-1+2+3) (Fig. S2B) and tested the ability of purified proteins (Fig. S2C) to bind with AT-rich or non-AT rich DNA. It is evident from the
EMSA experiments and their quantitation that mutations in AT-hook 1 and 3 (mAT-1 and mAT-3) show a significant reduction in their ability to bind with both AT-rich and non-AT rich DNA (Fig. S2, D to G). Our data further established that the presence of glycine and proline is not as important in the -RGRP- core, as the positively charged arginine, alternately present in the AT-hook motifs as earlier reported (25). Through our initial in silico, biochemical and cellular analysis, we suggest that dElys is canonical ELYS molecule despite low sequence identity.

**dElys is essential for normal development in Drosophila:**

After assigning features of a nucleoporin and determining the localization dynamics, we asked whether dElys is essential for development in Drosophila. We performed in vivo depletion of dElys using RNAi line (v103547) from Vienna Drosophila Resource Centre (VDRC) (dElys^KK). To validate phenotypes observed with the dElys^KK RNAi line, we also generated a TRiP based RNAi line (dElys^TRiP). The ubiquitous knockdown of dElys by the two RNAi lines led to severe consequences on viability and induced lethality with a varying extent at each developmental stage. While no adults emerged from the dElys^KK RNAi line, a few viable flies (~4%) emerged out in dElys^TRiP. dElys depleted embryos were largely unaffected (~4% lethality), but a significantly increased lethality occurred at the larval stage (~46%), and ~50% of pupae died in the late pupal stage of development (Fig. 2A). The quantitative PCR based assessment of knockdown in dElys transcript levels suggests ~70% and ~55% knockdown in dElys^KK and dElys^TRiP RNAi lines, respectively (Fig. 2B). Although dElys^KK has two predicted off-targets, CG15643 and CG7051 in addition to dElys, the expression level of CG15643 remained unperturbed while CG7051 levels decreased by ~20% during dElys knockdown (P<0.05) (Fig. S3, A and B). CG7051 is reported to have an exclusive expression in male germ cells (26) hence would not have contributed to phenotypes observed. We have used the dElys^KK line in all subsequent studies because it showed a comparatively significant knockdown and pVALIUM10 based TRiP line is relatively ineffective in knockdown during oogenesis as well as lack uniform knockdown (27).

Maternally contributed dElys will hinder the analysis for developing an understanding of dElys functions in early development. We thus used the mat-α-tub-GAL4 driver to deplete maternally contributed dElys before the induction of zygotic transcription to overcome this issue. Maternally depleted dElys (dElys^KK RNAi line) embryos have an abnormal shape, as well as, they show a very slow rate of development compared to control embryos (data not shown). Hatching rate analysis showed that maternal dElys depleted embryos showed ~62% lethality as compared to ~12% lethality observed with control embryos obtained by crossing wild-type flies with the driver line (Fig. 2C).

To avail more insights on the importance of dElys in development, we silenced dElys in a tissue-specific manner in eyes and wings using eyeless (Ey) and wingless (Wg) drivers, respectively. dElys depleted flies have a shrunken eye with a significant reduction in the area occupied by ommatidia. Also, the ommatidia shape was irregular, and their arrangement was in disarray, leaving behind an eye
cavity (Fig. 2D, first and second vertical panels). Moreover, a significant number of dElys depleted ommatidia show loss of eye bristles, duplication of bristles and an irregular orientation (Fig. 2D, third vertical panels, SEM-2.5K X). Similarly, dElys silenced wings look crumpled with signs of vein atrophy and significant cell death in the wing blade tissues (Fig. 2E). The severity of these phenotypes varied between the two different dElys RNAi lines used in this study, yet the observations made regarding eye and wing phenotypes were consistent. Our data thus suggests an essential requirement of dElys for the normal development of Drosophila.

Loss of dElys results in the nuclear pore and nuclear lamina assembly defects:

We next asked if the dElys is functionally conserved in Drosophila. We assessed the localization of various nucleoporins representing different sub-complexes of NPC when dElys is ubiquitously knocked down. When salivary gland nuclei from dElys knockdown flies were stained for Nup43, representing the Nup107 complex; Nup98, a mobile nucleoporin (Fig. S4, A and B), and mAb414 antibody recognizing FG-repeats, they were found to be largely absent from the nuclear membrane (Fig. 3, A and B, right panels). Concomitantly, a marked increase in the cytoplasmic staining for these nucleoporins was observed in the dElys depleted salivary gland tissues indicating a redistribution of nucleoporins. Quantitation of nuclear membrane intensities for different Nups also corroborates the fact that dElys affects efficient assembly of nucleoporins into NPC (dElys: Control- 1.00 ± 0.04; dElys\textsuperscript{KK}- 0.15 ± 0.01, Nup43: Control- 1.00± 0.08; dElys\textsuperscript{KK}- 0.29 ± 0.02, Nup98: Control- 1.07 ± 0.12; dElys\textsuperscript{KK}- 0.31 ± 0.02, FG-repeat nucleoporins: Control- 1.00 ± 0.07; dElys\textsuperscript{KK}- 0.36 ± 0.01) (Fig. S4, G to J). We have further confirmed with histone H3 staining that dElys depletion does not alter chromatin structure significantly (Histone H3: Control- 1.00 ±0.06; dElys\textsuperscript{KK}- 0.85 ± 0.03) (Fig. 3D) and hence could be used for normalization of intensities of every molecule tested. Detection of the protein levels of these nucleoporins in the larval head-complex lysate, suggests no significant decrease in their levels except for Nup98 (Fig. 3E and Fig. S4P). Our data thus establish a conserved role for dElys in post-mitotic NPC assembly in Drosophila.

ELYS regulates the incorporation of LBR in the nuclear envelope in a phosphorylation-dependent manner (17,28). So, we asked if the nuclear lamina assembly is also affected by dElys. Salivary gland tissues, ubiquitously depleted for dElys, when stained for Lamin B, Lamin B-receptor (LBR), and Lamin C showed reduced localization in the nuclear lamina (Fig. S4, C and D, and Fig. 3C respectively). Quantitation of nuclear lamina intensities for lamina-associated molecules suggests a clear decrease under dElys depleted conditions, which emphasizes the importance of dElys in the recruitment of these molecules to the nuclear lamina (LBR: Control- 1.02 ± 0.07; dElys\textsuperscript{KK}- 0.28 ± 0.01, Lamin B: Control- 0.98 ± 0.04; dElys\textsuperscript{KK}- 0.50 ± 0.04, Lamin C: Control- 0.99 ± 0.04; dElys\textsuperscript{KK}- 0.43 ± 0.01). Our data constitute the first-ever report highlighting the in vivo importance of dElys in the localization of Lamin B and Lamin C to the nuclear lamina. It is also important to note that during the dElys
depletion experiments, protein levels of lamins and LBR remained unaffected (Fig. 3F and Fig. S4Q). These observations suggested that dElys is indispensable for the maintenance of the nuclear lamina and ultimately the nuclear architecture.

While analyzing the nuclear periphery localization of nucleoporins and lamins, we noticed that the Ran-GTPase, required for the coordination of the nucleo-cytoplasmic transport of protein cargo, is mislocalized upon dElys depletion. In dElys depleted salivary gland tissues, Ran reactivity was lacking from the nuclear periphery and appeared redistributed throughout the cell (Ran: Control- 1.03 ± 0.09; dElysKK- 0.29 ± 0.05) (Fig. S4, E and N). Upon dElys knockdown, the absence of Ran-GTPase, Nups, and lamins prompted us to ask if everything associated with the nuclear periphery is re-distributed. TBP-associated factors that form TFIID show nuclear rim and strong cytoplasmic staining in the Drosophila embryo (29). Accordingly, TATA-box binding protein-1 (TBP1, dTBP in Drosophila) when stained with polyclonal anti-yeast TBP antibodies (as used in (30)), presented the characteristic nuclear periphery staining pattern even in the dElys depleted salivary gland tissues (dTBP: Control- 1.00 ± 0.11; dElysKK- 0.76 ± 0.12) (Fig. S4, F and O). Unaltered protein levels of these molecules indicate that dElys is required for their recruitment to the nuclear envelope, possibly during post-mitotic nuclear build-up where dElys is the first molecule associating with DNA. A similar loss of FG-nucleoporins from the nuclear rim was noticed in salivary glands when dElys was knocked down using TRiP based RNAi line (Fig. S5, A, bottom-most panels). To further confirm the dElys function in NPC assembly, we attempted to rescue dElys depletion phenotype by multiple gene copy approach, where we provided an extra copy of dElys (UAS-dElys) through transgene to exhaust the dsRNA generated by RNAi (dElysKK), leaving behind a significant amount of dElys to perform its function. The FG-repeat nucleoporins analyzed in this combination (dElysKK; UAS-dElys) showed partial rescue of its level on nuclear periphery (FG-repeat nucleoporins: Control- 1.00 ± 0.13, dElysKK- 0.43 ± 0.01, dElysKK; UAS-dElys- 0.71 ± 0.03) (Fig. S5, A, third horizontal panels and B), suggesting that the phenotypes observed were indeed a result of dElys depletion.

**dElys knockdown shows activated Dorsal pathway and induced apoptosis:**

To deduce the underlying molecular mechanism that drives dElys depletion phenotypes, we sought to check for perturbed nucleo-cytoplasmic traffic of various signaling molecules. Dorsal (NF-κB) is an important transcription factor, which stably associates with Cactus and remains inactive in the cytoplasm under normal conditions. The rapid shuttling of Dorsal into the nucleus requires a stimulus (31,32) and is accompanied by ubiquitination-dependent degradation of the Cactus, NF-κB inhibitor (IκB) in the cytoplasm (33,34). In salivary glands isolated from ubiquitous knockdown of control, the Dorsal signal was predominantly diffused in cytoplasmic (Fig. 4A, upper panels). In contrast, dElys depleted salivary gland tissues showed an intense Dorsal signal inside the nucleus (Fig. 4A, lower panels). Ratiometric quantification of the Dorsal intensities in cytoplasm and nucleus
further highlighted that Dorsal signals significantly increased inside the nucleus upon dElys depletion (Nuclear/Cytoplasmic Dorsal: Control- 1.00 ± 0.07; dElys<sup>KK</sup>- 4.93± 0.20) (Fig. 4D). Increased nuclear localization of Dorsal was also seen in salivary glands obtained from dElys knockdown using TRiP based RNAi line (Fig. S5, C, fourth horizontal panels). Upon dElys depletion, the increased retention of Dorsal inside the nucleus is not accompanied by an increase in dorsal transcript levels (Fig. S6A). Thus, dElys regulates the redistribution of Dorsal, but not its expression. The accumulation of Dorsal inside the nucleus prompted us to ask if the cytoplasmic level of Cactus has reduced. The cytoplasmic Cactus signal is significantly diminished in dElys depleted salivary gland tissues as compared to control tissues (Fig. 4B). The quantification of Cactus staining further corroborated the observation that dElys depletion may induce Cactus degradation and Dorsal accumulation inside the nucleus (Cactus: Control- 1.00 ± 0.03; dElys<sup>KK</sup>- 0.40 ± 0.01) (Fig. 4E). Our attempts to rescue Dorsal nuclear localization by providing an extra copy of dElys using transgene (UAS-dElys) led to receding of Dorsal from the nuclei of the third instar larval salivary glands, suggesting a specific role of dElys in nuclear Dorsal localization (Nuclear/Cytoplasmic Dorsal: Control- 1.00 ± 0.07; dElys<sup>KK</sup>- 7.03 ± 0.47, dElys<sup>KK</sup>; UAS-dElys- 1.33 ± 0.05) (Fig. S5, C, third horizontal panel and D). While Dorsal is known to be a pro-growth molecule, studies suggest that activated Dorsal can also induce apoptosis in cells (35-37). The phenotypes observed upon dElys depletion may be a consequence of the unfavorable accumulation of Dorsal inside the nucleus and thus could induce cell death.

We next probed if the abnormal development of the eye and wing tissues, as well as lethality observed upon dElys depletion, is due to apoptosis. We employed acridine orange staining as a primary assay to infer the induction of apoptosis in salivary glands. dElys depleted tissues accumulated significantly more acridine orange than normal tissues suggesting activation of cell death (data not shown). To further assert if dElys depletion can induce cell death (apoptotic response), we probed for cleaved Death caspase-1 (Dcp-1). Dcp-1 is critical for normal embryonic development, and an elevation in Dcp-1 levels is a hallmark for apoptosis (38-40). Dcp-1 staining of salivary gland tissues from ubiquitous dElys knockdown showed increased immunoreactivity and increased Dcp-1 positive puncta (Cleaved Dcp-1: Control- 1.00 ± 0.03; dElys<sup>KK</sup>- 4.79 ± 0.13) (Fig. 4, C and F). We next asked if nuclear accumulated Dorsal causes any alterations in the expression of apoptotic genes. We probed for the transcript levels of pro-apoptotic genes reaper and hid, and anti-apoptotic diap-1 (Drosophila inhibitor of apoptosis 1). cDNA was prepared from the third instar larval head complex isolated from the ubiquitous knockdown of control and dElys, and the transcript levels were assessed by quantitative real-time PCR. We observed a significant increase in the level of the pro-apoptotic reaper and hid expression while there was a complementary decrease in the level of anti-apoptotic diap-1 upon dElys depletion (Fig. 4G). The relative levels of diap-1, reaper, and hid are important determinants of cell survival or cell death. Together, the qPCR and Dcp-1
immunostaining data indicate an indisputable correlation towards apoptotic induction upon dElys depletion. Accumulation of Dorsal upon dElys knockdown should bring a change in the levels of its target genes. The quantitative PCR-based detection of snail, twist, rho, and sog levels revealed an increase in their expression. However, the levels of dpp (decapentaplegic), a gene suppressed by Dorsal, decreased in dElys depleted samples as compared to control (Fig. 4H). To rule out the possibility of activated Dorsal pathway under immune response, we checked for the expression of the anti-microbial peptide, drosomycin in dElys depleted cDNA. The unaltered drosomycin levels suggest that dElys depletion dependent activation of the Dorsal pathway does not have a microbial infection and immunity component to it (Fig. 4H).

Dorsal dependent expression of snail, twist, and sog during early development is important for cellularization (41). We used the third instar larval head complex for cDNA preparation, which is quite late in the development. Expression of snail, twist, and sog could be detected in the late larval stages, and that may be the cause of apoptosis in dElys depleted organisms inducing developmental defects. Studies indicate that Drosophila apoptotic response activators include snail, twist, dpp, and rho (42-45). It is thus not surprising to find resurrected expression of Dorsal targets during late developmental stages which could induce apoptosis and developmental defects observed under dElys depleted conditions.

Accumulation and re-activation of Dorsal in post-embryonic tissues is dElys specific:

We set out to investigate if dElys depletion dependent nuclear retention of Dorsal is a temporal event of later developmental stages or Dorsal is entrapped in the nucleus even after the embryonic stage. First, we analyzed the Dorsal levels in early embryonic stages (Stage 5) in control and dElys knockdown were driven by mat-α-tub-GAL4. The nuclear levels of Dorsal remain high and unperturbed in both dElys and control depleted embryos (Fig. 5A, first vertical panels). Significant nuclear Dorsal levels along with increased cytoplasmic localization of Dorsal was observed in presumptive embryonic salivary glands from late embryonic stages (Stage 15) of control and dElysGAL4 organisms (Fig. 5A, second vertical panel). We then looked for Dorsal signals in the nuclei of salivary glands isolated from each successive larval stage. A gradual but steady decrease in the nuclear Dorsal signal was observed in the first instar to third instar stage nuclei of the ubiquitous control knockdown organisms. The salivary gland nuclei from the first instar larva of ubiquitous dElys depletion have the Dorsal signal inside the nucleus that is comparable to control depletion (Fig. 5A, third vertical panels). However, we observed an increase in nuclear levels of Dorsal in the second instar larval stage of dElys depleted organisms, while control nuclei have only residual or no Dorsal signals (Fig. 5A, fourth vertical panels). Importantly, the salivary glands from the third instar larval stage of ubiquitous dElys depletion show a prominent nuclear accumulation and a complementary decrease in the Dorsal signal inside the cytoplasm. But the control third instar salivary gland nuclei had no Dorsal inside the nucleus, and the Dorsal levels remained restricted to
cytoplasm only (Fig. 5A, fifth vertical panel). Quantification of nuclear Dorsal signal intensities from control and dElys depleted salivary gland nuclei further establish that Dorsal starts to redistribute in the nucleus during post-embryonic developmental stages upon dElys depletion (Fig. 5B). To further support this observation, we followed the expression of three Dorsal targets, namely snail, twist, and sog, which are key determinants of dorsoventral polarity in successive developmental stages. Under dElys depleted conditions, the expression of snail, twist, and sog is high during the early embryonic stage, which diminished during the first instar larval stage after specifying dorsoventral polarity. But a resurrected expression of these key molecules was observed during the third instar stage when dElys was depleted. Under the control depletion condition, there is no significant expression observed during the late larval stages (Fig. 5C). Our results thus strongly suggest that dElys plays an important role in regulating the Dorsal signaling during post-embryonic stages and its temporal sequestration in the cytoplasm.

We next asked if this nuclear re-localization of Dorsal is specific to dElys depletion or a general consequence of a lack of properly assembled nuclear pores. To check this, we investigated Dorsal localization in salivary gland nuclei from the knockdown of critical nucleoporins nup160, nup133, nup107, and nup153. Members of the Nup107 complex play a key regulatory role in post-mitotic and interphase NPCs assembly, while Nup153 is a critical molecule for interphase NPCs assembly only (46-48). RNAi mediated depletion of nup160 and nup107 caused lethality at the first instar larval stage; however, when reared at 23°C organisms from these crosses could survive until third instar larva. The salivary gland nuclei from nup160 and nup107 depletion do not show significant accumulation of Dorsal inside the nucleus (Nuclear/Cytoplasmic Dorsal: Control- 1.00 ± 0.05; nup160GD- 0.95 ± 0.02, nup107KK- 1.05 ± 0.02) (Fig. 5D). When compared with control, nup153 depletion shows no significant difference in Dorsal signals inside nuclei. Only nup133 depletion showed little accumulation of nuclear Dorsal, still significantly lower than dElys (Nuclear/Cytoplasmic Dorsal: nup153TRiP- 1.11 ± 0.03, nup133KK- 1.57 ± 0.10) (Fig. 5D). The knockdown of these nucleoporins suggests a drastic loss in their expression (Fig. S6, B to E). mAb414 staining of salivary gland nuclei indicated that removal of these other nucleoporins had induced NPC assembly defects (Fig. S7). Accordingly, the mRNA export was also perturbed that led to accumulation of mRNA inside the nucleus (Nuclear mRNA: Control- 1.00 ± 0.11; dElysKK- 1.87 ± 0.23, nup160GD- 3.68 ± 0.28, nup107KK- 7.29 ± 0.59, nup153TRiP- 3.84 ± 0.28) (Fig. S8, A and B). Moreover, GFP-NLS import assay showed increased GFP-NLS signal in cytoplasm suggesting that import process is also affected (Cytoplasmic/Nuclear GFP-NLS: Control- 1.00 ± 0.08; dElysKK- 1.85 ± 0.07, nup160GD- 2.32 ± 0.19, nup107KK- 2.13 ± 0.15, nup133KK- 2.03 ± 0.08) (Fig. S8, C and D). Quantitation of nuclear intensities of the Dorsal signal demonstrates that the Dorsal signal inside the nucleus increased by ~5 fold upon dElys depletion, but the same remains largely unchanged upon depletion of other tested nucleoporins involved in NPC assembly (Fig. 5E). We
suggest that the resurrected residence of the Dorsal inside the nucleus is dElys specific and not the outcome of the absence of functional NPCs in the nuclear envelope.

**dElys depletion-induced apoptosis is Dorsal mediated:**

We next examined if the apoptosis and the nuclear localization of Dorsal are independent consequences of dElys depletion. We have created a genetic combination of dElys depletion in the dorsal null background, but the embryos showed lethality at the early embryonic stage (~stage 2) hence could not be used in any relevant study (Fig. S9A). Analysis of dElys depleted or dorsal null organisms revealed increased apoptosis (Dcp-1 staining) by the embryonic stage 11 as compared to control (Fig. S9B). Hence, we employed the combined knockdown of dElys and dorsal and analyzed the apoptotic response from co-depleted salivary glands. In control depletion, Dorsal remained cytoplasmic and no Dcp-1 staining was observed (Fig. 6, A first horizontal panels, B and C). But, dElys depletion induced accumulation of Dorsal and enhanced punctate staining of Dcp-1 was observed (Fig. 6, A second horizontal panels, B and C). When dElys and dorsal were co-depleted, nuclear levels of Dorsal and punctate Dcp-1 staining both reduced significantly in salivary gland tissues (Total Dorsal intensity: Control- 1.00 ± 0.09, dElysKK- 1.00 ± 0.07, dElysKK; dorsalKK- 0.56 ± 0.03) (Total Dcp-1 intensity: Control- 1.00 ± 0.09, dElysKK- 2.25 ± 0.08, dElysKK; dorsalKK- 1.14 ± 0.05) (Fig. 6, A third horizontal panels, B and C).

We next examined if apoptosis can be rescued by overexpressing diap-1 (diap-1OE) in the dElys knockdown. As ubiquitous overexpression of diap-1 is lethal, we used localized expression using salivary gland specific drivers, Fkh-GAL4; C-147-GAL4. Salivary gland specific dElys depletion elicited the Dcp-1 signal, but the diap-1 overexpression in dElys depletion background brings the Dcp-1 levels back to normal and subsiding the apoptotic response (Total Dcp-1 intensity: dElysKK; diap-1OE- 0.96 ± 0.04) (Fig. 6, A fourth horizontal panels, and C). We further intended to rescue this phenotype in adult wings by using nubbin-GAL4; Ubx-GAL4 for expressing diap-1 in dElys depletion background. We could partially rescue the apoptosis and wing phenotypes when diap-1 is expressed in wing imaginal discs (Fig. S10A) of dElys knockdown (dElysKK; diap-1OE) organisms. The disc shape appears normal, and the Dcp-1 staining is reduced in the wing pouch region of the wing imaginal disc from dElysKK; diap-1OE organisms as compared to dElysKK organisms (Total Dcp-1 intensity in Wing imaginal discs: Control- 1.00 ± 0.30, dElysKK- 6.42 ± 1.42, dElysKK; diap-1OE-3.41 ± 0.42) (Fig. S10, B, and C). The wings in adults arising from this genetic combination show improved morphology compared to dElys depletion (Fig. S10D). We attributed this partial rescue to the weak expression of Ubx-Gal4 in the wing-pouch area, which forms the major surface of wings. Together, our data strongly suggest that the induction of apoptosis upon dElys depletion is Dorsal mediated, and the abundance of DIAP-1 counters the apoptotic phenotypes of dElys depletion.
Discussion:

ELYS was found in stable association with the Nup107 complex of nuclear pores (6-8), and studies revealed that ELYS recruits Nup107 complex to the kinetochores and helps in regulation of Nup107 complex-mediated microtubule formation at the kinetochores (12,49,50). The biochemical characterization of AT-hook like motif in Drosophila ELYS orthologue (dElys) encoded by CG14215 has echoed the view that positively charged amino acid residues play an important role in the DNA binding (Fig. 1), similar to what has been observed with non-canonical AT-hook of Drosophila TAF-1 (25). The DNA binding ability enables ELYS to initiates NPC assembly at a precise location and not at ectopic sites (11,49,51). The conserved localization of dElys to the nuclear rim in interphase also suggests a conserved function of dElys in Drosophila. However, unlike other characterized ELYS molecules (8,12,49,50), dElys is absent from kinetochores and appears to envelop chromatin in mitosis (Fig. 1). The absence of dElys from the kinetochores provides a suitable explanation for the lack of Drosophila Nup107 complex from kinetochore during mitosis (23). Although dElys and Sec13 (a dNup107 complex member) localize to the active chromatin regions (52), it remains to be elucidated if they both interact physically.

The ubiquitous knockdown of dElys, induced developmental defects, and lethality in Drosophila (Fig. 2) mirroring the defects observed in other organisms following the conserved function of ELYS molecules (6,11,53,54). The dElys phenocopies nucleoporin functions of ELYS molecule characterized in other organisms and is involved in nucleo-cytoplasmic transport regulation, NPC assembly and interacts with nuclear lamina proteins (Fig. 3) as reported in mammalian cell lines, C. elegans and Xenopus egg extract studies (6,7,9,16,28,53), suggesting a conserved functional role in Drosophila. Nucleocytoplasmic transport defect observed in dElys depletion highlights its conserved role as a nucleoporin (7,10,55-57). We observed a conserved role for dElys in Lamin B receptor (LBR) recruitment to help organize the nuclear lamina, similar to what has been reported previously (17,28). Importantly, we report the mislocalization of both Lamin B and C from the nuclear lamina upon dElys depletion (Fig. 3). We attribute this mislocalization of Lamin B and C to the loss of Ran from the nuclear periphery due to the probable reduction of Nup358 upon dElys loss. RanGTP helps in the dissociation of lamin from the lamin-Importin α/β complex for incorporation into nuclear lamina (58). Loss of Ran from the nucleus in dElys depletion might have hampered the dissociation of lamin-importin α/β complex, in turn affecting nuclear lamina assembly in these non-dividing salivary gland cells. This aspect of nuclear lamina assembly regulation by dElys requires detailed investigation.

ELYS is known to initiate post-mitotic NPCs assembly across different model organisms (6-11,15,49). Assembly of the NPCs, in turn, regulates the proper distribution of inner nuclear membrane proteins leading to correct nuclear architecture regulating proper nuclear transport (28,59). So, NPC assembly, LBR localization, lamina assembly, and nuclear transport can be linked to nuclear pore associated function of ELYS in Drosophila.
Dorsal (NF-κB) is a developmentally regulated key transcription factor, its nuclear localization, and regulated activation of its downstream pathways are critical for normal development (34). The induction of the Dorsal signaling pathway plays an important role in the growth and development of the organism (60,61). The ectopic presence of Dorsal inside the nucleus of dElys depleted salivary gland tissues (Fig. 4) is in direct contrast to the presence of Dorsal inside the nucleus only during early embryonic stages or a microbial infection. It is rather uncommon for Dorsal to be present inside the nucleus in later stages of Drosophila development and could suggest an uncharacteristic Dorsal pathway activation. The Dorsal present inside the dElys depleted third instar salivary gland nuclei is active and transcribes growth-promoting target genes snail, twist, and sog. Expression of these genes is generally restricted to embryonic stages of development. Besides, the expression of pro-apoptotic genes reaper and hid is also induced (Fig. 4). The snail, twist, and sog genes are known to induce apoptosis when expressed in an unregulated manner (43,44). In accordance, an increase in apoptosis is evident in dElys depleted salivary gland tissues (Fig. 4). The two contrasting paradigms of gene expression can generate a developmental imbalance resulting in apoptosis by a hitherto unknown mechanism. Dorsal can induce apoptotic responses, and emerging ideas suggest that Dorsal, depending on cues, helps in growth versus apoptotic fate choices (62). Thus, dElys depletion can regulate effective nucleocytoplasmic transport of important transcriptional regulators to induce apoptosis. Importantly, no other nucleoparin tested in our study, when depleted, phenocopied the Dorsal accumulation inside the nucleus (Fig. 5). These results indicate strongly towards nuclear pore independent function of dElys in Dorsal localization. A detailed analysis exploring interactions between dElys, Dorsal, and upstream players of the Dorsal pathway will shed more light on nuclear pore independent functions of dElys. The apoptotic consequence of an aberrant Dorsal pathway activation seems to be a signature of dElys nucleoporin in Drosophila. Nucleoporins depletion and apoptosis are not uncommon, and our observation aligns with the apoptosis induction in astrocytoma cells when nup107 is depleted (63).

Our observation regarding Dorsal pathway activation is in direct contrast to what is known for another nucleoporin mbo (Nup88) in Drosophila. First, the deletion of the mbo did not affect the general nuclear import/export and nuclear lamina assembly. Nup88 interacted feebly with Dorsal, but more importantly, nuclear levels of activated Dorsal were reduced in response to infection when Nup88 was deleted (64). Our observation can imply that in addition to genetic interaction, dElys may possess a physical interaction with Dorsal or Cactus. Nup88, along with other nucleoporins like Nup62, Nup153, and Nup214, play important roles in developmental pathways (65-67), but a detailed mechanistic understanding of selective nuclear translocation of activated signals are lacking. ELYS targets protein phosphatases to the nucleus, which can dephosphorylate and exports molecules back to the cytoplasm (18). Structural and functional conservation of dElys may suggest that
dElys perhaps regulates the localization of an orthologous phosphatase to control the nucleo-cytoplasmic shuttling of Dorsal in *Drosophila*. It is also very likely that through phosphatases, dElys may regulate the phosphorylation mediated degradation of Cactus, resulting in the nuclear translocation of Dorsal. It will be of greater interest to identify if such a phosphatase is involved in the shuttling process of Dorsal in *Drosophila*. Our results highlight the key difference between dElys and other nucleoporins and affirm a role for dElys in developmental signaling in addition to NPC and nuclear lamina organization.

NF-κB family transcription factors play a critical role in normal growth and development. Their dysregulation leads to cancer, chronic inflammatory disease and developmental defects (68,69). WntD, the product of Dorsal/Twist/Snail Signalling, is a feedback inhibitor of Dorsal signaling. WntD inhibits the nuclear translocation of Dorsal, thus inactivating expression of downstream targets (70,71). Our report highlights the novel role of the nucleoporin, dElys in Dorsal regulation during the later stages of development is an addition to the growing pool of reports for molecules involved in the regulation of Dorsal signaling. Unlike WntD, dElys is a novel intracellular regulator of Dorsal signaling.

We suggest that dElys levels help maintain a tight balance between pro-growth and pro-apoptotic responses to achieve normal growth and development in *Drosophila*. dElys does regulate development and could contribute to normal growth in post-mitotic, non-dividing cells by keeping the NF-κB trigger under control in a spatiotemporal manner (Fig. 6).

Our study thus provides an initial hint to a novel but yet-to-be-identified pathway which involves non-canonical AT-hook like motifs containing dElys to regulate the spatiotemporal distribution of Dorsal to ensure proper growth and development in *Drosophila*. Suppression of Dorsal signaling after the embryonic stage by dElys is an added layer of regulation to ensure proper growth and development. Moreover, there may be additional signaling cascades that are perturbed by changes in dElys levels, which require further elucidation.

### Materials and Methods:

**In silico analysis:**

An orthologue of ELYS in *Drosophila* was identified by using mouse ELYS as the reference sequence. PSI-BLAST with three iterations using mouse ELYS and its AT-hook motif sequence was used against the non-redundant protein database at NCBI with default parameters. ELYS orthologues in other organisms were identified by similar searches in the NCBI database. T-coffee, a multiple sequence alignment server was used for alignment (72). The aligned sequence was then used for visualization in Jalview and highlighted with a Clustal X color scheme. The phylogenetic tree was inferred from this analysis using Jalview (73). The phylogenetic tree was inferred from this analysis using Jalview (73). Protein sequence motifs were identified using the SMART database for the representative sequence from each organism (74). IBS was used to draw a scaled protein illustration for each protein (75). Percentage identity was calculated against mouse ELYS as a
reference for each ELYS like molecules. Secondary structure for CG14215 was predicted using PSIPRED v3.3, DisoPRED and DomPRED (76). Predicted secondary structure for N-terminal and central helical domain was compared with the experimentally proven secondary structure of human and mouse ELYS (3). 3D structure of NTD of CG1425 was modeled using a Phyre2 algorithm and mouse ELYS NTD structure as a template (21).

Fly strains and genetics:

All flies were reared at 25°C on standard corn meal-yeast-agar medium. RNA interference crosses were grown at 28°C for better expression of GAL4. nup160GD and nup107GD crosses were grown at 23°C to obtain third instar larva. RNAi line (KK 103547) for CG14215 was obtained from the Vienna Drosophila Resource Centre (VDRC). Other fly lines used in this study were obtained from the Bloomington Drosophila Stock Centre (BDSC) at Indiana University or VDRC. Controls used in this study are F1 progeny from Driver line crossed with W1118 flies. Fly lines used in this study are mentioned in table S1. All the combination and recombination are made with standard fly genetics. Tissue-specific knockdown of dElys was achieved by Act5C-GAL4, Ey-GAL4, Wg-GAL4, Fkh-GAL4, mat-a-tub-GAL4 drivers obtained from BDSC. For maternal knockdown F1, virgin females from the cross of RNAi and mat-a-tub-GAL4 was crossed with RNAi males, and early embryos were collected from this cross.

Cloning and Transgenic fly generation:

We obtained the clone LD14710 containing the full-length CG14215 coding sequence in the pBluescript SK (-) vector from Drosophila Genomics Resource Centre (DGRC). This clone was used as a template for cloning full-length CG14215 into pTVW (Vector. No. 1091), a fly transformation vector with gateway cassette, UASt promoter, and N-terminal EYFP tag as well as in pTW (Vector. No. 1129) vector containing gateway cassette and UASt promoter (obtained from DGRC gateway-1 collection). Cloning was done using the Gateway cloning kit as per the manufacturer’s instructions (Thermo Fisher Scientific). RNAi lines against CG14215 was generated by cloning 503 bps from exon 7 of CG14215 predicted using SnapDragon algorithm at Drosophila RNAi screening center (DRSC) website (FlyRNAi.org) which do not have any predicted off-targets (77) and cloned into gateway based pVALIUM10 RNAi vector obtained from DRSC (TRiP). Transgenic flies were generated at Fly facility of Centre for Cellular and Molecular Platforms at the National Center for Biological Sciences (C-CAMP-NCBS), Bangalore, India. Primers used in this study are mentioned in table S2.

Drosophila S2 cell culture and transfections:

Drosophila S2 cells were grown in Schneider’s Drosophila medium (Gibco, Thermo Fisher Scientific) supplemented with 10% Foetal bovine serum (Gibco, Thermo Fisher Scientific) and 50 U/ml penicillin, 50 U/ml streptomycin and 25 µg/ml amphotericin B (Gibco, Thermo Fisher Scientific) at 25°C in CO2 free incubator. Full-length CG14215 was cloned in pAVW (Vector No. 1087) from DGRC. S2 cells were transfected at 50% confluency with Effectene transfection reagent (Qiagen). Cells
were grown for 3 days post-transfection. S2 cells were arrested in metaphase of mitosis by the addition of 20 μM MG-132 (RTU solution, Sigma) for 2 hours at 25°C.

**Antibody generation and Western blotting:**

C-terminal 343 amino acid of CG14215 which is a unique, most antigenic and hydrophilic part was amplified from LD14710 clone and sub-cloned into pET28a (+) vector. Protein was expressed in *E. coli* BL21 (DE3) cells, induced using 200 μM IPTG (Sigma) and incubated at 18°C overnight. Cells were pelleted down, lysed in 100 mM NaH₂PO₄, 10 mM Tris-HCl buffer, pH 8.0 containing 8 M urea and 1% Triton X-100, 50 μg/ml lysozyme and 1X protease inhibitor cocktail (Roche). The recombinant protein was purified over Ni-NTA beads and eluted with low pH at 4.5 in 100 mM NaH₂PO₄, 10 mM Tris-HCl buffer, pH 8.0 containing 8 M urea. Protein was dialyzed against 100 mM NaH₂PO₄, 10 mM Tris-HCl buffer, pH 8.0 containing a decreasing amount of urea up to 2 M. Protein was concentrated using centrifugal concentrator of 30 kDa (Amersham, GE Healthcare Life sciences). Protein was flash-frozen in liquid nitrogen and stored at -80°C. Polyclonal antibody against CG14215 was generated in the rabbit at Abgenex, Bhubaneswar, Odisha. Antibodies were affinity-purified over purified antigen chemically cross-linked to N-hydroxysuccinimidyl-sepharose (NHS) beads (Sigma). Eluted with low pH, neutralized and dialyzed against PBS overnight at 4°C. Antibody against full-length dNup43 was also generated using an identical protocol.

Larval head complexes were dissected in cold PBS from third Instar larva and lysed in laemmli buffer. A total of two head complexes equivalent were loaded in each well of the gel. *Drosophila* S2 cells were pelleted down and lysed in 50 mM Tris-HCl pH 7.6, 150 mM NaCl, 1 mM MgCl₂, 1 mM EDTA, 10% glycerol, 0.4% sodium deoxycholate, 1% Triton X-100, 0.5% SDS, 1% NP-40, 2X PIC (modified from (78)). 5 and 10 μg of total protein was resolved on to 8% SDS-PAGE and transferred to methanol activated PVDF membrane (Merck-Millipore). Polyclonal, anti-dElys antibody was used at 1:1000, anti-Nup43 antibody (1:500), anti-Nup98 (1:2500), anti-Lamin B (1:2500), anti-Lamin C (1:2500), anti-mAb414 (1:5000), anti-Ran (1:5000) and anti-α-tubulin (1:5000) dilutions with overnight incubation at 4°C. The HRP-coupled secondary antibody was used at 1:15,000 dilutions for the detection of proteins in Western blot developed with super signal west Pico chemiluminescent substrate (Pierce, Thermo Fisher Scientific).

**Immunostaining:**

**In vivo** localization of dElys was revealed by Immunostaining of *Drosophila* embryos. *mat-α-tub-GAL4* driven W¹¹¹⁸ and dElys KK staged syncytial blastoderm embryos were collected on apple juice agar plates for 1 hr and aged to 2.5-3 hours at 25°C. Embryos were processed, as mentioned elsewhere (79,80). Briefly, embryos were dechorionated in 1:1 solution of sodium hypochlorite (~4% w/v available chlorine) and water until appendages from 80% of embryos disappear. Embryos were washed thoroughly in embryo wash buffer containing 0.2% NaCl and 0.05% Triton X-100 and fixed.
with 4% formaldehyde in heptane for 45 min at room temperature. Embryos were then devitellinized in 100% methanol for 30 min at room temperature. Embryos were blocked in 5% neutralized goat serum (Jackson Laboratories). Processed embryos were then used for immunostaining with the anti-\( \text{dElys} \) antibody (1:1000) and mAb414 (1:500, Bio-legend), anti-Dorsal (1:20, 7A4, DSHB). Embryos were mounted in vectashield mounting medium (Vector Laboratories).

For immunostaining of *Drosophila* salivary glands, ubiquitous Act5C-GAL4 driven first, second or third instar larva was dissected in cold PBS for isolation of salivary glands. Glands were fixed in freshly prepared 4% formaldehyde for 30 min at room temperature and washed thoroughly with 0.2% PBST (PBS+0.2% Triton X-100). Salivary glands were blocked with 5% neutralized goat serum (Jackson Laboratories) and stained with anti-\( \text{dElys} \) (1:1000), anti-\( \alpha \)-Nup43 (1:250), mAb414 (1:500, Bio-legend), anti-Ran (1:5000, BD bioscience), anti-Lamin Dm0 (1:1000, ADL67.10, DSHB, deposited by Prof. Paul A. Fisher, Stony Brook University, USA (81)), anti-LBR (1:500, gift from Prof. George Krohne, University of Wurzburg, Germany, (82)), anti-Nup98 (1:1000, gift from Dr. Cordula Schulz, University of Georgia, USA (83)), anti-TBP (1:200, (30) for overnight at 4°C also probed with anti-Dorsal (1:20, 7A4, DSHB, (34), 1:50 for Dorsal antibody gift from Dr. Zeitlinger, Stowers Institute, USA), anti-Cactus (1:50, 3H12, DSHB,(34)). For apoptotic induction, salivary glands were probed with an anti-cleaved-Dcp-1 antibody (1:100, Cell signaling technology, #9578 (38)). Eye and wing imaginal discs were dissected from third instar larva and processed as described above for probing of apoptotic induction with the Dcp-1 antibody. Secondary antibodies used were anti-rabbit Alexa Fluor 568 (1:800, Thermo Fisher Scientific), anti-mouse Alexa Fluor 488 (1:800, Thermo Fisher Scientific), anti-guinea pig FITC (1:400, Jackson laboratories), anti-mouse Cy5 (1:500, Jackson Laboratories). DNA was stained with DAPI (1:5000, Thermo Fisher Scientific). Salivary glands were mounted in vectashield mounting medium (Vector Laboratories).

*Drosophila* S2 cells were immunostained by immobilizing on 0.25 mg/ml concanavalin A coated coverslips for 2 hours at 25°C. MG-132 arrested cells were fixed by 100% methanol at -20°C, blocked with 5% neutralized goat serum (Jackson laboratories) and immunostained anti-\( \text{dElys} \) (1:1000), anti-\( \alpha \)-tubulin (1:1000, DSHB), anti-CID (1:1000, a gift from Prof. Steve Henikoff, FHCRC, USA, (22)). All the steps of immunostaining of S2 cells were followed as per (84).

All the samples were imaged on Carl Zeiss LSM 780 up-right confocal microscope equipped with a 63X/1.4 N.A. oil immersion lens. Super-resolution microscopy of anti-CID antibody labeled, EYFP-\( \text{dElys} \) transfected cells were done with Carl Zeiss LSM 800 Airyscan inverted microscope. Images were processed for super-resolution in the *inbuilt* mathematical algorithm of Zen.2 software of LSM 800 Airyscan. Images were processed with ImageJ (NIH) or Fiji software and Adobe Photoshop CS6 (Adobe Corporation).
**Intensity Quantification:**

Average signal intensities for each molecule were measured by using Zen-2012 (Carl-Zeiss, Image analysis software) or Fiji software and graphs were plotted with GraphPad software (Prism). For the quantification of Dorsal and GFP-NLS nuclear localization, five different regions of interest (ROI) were defined per nucleus and cytoplasm around it. The average intensity from each ROIs was measured using Zen-2012 (Carl-Zeiss, Image analysis software), and the mean of those intensities was taken per nucleus and cytoplasm around it. The ratio of the nucleus and cytoplasmic mean intensity was calculated for the final graph. Dcp-1 signal intensities were measured using five ROIs per cell and the mean of that is used for the final graph. Nuclear rim localization of different molecules was calculated as average intensity per nucleus including the nuclear rim. All the experiments were performed as at least three independent replicates.

**Bright-field microscopy:**

For imaging of eye and wing phenotype of dElys knockdown flies, three days old flies were anesthetized with diethyl ether (Merck) and immobilized on sticky gum for proper orientation of *Drosophila* eye. At least 10 flies from each knockdown experiment were imaged from three independent experiments for each genotype. *Drosophila* eyes were imaged using Leica fluorescent stereomicroscope M205 FA using a 123X magnification of particular equipment. For wing imaging in case of wing phenotypes with a dElys knockdown and dElys<sup>KK</sup>, diap-1<sup>OE</sup>, three-day-old fly wings from each genotype were dissected and placed on a glass slide under a coverslip and imaged directly under Leica upright light microscope DM2500 with 10X magnification (85). At least 15 pairs of wings from each genotype were examined from three independent crosses.

**Scanning electron microscopy (SEM):**

*Drosophila* eyes from eye-specific knockdown of dElys were imaged using a scanning electron microscope for detailed analysis of eye structure perturbations. Three days old flies from each RNAi cross was processed as per (86). Briefly, flies were anesthetized and fixed with 2.5% glutaraldehyde in phosphate-buffered saline for 2 hours at 4°C. Flies were washed thoroughly twice with PBS+ 4% sucrose. Flies were dehydrated through a graded ethanol series and were subjected to critical point drying. Samples were mounted on aluminum stubs with carbon conductive tape. Flies were coated with gold particles in a sputter coating apparatus. Samples were imaged using a Carl Zeiss Gemini II FESEM microscope. At least 10 flies from each genotype were imaged from three independent experiments.

**In vitro DNA binding experiment:**

To analyze the DNA binding activity of AT-hook like motifs of dElys, we purified fragment spanning all three AT-hook like DNA binding motif by cloning C-terminal 104 amino acid (1858-1962) from clone LD14710 into pET28a (+) vector. (His)<sub>6</sub> tagged AT-hook motif fragment was purified by expressing in *E.coli* BL21 (DE3) cells. Protein was induced by 200 µM IPTG (Sigma) overnight at 18°C. Cells were lysed in lysis buffer containing 50 mM Tris-HCl pH8.0, 150 mM NaCl, 20 mM imidazole, 1% Triton
X-100, and 50 µg/ml lysozyme on ice. Protein was purified over Ni-NTA beads by eluting in 300 mM imidazole in 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM MgCl₂ and 0.5 mM EDTA. Protein was dialyzed overnight against dialysis buffer, 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM MgCl₂ and 0.5 mM EDTA.

For site-directed mutagenesis of arginine residues, we used Q5 site-directed mutagenesis kit (NEB Bio-labs) with primers coding mutated nucleotides. Primers used for mutagenesis were mentioned in Table S2. The clone described above was used as a template for site-directed mutagenesis where conserved arginines were mutated to alanine as shown in Fig. S2. Mutations were confirmed using DNA sequencing (IISER-Bhopal sequencing facility). Clones were transformed into E.coli BL21 (DE3) cells and proteins were purified as mentioned earlier.

In vitro DNA binding experiment was performed by incubating template which contains 50% AT richness and 90% AT richness with purified wild-type and mutant proteins. A known cytosolic protein which does not bind to DNA was used as a negative control. DNA binding experiment was performed in binding buffer containing 100 mM Tris, 500 mM KCl, 10 mM DTT pH 7.5 for 30 min at room temperature and analyzed on to 0.8% agarose gel and detected by UV transilluminator (UVP). Purified DNA used for the binding experiment is 5 nM and the total purified protein was used at 0.5 µM concentrations. EMSA was performed by using the Lightshift Chemiluminescent EMSA Kit (Pierce, Thermo Scientific) by using Sdic DNA oligos as described by (25).

Total mRNA fluorescence in situ hybridization (mRNA-FISH):

Total mRNA FISH was performed as per (87) with some modifications. Salivary glands from third instar larva were dissected and fixed in 4% paraformaldehyde for 20 min at room temperature followed by the second fixation in 100% methanol for 10 min at 4°C. Tissues were dehydrated in graded ethanol series for 5 min each. Cy3-labelled poly (dT) oligos (IDT) were denatured in hybridization buffer containing 50% formamide, 2X SSC (Saline sodium citrate), 10% Dextran Sulfate and 0.05% BSA at 70°C for 5 min. Denatured poly (dT)-Cy3 were added at 0.1 pmol/µl concentration to the fixed tissue and incubated overnight at 37°C for hybridization. Tissues were washed in 2XSSC twice. DAPI was added for staining DNA and mounted in vectashield. Tissues were imaged using the LSM 780 laser scanning confocal microscope.

Quantitative-PCR:

Total RNA was isolated from Control and dElys knockdown with either mat-a-tub-GAL4 driven early embryos or ubiquitous Act5C-GAL4 driven first and third instar larva head complex using total tissue RNA isolation kit (Favorgen Biotech). One µg of total RNA was used to synthesize cDNA using iScript cDNA synthesis (Bio-Rad). cDNA was diluted 5 times, and 1 µl of cDNA from each genotype was used as a template. Semi-quantitative PCR was done using gene-specific primers and RpL49 as control. Real-time PCR on the same cDNA was done in Roche Lightcycler 480 at standard cycling conditions and probed with SYBR-green (Bio-Rad) in real-time using dElys and Actin RT
primers. Quantification of each reaction was done by calculating ΔCT values. ΔCT was normalized against C^T values of actin. The graph was plotted as fold change in dElys expression using GraphPad software (Prism). Primers used in quantitative PCR are mentioned in table S2.

**Hatching rate analysis:**

Embryos from control and dElys RNAi driven with *mat-a-tub-GAL4* was collected for 5 hrs and aged at 28°C for 48 hrs after counting the total number of embryos. After 48 hrs number of intact, unhatched embryos were counted manually under the stereoscope. The percentage of unhatched embryos was determined by the ratio of unhatched embryos to the total number of embryos collected. Each experiment was performed at least three independent replicates.
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Author Contributions:
Conceptualization: RKM and SJM. Methodology: SJM, RKM, and VK; Formal Analysis: SJM, RKM, and VK; Investigation: SJM; Resources: RKM and VK; Writing – original draft: SJM and RKM; Writing - review & editing: SJM, RKM, and VK; Visualization: SJM; Supervision: RKM and VK; Project administration: RKM; Funding acquisition: RKM

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Conflict of Interests:

The authors declare no competing or financial interests.
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Figure 1: ELYS is conserved in *Drosophila*

(A) Graphical representation of sequence identity of dElys with mouse and human ELYS. dElys is 20.9% and 20.2% identical with mouse and human ELYS, respectively. Domain-wise identity is mentioned in each domain. Conserved secondary structures between *Drosophila*, mouse and human ELYS are represented. β-propeller N-terminal domain, α-helical central domain, and disordered C-terminal regions are conserved in dElys as in higher orthologues.
(B) The antibody generated against dElys identified a band of ~215 kDa in lysates obtained from third instar larva head complex from wild-type control and ubiquitous dElys knock down lines as indicated. α-tubulin was used as a loading control.

(C) Syncytial Drosophila embryos stained with dElys antibody (red), mAb414 (green) marking FG-nucleoporins, and DNA is stained with DAPI. (Scale bar: 5 µm)

(D) A high-resolution image of metaphase-arrested Drosophila S2 expressing EYFP-tagged dElys (green) stained for kinetochores with an anti-centromere identifier (CID) antibody (red). (Scale bar: 2 µm)
Figure 2: dElys is essential for the normal development of Drosophila

(A) Quantification representing the lethality stages of the dElys knockdown organism. RNAi lines were driven with ubiquitous Act5C-GAL4 driver. Control represents wild-type flies crossed with the Act5C-GAL4 driver.

(B) Quantitative PCR for dElys knockdown using two different RNAi lines. Data are represented from at least three independent experiments. Statistical significance
derived from one way ANOVA followed by Tukey’s post-hoc test. Error bars represent SEM. *** represents p<0.0001 and ** represents p<0.001.

(C) Hatching rate analysis of maternal depletion of control and dElys in embryos using mat-a-tub-GAL4>>RNAi driver.

(D) Eye-specific knockdown of dElys using the Ey-GAL4 drivers. The first column shows the stereomicroscopic image, the second column represents the SEM image (500 X), and the third column shows an SEM image (2.5K X). Observations were made from at least three independent experiments (Scale bar: 200 µm in the stereomicroscopic image, 20 µm in SEM column 1 and 2 µm in SEM column 3). Control represents wild-type flies crossed with Ey-GAL4 driver.

(E) dElys depletion in the wing using the Wg-GAL4 driver. Representative images are shown for wing phenotypes. Observations were made from at least three independent experiments (Scale bar: 200 µm). Control is wild-type flies crossed with Wg-GAL4 driver.
Figure 3: dElys is essential for the nuclear pore complex and nuclear lamina assembly in Drosophila

(A-B) Detection of dElys (A), and FG-repeat nucleoporins (detected by mAb414, B), on the nuclear rim assessed in third instar larval control, (first and second vertical panels) and dElys depleted (ubiquitous Act5C-GAL4 driven, third and fourth vertical panels) salivary gland nuclei. DNA is stained with DAPI (Scale bar: 5 µm). Control is wild-type flies crossed with the Act5C-GAL4 driver.

(C) Assessment of Lamin C localization in third instar larval control and dElys depleted (ubiquitous Act5C-GAL4 driven) salivary gland nuclei as mentioned in A and B. (Scale bar: 5 µm)

(D) Representative images of histone H3 (red) staining in control and dElys depleted (ubiquitous Act5C-GAL4 driven) third instar larval salivary gland nuclei. DNA is stained with DAPI (Scale bar: 5 µm). Control is wild-type flies crossed with the Act5C-GAL4 driver.

(E) Western blot analysis of indicated molecules tested in control and dElys knockdown (Ubiquitous Act5C-GAL4 driven) third instar larva head lysate. * indicates detection with mAb414 antibodies. α-tubulin was used as a loading control. Control is wild-type flies crossed with the Act5C-GAL4 driver. Each western is performed at least three times. Stripes separated from the same experiment were probed with additional antibodies as mentioned in Fig. S4P.

(F) Western blot analysis for nuclear lamina molecule Lamin C as done in (E). α-tubulin was used as a loading control. Stripes separated from the same experiment were probed with additional antibodies as mentioned in Fig. S4Q.
Figure 4: Dorsal is activated in dElyș depletion and show induced apoptotic response

(A) Detection of Dorsal with anti-Dorsal (green) and dElyș antibodies (red) in control (upper panels) and dElyș RNAi (ubiquitous Act5C-GAL4 driven, lower panels) third instar larval salivary gland nuclei. DNA is stained with DAPI (Scale bar: 5 µm). Control is wild-type flies crossed with the Act5C-GAL4 driver.

(B) Detection of Cactus in third instar larval salivary gland cells with anti-Cactus (green) as mentioned in A. (Scale bar: 5 µm)

(C) Detection of apoptosis in third instar larval salivary gland cells with anti-Drosophila caspase-1 antibody (red) and FG-nucleoporins by mAb414 antibodies in control (upper
panels) and dElys depletion (ubiquitous Act5C-GAL4 driven, lower panels). DNA is stained with DAPI (Scale bar: 5 µm). Control is wild-type flies crossed with the Act5C-GAL4 driver.

(D-F) Quantification of intensities of indicated molecules in control and dElys RNAi condition. The intensity of each molecule was normalized to the intensity of DAPI. Data are represented from at least three independent experiments. At least 45 nuclei were analyzed from 7-8 pairs of salivary glands. Statistical significance derived from the student’s t-test. Error bars represent SEM. *** represents p<0.0001.

(G) Expression analysis of apoptosis regulatory genes, reaper, hid and diap-1 by quantitative PCR in cDNA from third instar larval head complex of control and dElys RNAi (ubiquitous Act5C-GAL4 driven). Data is represented from at least three independent experiments. Statistical significance derived from the student’s t-test. The error bars represent the standard deviation. *** represents p<0.0001 and ** represents p<0.001.

(H) Expressions of Dorsal target genes snail, twist, rho, dpp, short gastrulation (sog), and drosomycin analyzed by quantitative PCR in control and dElys RNAi (ubiquitous Act5C-GAL4 driven) third instar larval head complex lysate. Data are represented from at least three independent experiments. Statistical significance derived from the student’s t-test. The error bars represent the standard deviation. *** represents p<0.0001 and ** represents p<0.001 * represents p<0.05, ns is non-significant.
Figure 5: dElys depletion re-distributes Dorsal in the nucleus during the larval stage and specific to dElys

(A) Nuclear localization of Dorsal (green) assessed in control and dElys depletion in each stage of development. The first two vertical panels show early and late embryonic stages while the next three vertical panels show each successive larval stage salivary glands. DNA is stained with DAPI (Scale bar: 5 µm). Early embryonic depletion was driven with mat-α-tub-GAL4. Control is wild-type flies crossed with the mat-α-tub-GAL4 (for early embryos) and ubiquitous Act5C-GAL4 (for late embryo and later developmental stages) driver respectively. Stages of embryonic development are mentioned.
(B) Quantitation of nuclear/cytoplasmic intensity ratio of Dorsal measured through developmental stages of *Drosophila* and plotted for each stage showing the distribution of Dorsal in embryos and salivary gland cells. Data represent at least three independent experiments. Data are represented from at least three independent experiments. Error bar represents SEM.

(C) Dorsal target genes *snail, twist* and *sog* expression was analyzed in successive developmental stages by quantitative-PCR. The red bar represents analysis in whole early embryos, the grey bar represents analysis in head complex tissues of the first instar larva, and the blue bar represents analysis in head complex tissues of third instar larva. Control and *dElys* depletion graphs are mentioned with different shades in the image. Data are represented from at least three independent experiments. The error bar represents the standard deviation. *** represents $p<0.0001$, ** represents $p<0.001$ and * represents $p<0.05$.

(D) Dorsal nuclear localization assessed in control and nucleoporin knockdown third instar larval salivary glands (ubiquitous Act5C-GAL4 driven). DNA is stained with DAPI (Scale bar: 5 µm). Control is wild-type flies crossed with the Act5C-GAL4 driver. RNAi mediated knockdown of Nup160*GD* and Nup107*GD* was carried out at 23°C to obtain third instar larva.

(E) Quantitation of nuclear/cytoplasmic intensity ratio of Dorsal in control and nucleoporins knockdown salivary gland nuclei (ubiquitous Act5C-GAL4 driven). Data are represented from at least three independent experiments. At least 45 nuclei were analyzed from 7-8 pairs of salivary glands. Statistical significance derived from one way
ANOVA followed by Tukey’s post-hoc test. The error bar is SEM. *** represents $p<0.0001$ and * represents $p<0.05$, ns is non-significant.
Figure 6: *dorsal* depletion and *diap-1* overexpression can rescue *dElys* depletion apoptotic effects

(A) Apoptotic induction assessed in control (first horizontal panels), *dElys* RNAi (second horizontal panels) and *dElys*, *dorsal* co-depletion (ubiquitous Act5C-GAL4 driven, third horizontal panels) and *dElys* RNAi; *diap-1* over-expressed (salivary gland-specific expression, fourth horizontal panels). Third instar larval salivary gland tissues isolated from an above genetic combination of organisms were stained with Dorsal (green) and Dcp-1 (red) antibodies. DNA is stained with DAPI (Scale bar: 5 µm). Control is wild-type flies crossed with the Act5C-GAL4 driver as well as salivary gland-specific drivers.

(B and C) Quantitation of signal intensity of Dorsal (B) and Dcp-1 (C) in indicated samples. Data is derived from at least three independent experiments. At least 45 nuclei were analyzed from 7-8 pairs of salivary glands. Statistical significance derived from one way ANOVA followed by Tukey’s post-hoc test. Error bars represent SEM. *** represents p<0.0001 and ns is non-significant.

A theoretical model integrating the observation of the study. (D) Under normal conditions, *dElys* initiates postmitotic NPC assembly leading to functional nuclear pore in the nuclear membrane and normal organization of the nuclear lamina. Nuclear-cytoplasmic shuttling of Dorsal occurs conventionally leading to normal development. Dorsal is transported back to the cytoplasm through functional nuclear pores after completing the transcriptional regulation of key target genes in the nucleus. Inside cytoplasm, Dorsal is kept inactive by binding with Cactus, and by certain post-translational modifications or may be degraded.
Under dElys depleted condition, nucleoporins and nuclear lamina components are mislocalized. The NPC assembly and functional nuclear pore formation are perturbed, the nuclear lamina organization too is compromised. Dorsal translocate into the nucleus during early developmental stages exits as normal but during larval stage re-enters in the nucleus may be due to the defective nucleo-cytoplasmic shuttling of certain post-translational regulators of Dorsal in the nucleus or loss of its independent interaction with dElys. Resurrected Dorsal inside nucleus activates transcription of Dorsal target genes which rather remain shut during the post-embryonic stage. The mistimed transcription of developmentally regulated proteins may induce apoptosis resulting in lethality and developmental defects. Thus, the presence of dElys is critical for Dorsal localization, growth vs. apoptosis fate choice and to the conventional developmental program of the organism.
