Developmental Biology
Genetics and Genomics

Nup107 is a crucial regulator of torso-mediated metamorphic transition in *Drosophila melanogaster*

Reviewed Preprint
v1 • February 28, 2025
Not revised

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eLife Assessment

This **useful** study presents findings on the developmental roles of Nup107, a key nucleoporin, in regulating the larval-to-pupal transition in Drosophila melanogaster through its involvement in ecdysone signaling. The evidence supporting the authors' claims is **solid**, with robust experimental approaches including RNAi knockdown and rescue experiments. The findings highlight Nup107's function in regulating ecdysone biosynthesis, specifically through the regulation of EcR levels and Halloween genes expression in the prothoracic gland; additionally, rescue experiments suggest that the RTK PTTH/Torso signaling pathway is disrupted upon Nup107 depletion, further emphasizing its role in ecdysone regulation. However, finding a mechanism, addressing potential off-target effects of RNAi, and exploring alternative mutant models would strengthen the findings as the currently proposed mechanism is not fully supported by the data.

https://doi.org/10.7554/eLife.105165.1.sa4

Abstract

Nuclear pore complexes (NPCs), composed of nucleoporins (Nups), affect nucleocytoplasmic transport, thus influencing cell division and gene regulation. Nup107 subcomplex members have been studied in housekeeping functions, diseases, and developmental disorders.

We report a unique regulatory function for Nup107 in metamorphic transition during *Drosophila* development. RNAi-mediated *Nup107* depleted larvae were arrested in the third-instar larval stage and completely ceased to pupariate. The pupariation defect is primarily due to inhibited nuclear translocation and transcriptional activation by EcR. We unequivocally demonstrate the involvement of Nup107 in the transcription of the *Halloween* genes, modulating ecdysone biosynthesis and the EcR pathway activation. The regulation of EcR-mediated metamorphosis by the receptor tyrosine kinase, *torso*, is well documented. Accordingly, overexpression of the *torso* and MAP-kinase pathway activator, ras^{V12}, in the *Nup107* depletion background rescues the phenotypes, implying that Nup107 is an epistatic regulator of Torso-mediated activation of EcR signaling during metamorphosis.

Introduction

The multi-protein assembly of nucleoporins (Nups) constitutes nuclear pore complexes (NPCs). In eukaryotic cells, NPCs serve as molecular conduits for the trafficking of proteins and RNAs between the nucleus and the cytoplasm. The NPC composition was revealed from proteomic analyses conducted in yeasts and vertebrates, have contributed to our understanding, revealing that Approximately 30 distinct Nups are arranged in sub-structures and are distributed on different faces of NPCs (Cronshaw et al., 2002; Rout et al., 2000). The substructures are the outer cytoplasmic and inner nuclear rings, which surround a central inner ring called the scaffold ring, primarily aiding in the process of nucleocytoplasmic transport (Lin & Hoelz, 2019).

The largest Nup107 sub-complex, also called the Y-complex, is symmetrically located on both sides of the nuclear membrane. In metazoans, the Y-complex is composed of ten distinct Nups (ELYS, Nup160, Nup133, Nup107, Nup96, Nup85, Nup43, Nup37, Sec13, and Seh1). ELYS is a sub-stoichiometric Y-complex member and is present only on the nucleoplasmic side (D'Angelo & Hetzer, 2008; Morchoisne-Bolhy et al., 2015). Nup107, along with Nup133, forms the stalk of the Y-complex and is critically required for the Y-complex stability. The Nup107 complex plays a pivotal role in facilitating ELYS-coordinated post-mitotic NPC assembly (Boehmer et al., 2003; Walther et al., 2003) and messenger RNA (mRNA) export (Bai et al., 2004). Additionally, The Nup107 complex members actively participate in mitosis, contributing to the regulation of kinetochore-microtubule polymerization (Mishra et al., 2010; Zuccolo et al., 2007). The multitude of cellular processes where the Y-complex performs vital functions suggests it to be a central component in maintaining cellular homeostasis.

As a stable constituent of NPC, many Nups, including the members of the Y-complex, associate with chromatin and exert transcriptional regulation. Notably, interactions between active genes and Nups occurring predominantly within the nucleoplasm have been reported for dynamic Nups such as Nup98, ELYS, and Sec13 (Capelson, Liang, et al., 2010; Kalverda et al., 2010; Kuhn et al., 2019). In *Drosophila*, the dual nucleoporin, ELYS, governs the development, and ELYS RNAi-induced developmental defects are due to the reactivation of the Dorsal (NF-κB) pathway even during the late larval stages (Mehta et al., 2020).

Nup107 is associated with actively transcribing genes at the nuclear periphery (Gozalo et al., 2020). The disruption of Nup107 in zebrafish embryos leads to significant developmental anomalies, including the absence of the pharyngeal skeleton (Zheng et al., 2012). Moreover, the biallelic Nup107 mutations (D157Y and D831A) correlate well with clinical conditions such as microcephaly and steroid-resistant nephrotic Syndrome (Miyake et al., 2015). In *Drosophila*, Nup107 co-localizes with Lamin during meiotic division, and Nup107 depletion perturbs lamin localization, leading to a higher frequency of cytokinesis failure during male meiosis (Hayashi et al., 2016). Nup107 influences the regulation of cell fate in aged and transformed cells by modulating EGFR signaling and the nuclear trafficking of ERK protein (Kim et al., 2010).

Drosophila undergoes elaborate metamorphosis initiated by the neuropeptide prothoracicotropic hormone (PTTH) (Rewitz et al., 2013). Bilateral neurons projecting into the prothoracic gland (PG), when stimulated by the PTTH, induce Ecdysone production, which is subsequently released into the circulatory system for conversion by peripheral tissues into its active form, 20-hydroxyecdysone (20E) (Johnson et al., 2013; McBrayer et al., 2007; Shimell et al., 2018). The 20E binds to the ecdysone receptor, and the whole complex translocates into the nucleus and binds to chromatin to activate ecdysone inducible genes (Johnston et al., 2011; Kozlova & Thummel, 2002; Tennessen & Thummel, 2011). In the prothoracic gland, the primary neuroendocrine organ, PTTH signals through the receptor tyrosine kinase (RTK), Torso. The Torso-dependent activation of the MAP-kinase pathway is responsible for the production and release of

ecdysone hormone. However, ecdysone synthesis can also be regulated by the EGFR pathway (Cruz et al., 2020; Yamanaka et al., 2013). While Nup107 modulates EGFR pathway activation, the involvement of EGFR and torso pathways in ecdysone-dependent metamorphosis is undeniable. We dissect the involvement of Nup107 in Torso-mediated signaling and underlying mechanisms during *Drosophila* metamorphosis.

In a reverse genetic RNAi screening for Nup107 complex members, we noted that *Nup107* RNAi induces a significant developmental arrest at the third instar larval stage. Further analysis revealed that the Ecdysone Receptor (EcR) signaling pathway is perturbed, and EcR fails to translocate into the nucleus in *Nup107* knockdown. The failure of the EcR nuclear localization upon *Nup107* depletion is due to significantly reduced levels of the ecdysone hormone during the late third instar larval stage. Interestingly, overexpression of the *torso* and the ras^{V12} in *Nup107* depleted larvae rescued the developmental arrest and subsequently initiated pupation. We propose that Nup107, an epistatic regulator of torso-pathway activation in the PG, enables ecdysone surge for efficient metamorphic transition.

Results

Nup107 is essential for larval to pupal metamorphic transition

Cell biological analyses in the mammalian cell culture system has shed light on critical regulatory roles of Nup107 in vertebrates. Yet its importance in development is poorly understood. In this context, we started the characterization of *Drosophila* Y-complex member Nup107 in greater detail. Utilizing RNAi lines ($Nup107^{KK}$ and $Nup107^{GD}$), we performed ubiquitous depletion through *Actin5C-GAL4*. Interestingly, *Nup107* depletion, leading to larvae arrest at the third instar stage, causing complete cessation of pupariation (120 h AEL, right panel, **Figure 1A**), was accompanied by an extension of larval feeding and growth periods. Quantitative assessment of *Nup107* transcript levels in the $Nup107^{GD}$ and $Nup107^{KK}$ RNAi lines suggested efficient *Nup107* knockdown (approximately 60-70%, **Figure 1B**). For further analyses, we generated polyclonal antibodies against the Nup107 amino-terminal antigenic fragment (amino acids 1–210; see methods for details). Purified anti-Nup107 polyclonal antibodies detected a band of approximately ~100 kDa in lysates prepared from the control larval brain complex. The intensity of this ~100 kDa band was significantly reduced in lysates prepared from organisms where *Nup107* was knocked down ubiquitously (**Figure 1C**) using *Actin5C-GAL4* driving *Nup107* RNAi (denoted as ubiquitous hereon). Further, the immunostaining with Nup107 antibodies identified a conserved and robust nuclear rim staining pattern overlapping with mAb414 antibodies recognizing FG-nucleoporins (**Figure S1A**) and mRFP-tagged Nup107 expressed through its endogenous promoter (**Figure S1B**) in salivary gland tissues. These observations confirm the efficacy of *Nup107* knockdown and provide a handle to assess levels and localization of Nup107 in affected tissues. To further investigate the role of Nup107 in development, we generated a *Nup107* null mutant using CRISPR-Cas9-mediated gene editing. This comprehensive approach involving RNAi-mediated knockdown and CRISPR-Cas9 gene editing is expected to provide valuable insights into the significance of *Nup107* in *Drosophila* development. The gRNAs targeting regions close to the start and stop codon of the *nup107* gene generated knockout ($Nup107^{KO}$) mutants, which were confirmed by sequencing and PCR (**Figures S2A** and **S2B**). However, the $Nup107^{KO}$ mutants could not be used as the $Nup107^{KO}$ homozygous shows lethality at the embryonic stage. So, we carried out most of the analyses hereon with *Nup107* RNAi lines.

Nup107 contributes significantly to ecdysone signaling

The depletion of *Nup107* in *Drosophila* resulted in a distinct halt in growth and a developmental arrest at the third instar stage (**Figure 1A**). To discern the pathways affecting juvenile to adult developmental transition in *Drosophila*, we focused on levels of the sole insect steroid hormone,

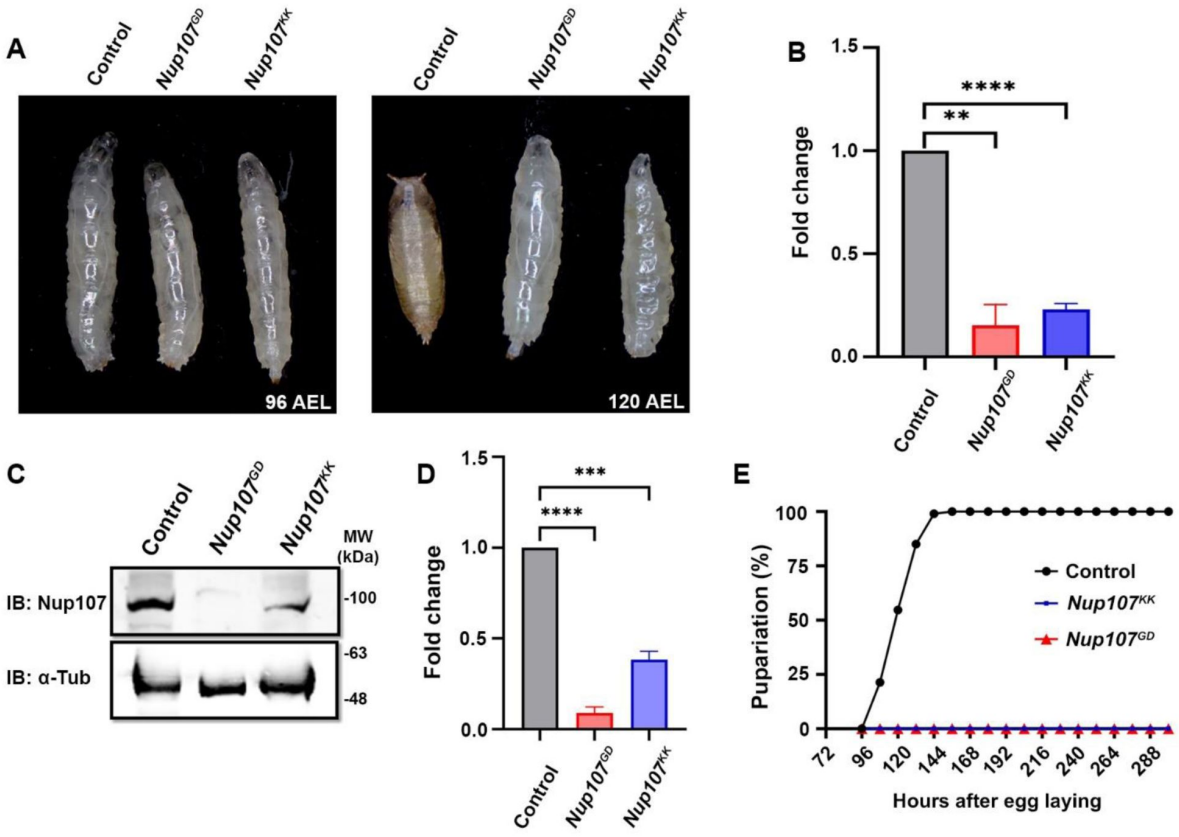

Figure 1.

***Nup107* depletion impairs metamorphosis.**

(A) Growth profile of third instar larvae from *Actin5C-Gal4* driven control and *Nup107* knockdowns ($Nup107^{GD}$ and $Nup107^{KK}$ RNAi lines) at 96 h AEL (after egg laying) and 120 h AEL.
(B) Quantitation of *Nup107* knockdown efficiency. Data are represented from at least three independent experiments. Statistical significance was derived from the Student's t-test. Error bars represent SEM. $^{**}p$ = <0.001 and $^{****}p$ = <0.0001.
(C) Immunodetection of Nup107 protein levels in third instar larval brain-complex lysates from control and *Nup107* knockdown.
(D) Quantification of Nup107 protein levels seen in (C). Data are represented from at least three independent experiments. Statistical significance was derived from the Student's t-test. Error bars represent SEM. $^{***}p$ = <0.0002 and $^{****}p$ = <0.0001.
(E) Comparison of pupariation profiles of control and *Nup107* knockdown organisms.

ecdysone.

We examined the localization of EcR in the late third instar salivary glands of both the control and *Nup107*-depleted larvae (using the $Nup107^{KK}$ line). Wild-type larvae exhibited normal EcR localization within the nucleus, but the nuclear translocation of EcR is perturbed in the *Nup107*-depleted larvae, with the bulk of the signal retained in the cytoplasm (**Figures 2A** and **2B**). Quantitative analysis of EcR intensities in the cytoplasm and nucleus further established a significant decrease in EcR signals inside the nucleus when *Nup107* is depleted (**Figure 2C**). This observation suggests that the Nup107 is required for EcR nuclear localization to mediate critical larval to pupal developmental transition. Furthermore, we noticed that significantly smaller size salivary glands and the brain complex in *Nup107* depleted larvae (**Figure S3**).

Prompted by the cytoplasmic accumulation of EcR, we investigated whether the mRNA levels of ecdysone-inducible genes were affected. When 20E binds with EcR, the activated EcR with enhanced affinity occupies the ecdysone response element (ERE) region in gene promoters. The ecdysone receptor is thought to function in a positive auto-regulatory loop, which may elevate EcR levels and sustain ecdysone signaling (Varghese & Cohen, 2007). Firstly, we examined the EcR transcript levels, revealing a reduction under *Nup107*-depletion conditions (**Figure 2D**). Subsequently, we measured the mRNA levels of known EcR target genes, *Eip75A* and *Eip74EF*, to find that the expression of each of these two target genes is reduced and correlates with *Nup107* knockdown levels (**Figures 2E** and **2F**). Similar results were observed in the depletion of *Nup107* using the GD-based RNAi line (**Figure S4**). The defects observed during metamorphic transitions can be attributed to impaired ecdysone signaling upon *Nup107*-depletion, which prompted a closer examination of their regulatory relationship.

Nup107 is dispensable for the nuclear import of EcR in the target tissue

We established that the nuclear localization of EcR is impaired upon *Nup107* knockdown (see **Figure 2B**). Typically, EcR nuclear localization follows an intricate mechanism where 20E binding to EcR allows its nuclear translocation and subsequent activation of its target genes (Cronauer et al., 2007; Johnston et al., 2011; Lenaerts et al., 2019). Biosynthesis of ecdysone hormone from dietary cholesterol requires a group of P450 enzymes coded by the *Halloween* genes, is a prerequisite for EcR activation (Kannangara et al., 2021; Niwa & Niwa, 2014). Moreover, the involvement of nucleoporin, Nup358, in facilitating nuclear transport of Met juvenile hormone receptors is well documented (He et al., 2017). Consequently, we posited the hypothesis that Nup107 either disallows active 20E-EcR complex formation by affecting 20E biosynthesis in ubiquitous *Nup107* knockdown scenarios or directly regulates EcR nuclear translocation in the target tissue.

We chose salivary glands to address these hypotheses and depleted *Nup107* using salivary glands-specific *AB1-GAL4* and PG-specific *Phm-GAL4*. Surprisingly, in contrast to the ubiquitous knockdown of *Nup107*, nuclear localization of EcR remained unaffected in salivary gland-specific *Nup107* knockdown (**Figures 3A, 3B**, **S5A** and **S5B**). Interestingly, the PG-specific *Nup107* knockdown phenocopied ubiquitous *Nup107* knockdown induced EcR nuclear localization defects (**Figures 3C** and **S5C**).

Quantification of EcR signals from *Nup107* depleted late third instar salivary gland cells and comparison with control salivary glands indicates that the nuclear/cytoplasmic ratios are drastically reduced in case of PG-specific depletion but unaltered in salivary gland-specific *Nup107* depletion (**Figures 3D** and **S5D**). Consistent with these observations, expression levels of ecdysone-inducible genes *Eip75A* and *Eip74EF* were significantly reduced in PG-specific *Nup107* knockdown (**Figures 3E, 3F**, **S5E** and **S5F**). In accordance with unperturbed EcR nuclear translocation, salivary glands-specific depletion of *Nup107* yielded no discernible differences in

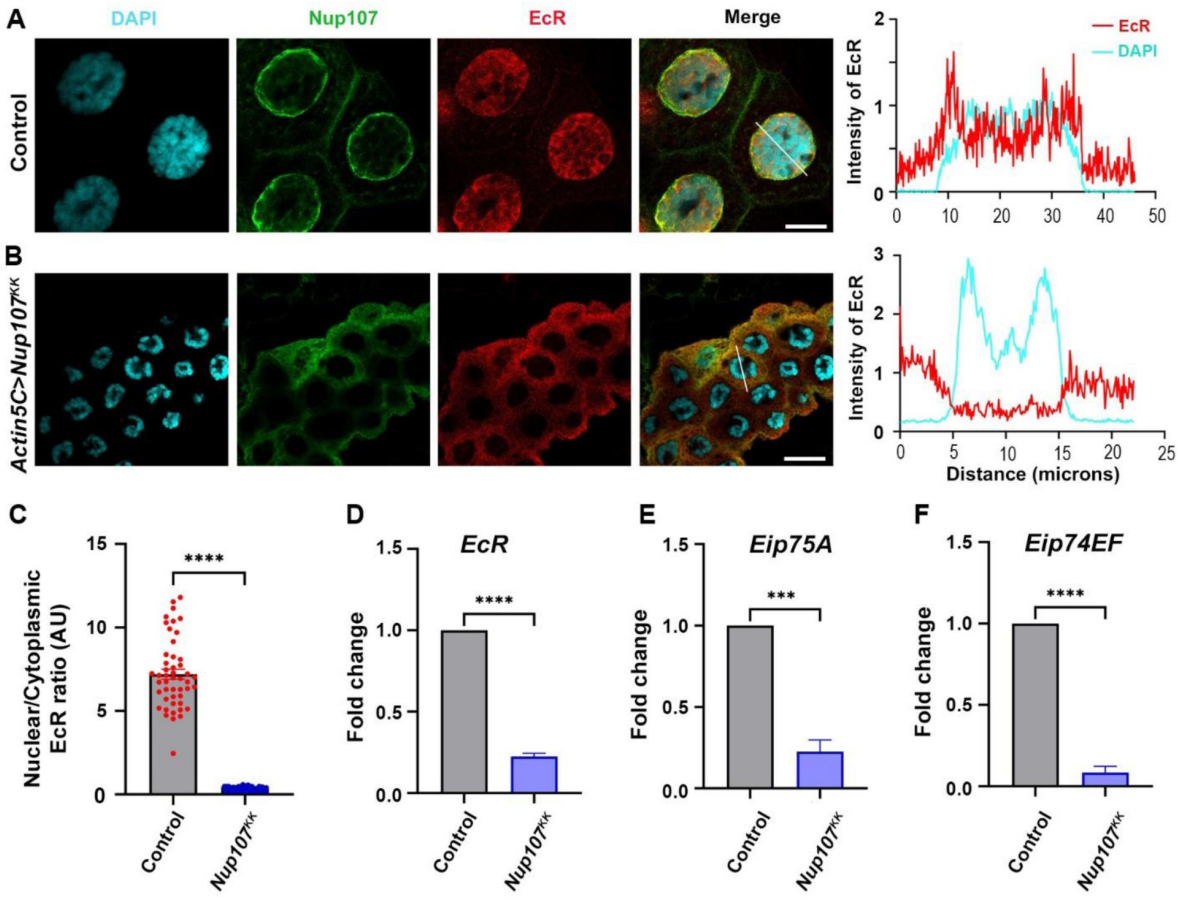

Figure 2.

Ubiquitous knockdown of *Nup107* disrupts ecdysone signaling.

(A-B) Staining of third instar larval salivary glands from control (A) and ubiquitous *Nup107* knockdown (B) with Ecdysone receptor (anti-EcR antibody, red) and Nup107 (anti-Nup107 antibody, green). DNA is stained with DAPI. Scale bars, 20 µm. Charts represent the line scan intensity profile of EcR (Red) and DAPI (Cyan) in the salivary gland nucleus region.
(C) Quantification of the nucleocytoplasmic ratio of EcR under control and *Nup107* knockdown conditions. At least 45 nuclei were analyzed from 7 to 8 pairs of salivary glands. Statistical significance was derived from the Student's t-test. Error bars represent SEM. ****p = < 0.0001.
(D-F) Analysis of ecdysone-inducible genes, *EcR* (D), *Eip75A* (E), and *Eip74EF* (F) expression, respectively, at the onset of metamorphosis (late third instar larvae stage).
Data are represented from at least three independent experiments. Statistical significance was derived from the Student's t-test. The error bars represent the SEM. ***p = <0.0004 and ****p = <0.0001.

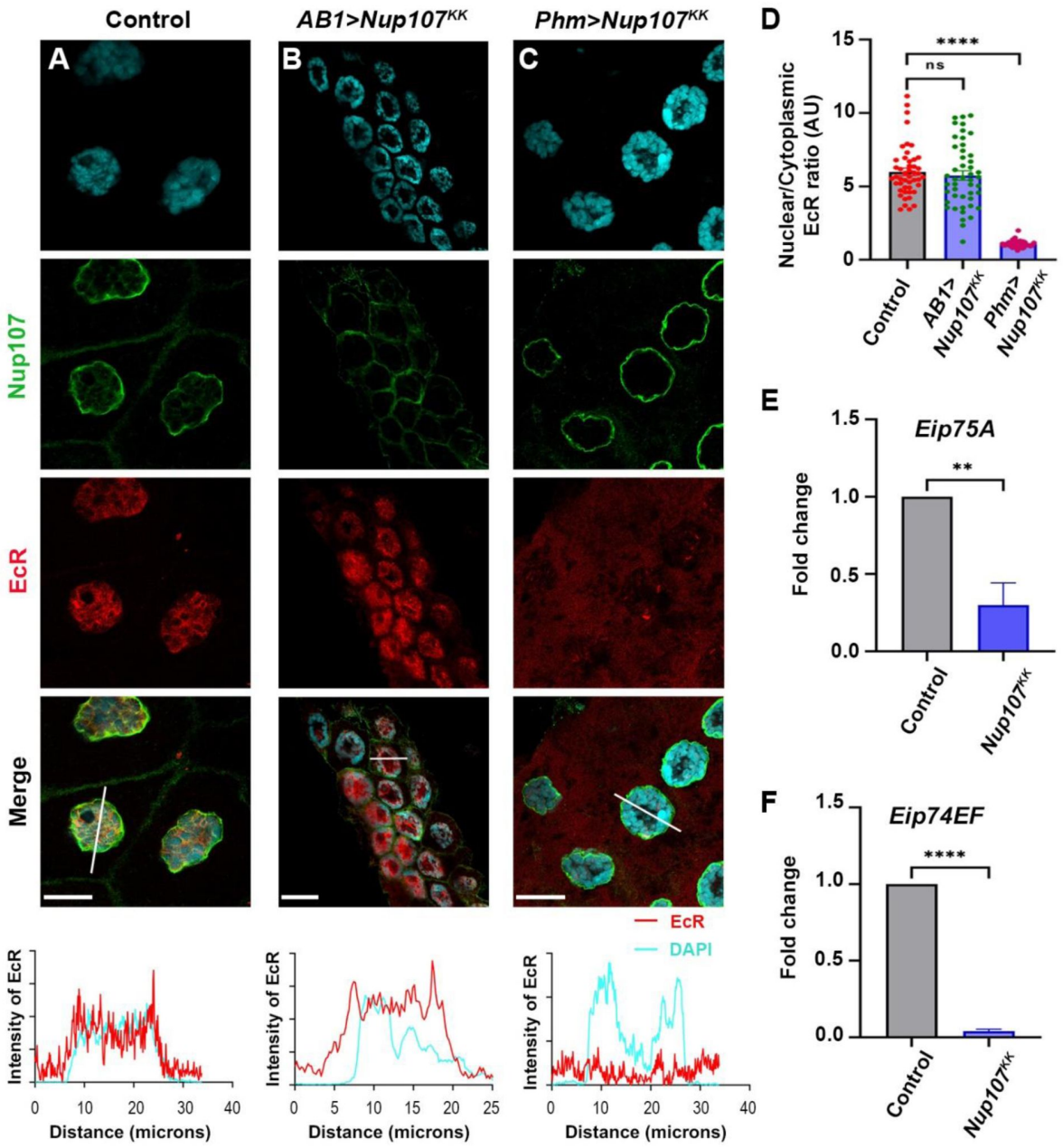

Figure 3.

***Nup107* regulates Ecdysone receptor-dependent signaling.**

(A-C) Detection of and quantitation of nucleocytoplasmic distribution of EcR (anti-EcR antibody, red) and Nup107 (anti-Nup107 antibody, green) in control (A), salivary gland-specific *Nup107* depletion (B), and prothoracic gland-specific *Nup107* depletion (C) from third instar larval salivary gland nuclei. DNA is stained with DAPI. Scale bars, 20 μm. Charts represent the line scan intensity profile of EcR (Red) and DAPI (Cyan) in the salivary gland nucleus region.
(D) EcR nucleo-cytoplasmic quantification ratio from the salivary gland and prothoracic gland-specific *Nup107* knockdown, respectively. At least 45 nuclei were analyzed from 7 to 8 pairs of salivary glands. Statistical significance was derived from the Student's t-test. Error bars represent SEM. ****p = <0.0001, and ns is non-significant.
(E-F) Quantitation of expression of *Eip75A* (E) and *Eip74EF* (F) ecdysone-inducible genes at the onset of metamorphosis (RNA isolated from late third instar larvae of control and prothoracic gland-specific *Nup107* depletion). Data are represented from at least three independent experiments. Statistical significance was derived from the Student's t-test. Error bars represent SEM. **p = <0.008 and ****p = <0.0001.

larval growth and pupariation compared to the control (**Figures S6A** and **S6C**). However, the PG-specific knockdown induced an extended third instar stage lifespan (10-12 days after egg laying, **Figures S6B** and **S6C**). The observed decrease in ecdysone-inducible gene expression during late third-instar developmental stages can explain the potential impairment of metamorphosis induction seen upon ubiquitous or PG-specific *Nup107* knockdown. In addition to the reduced size of the salivary gland and brain complex, we also noticed a compromise in the size of the Prothoracic gland upon *Nup107* knockdown (**Figure S7**).

These observations suggest that Nup107 exerts a regulation on ecdysone biosynthesis and active 20E-EcR complex formation rather than playing a direct role in EcR nuclear translocation.

Nup107 exerts control on the EcR pathway through Ecdysone level regulation

We reasoned that Nup107 may regulate the ecdysone biosynthesis in PG to induce the larval stage growth arrest. We delved into analyzing the effect of *Nup107* knockdown on ecdysone production. The considerable decrease in PG size due to *Nup107* knockdown (**Figure S7**) can potentially reduce 20E production. This prompted us to explore the potential role of Nup107 in influencing ecdysone production, and we assessed the impact of *Nup107* knockdown on ecdysone biosynthesis.

Utilizing an ELISA-based detection method, we assessed 20E levels in larvae at 96 hours and 120 hours after egg laying (AEL). Larvae from different experimental conditions, including control, ubiquitous *Nup107*-depletion, and PG-specific *Nup107*-depletion, were used in this analysis. Strikingly, the results indicated a substantial decrease (~ 3-fold with ubiquitous and ~10-fold for PG-specific knockdown) in total 20E levels upon *Nup107* knockdown, particularly at 120 AEL which coincides with the 20E surge seen during metamorphosis (**Figures 4A** and **S8A**). This observation is crucial and suggests a potential defect in ecdysone biosynthesis in *Nup107*-depleted organisms.

As depicted in **Figure 4B**, the PTTH hormone signaling in the PG upregulates the expression of *Halloween* genes (*spookier*, *phantom*, *disembodied*, and *shadow*) responsible for ecdysone biosynthesis, and the *shade* gene product is required for active 20E generation in peripheral tissues (Christensen et al., 2020; McBrayer et al., 2007; Shimell et al., 2018). We analyzed the *Halloween* genes transcript level in *Nup107*-depleted larvae and observed a significant downregulation for each of the *Halloween* quartet genes mentioned earlier. (**Figures 4C-4F** and **S8B-S8E**). Further, the level of the *shade* gene required for converting precursor ecdysone to active 20E was also reduced twofold in *Nup107*-depleted larvae (**Figures 4G** and **S8F**).

20E rescues Nup107-dependent EcR localization defects

The observed correlation between the expression levels of ecdysone biosynthetic genes and reduced levels of 20E upon *Nup107* knockdown strongly suggests a role for Nup107 in 20E biosynthesis, active 20E-EcR complex formation, and its nuclear translocation. It is not surprising in this context that exogenous ecdysone supplementation through larval food rescues pupariation blocks arising from ecdysone deficiency (Garen et al., 1977; Ou et al., 2016; Shimell et al., 2018). We experimented the same under *Nup107*-depletion conditions and supplemented exogenous ecdysone to PG-specific *Nup107*-depleted larvae by feeding them with a diet enriched with 20-hydroxyecdysone (0.2 mg/ml). Supplementation of 20E to *Nup107*-depleted larvae significantly (70-80%) alleviated the developmental arrest, and the onset of pupariation was comparable to the control (**Table S1**). However, none of the pupae could eventually eclose successfully, probably due to other effects of *Nup107* depletion. We asked if nuclear translocation of EcR can also be rescued by exogenous supplementation of 20E. While incubation of salivary glands of late third instar larva in S2 media control alone did not rescue EcR localization in any of

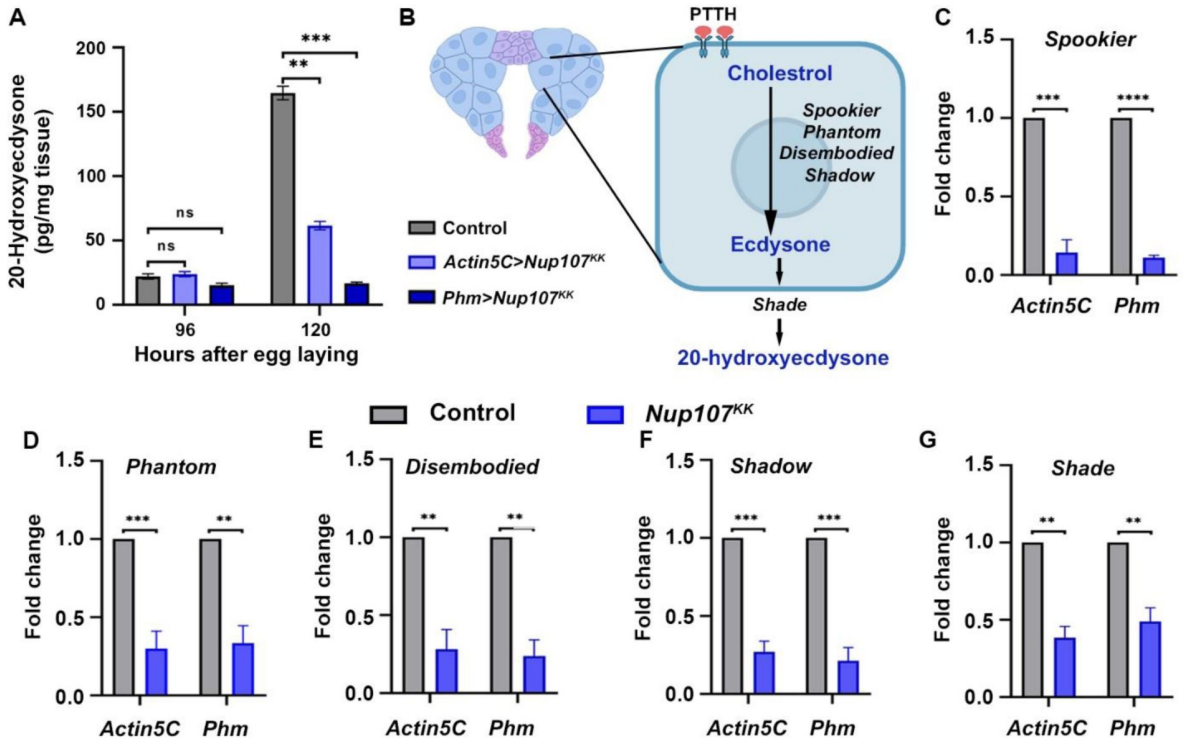

Figure 4.

***Nup107* critically regulates the expression of ecdysone-biosynthetic genes.**

(A) ELISA measurements of whole-body 20-hydroxyecdysone (20E) levels in control, ubiquitous (*Actin5C-Gal4*), and prothoracic gland-specific (*Phm-Gal4*) *Nup107* depletion at 96 and 120 h AEL. Data are represented from at least three independent experiments. Statistical significance was derived from the Student's t-test. Error bars represent SEM. ***p* = <0.0013, ****p* = < 0.0009 and ns is non-significant.

(B) Schematic representation of a Prothoracic gland cell showing genes involved in ecdysone biosynthesis from cholesterol.

(C-G) Quantification of ecdysone-biosynthetic gene expression levels of *Spookier* (C), *Phantom* (D), *Disembodied* (E), *Shadow* (F), and *Shade* (G) in cDNA isolated from control, and *Nup107* knockdown late third instar larvae. Data are represented from at least three independent experiments. Statistical significance was derived from the Student's t-test. Error bars represent SEM. ***p* = <0.001, ****p* = <0.0005 and, *****p* = <0.0001.

the *Nup107*-knockdown genotypes (**Figures 5A-5C** and **S9A**), we noticed a complete EcR nuclear translocation rescue in ubiquitous as well as PG-specific *Nup107*-depleted salivary glands when incubated with 20E (**Figures 5D-5F** and **S9B**).

The exogenous supplementation of 20E leads to active 20E-EcR complex formation as mRNA levels of the *Eip75A* and *Eip74EF* target genes were rescued significantly back to normal levels (**Figures 5G** and **5H**). Overall, these findings highlight the important regulatory function of Nup107 in the ecdysone signaling pathway.

Torso is an effector of *Nup107* mediated functions in metamorphosis

Next, we explored the mechanism of Nup107-driven regulation of 20E levels and metamorphosis. Receptor tyrosine kinase family of cell surface receptors signal by binding to ligands (growth factors and hormones) and participate in metabolism, cell growth, and development. Among these RTKs, the Torso, belonging to the platelet-derived growth factor receptor (PDGFR) class, plays a significant role during metamorphosis by serving as a receptor for the neuropeptide PTTH in the *Drosophila* brain (Sopko & Perrimon, 2013).

Functional engagement of PTTH-Torso activates the MAP kinase pathway, involving components of Ras, Raf, MEK, and extracellular signal-regulated kinase (ERK), thereby initiating metamorphosis (**Figure 6A**). The *torso* knockdown in the prothoracic glands resulted in the developmental arrest phenotype synonymous with what is observed with *Nup107* knockdown. Feeding 20E to *torso*-depleted larvae completely rescued developmental delay and normal growth phenotypes (Rewitz et al., 2009). We observed a significant decrease in the *torso* levels (~ four-fold) when *Nup107* was depleted ubiquitously using *Actin5C-GAL4* (**Figure 6B**), suggesting an epistatic regulation by Nup107 on the torso. Further, the phenotypic similarity between *Nup107* and *torso*-depletion scenarios and diminished *torso* level upon *Nup107*-depletion prompted us to investigate whether the *torso* is an effector of Nup107.

Overexpression of the *torso* and or ras^{V12} have been utilized to rescue torso pathway-mediated defects (Cruz et al., 2020; Rewitz et al., 2009). We probed the possibility of *Nup107* phenotype rescue by ubiquitous or PG-specific overexpression of the *torso* and ras^{V12}. Overexpression of either *torso* or ras^{V12} in *Nup107*-depletion background completely rescued the pupariation defects (**Figures 6C** and **6D**). The *torso* overexpression-mediated rescue of Nup107-depletion phenotypes is specific as overexpression of *Egfr* (another RTK implicated in metamorphosis) and *Usp* (co-receptor with EcR) in *Nup107* depletion background could not rescue the pupariation defects (data not shown). The remarkable rescue demonstrated by *torso* overexpression prompted us to analyze the status of EcR nuclear translocation, ecdysone biosynthesis, and ecdysone-inducible gene expression. The restoration of EcR nuclear translocation (**Figures 6E-6G**), the ecdysone biosynthesis genes, *spookier*, *phantom*, *disembodied*, and *shadow* (**Figures S10B-S10E**), and ecdysone target genes *Eip75A* and *Eip74EF* (**Figures 6H** and **6I**) to control levels indicated that *torso* could efficiently rescue *Nup107* phenotypes.

These observations suggest that the *torso* is a downstream effector of Nup107 functions, and torso-dependent signaling, responsible for 20E synthesis in PG and metamorphosis, is regulated by Nup107 levels.

Discussion

Apart from its importance in mRNA export and cell division at the cellular level, the NPCs Y-complex member Nup107, when mutated, correlates with developmental abnormalities, microcephaly, and nephrotic syndrome (Miyake et al., 2015; Zheng et al., 2012). In several

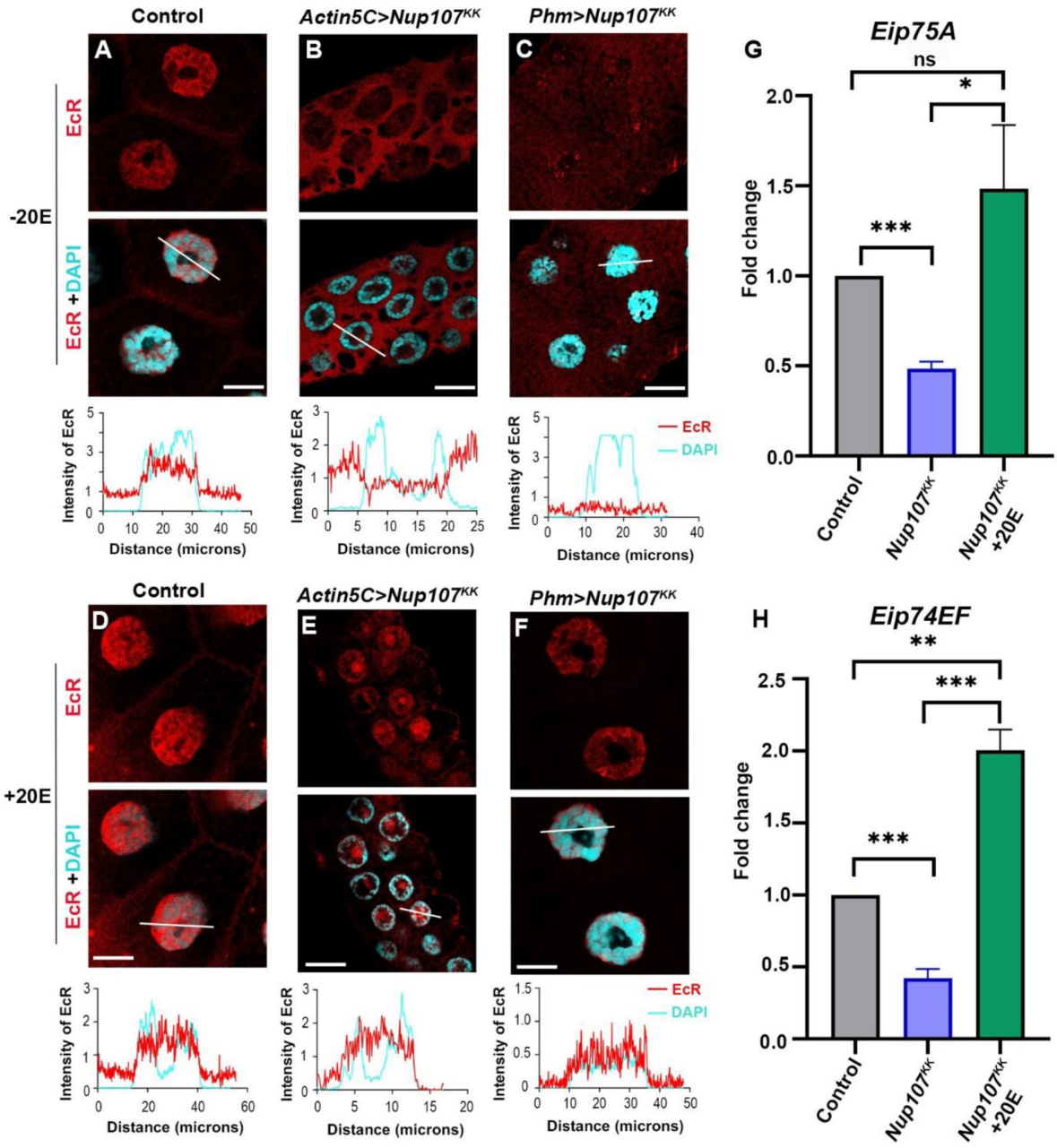

Figure 5.

20E supplementation rescues *Nup107* depletion-specific EcR signaling defects.

(A-F) Visualization of the nucleocytoplasmic distribution of EcR (anti-EcR antibody, red) without 20E (A-C) and with 20E (D-F) treatment in larval salivary glands of control, ubiquitous (*Actin5C-Gal4*) *Nup107* knockdown, and prothoracic gland-specific (*Phm-Gal4*) *Nup107* knockdown. DNA is stained with DAPI. Scale bars, 20 µm. Charts represent the line scan intensity profile of EcR (Red) and DAPI (Cyan) in the salivary gland nucleus region.

(G-H) Comparative quantification of expression ecdysone-inducible genes *Eip75A* (G) and *Eip74EF* (H) from 20E treated salivary glands. Data are represented from at least three independent experiments. Statistical significance was derived from the Student's t-test. Error bars represent SEM. *p = <0.02, **p = <0.002, ***p = <0.0008 and ns is non-significant.

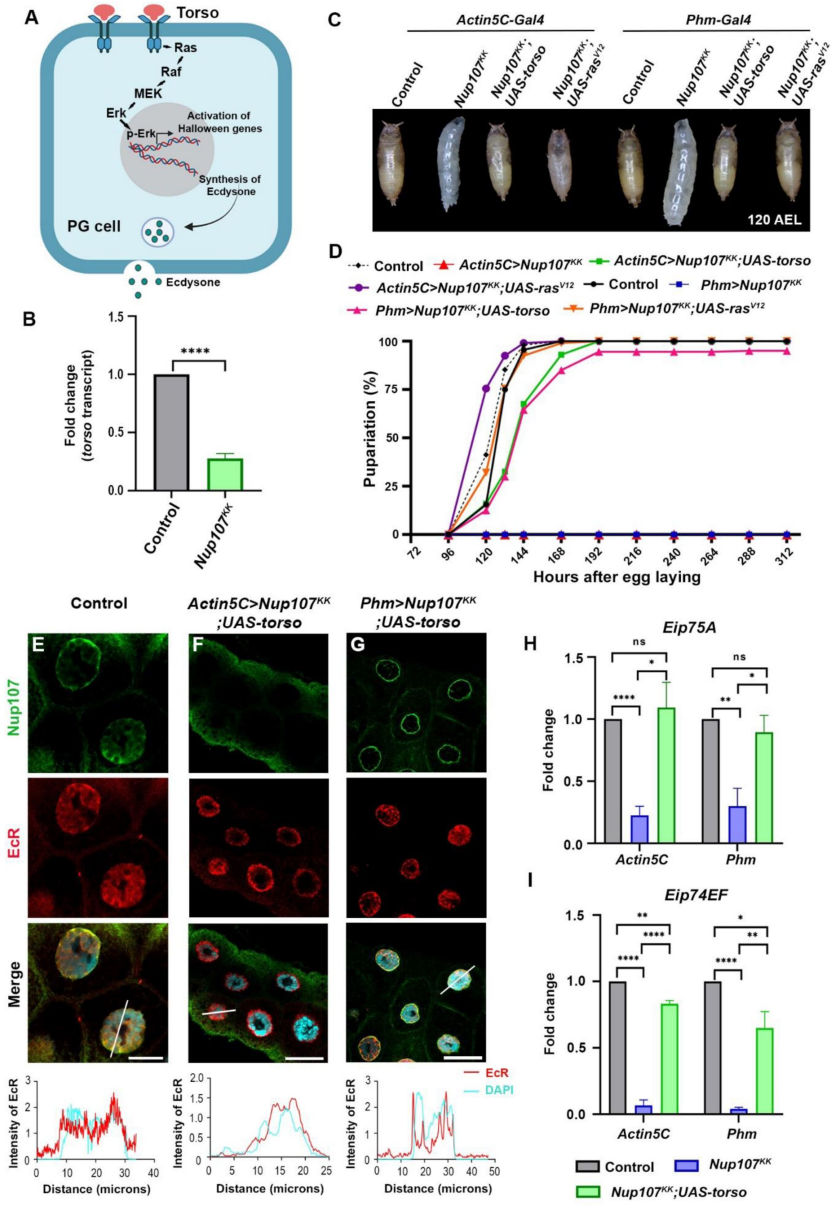

Figure 6.

Torso and *Nup107* act synergistically to activate the ecdysone signaling.

(A) A model of the Torso pathway and its components.
(B) Quantitation of *torso* transcript levels from control and *Nup107* depleted larvae (ubiquitous depletion using *Actin5C-Gal4*). Data are represented from at least three independent experiments. Statistical significance was derived from the Student's t-test. Error bars represent SEM. ****p = <0.0001.
(C-D) Comparison of pupariation profiles of control, *Nup107* knockdown, and *torso* and *ras*^{V12} overexpressing rescue organisms.
(E-G) Detection and quantitation of nucleocytoplasmic distribution of EcR (anti-EcR antibody, red) and Nup107 (anti-Nup107 antibody, green) in control, *torso* overexpressing ubiquitous *Nup107* knockdown (*Actin5C-Gal4>Nup107*^{KK}*; UAS-torso*) and *torso* overexpressing PG-specific *Nup107* knockdown (*Phm-Gal4>Nup107*^{KK}; *UAS-torso*) third instar larval salivary gland nuclei. DNA is stained with DAPI. Scale bars, 20 µm. Charts represent the line scan intensity profile of EcR (Red) and DAPI (Cyan) in the salivary gland nucleus region.
(H-I) Quantification of expression of *Eip75A* (H) and *Eip74EF* (I) ecdysone-inducible genes, respectively. Data are represented from at least three independent experiments. Statistical significance was derived from the Student's t-test. Error bars represent SEM. *p = <0.03, **p = <0.008, ****p = <0.0001 and ns is non-significant.

human cancer types, Nup107 exhibits high expression and serves as a biomarker for hepatocellular carcinoma. A strong structural and functional conservation between human and *Drosophila* Nup107 proteins allows *Drosophila* to be used to model to gain mechanistic insights in Nup107 functions in human diseases (Shore et al., 2022; Weinberg-Shukron et al., 2015). We observe that Nup107 is involved in critical developmental transitions from larva to pupa during *Drosophila* development as pupariation is completely arrested (**Figure 1**). The foraging third instar larva must acquire a critical weight and sufficient energy stores before it can metamorphose into a non-feeding pupa, molting into a healthy adult. The activation of the Torso receptor and sequent surge in ecdysone synthesis is essential for larval to pupal transition (Christensen et al., 2020; Hao et al., 2021; Luo et al., 2024). The elevated ecdysone levels trigger the translocation of the heterodimeric ecdysone receptor [in complex with Ultraspiracle (Usp)] into the nucleus to induce pupariation-specific transcriptional programming (Johnston et al., 2011). Similarly, when *Nup107* is depleted, nuclear translocation of EcR is abolished, arresting metamorphosis at the level of the third instar larval stage (**Figure 2**).

Translocation of nuclear receptor proteins like the ecdysone receptor to the nucleus is a crucial step for transcriptional activation. Nup358, present at the cytoplasmic filament face of NPC, plays a vital role in the nuclear import of Met (juvenile hormone receptor) along with Hsp83 (He et al., 2017) to help maintain the larval stages of development. The marked absence of EcR from the nucleus was intriguing as Nup107 generally participates in the nuclear export process. Perhaps Nup107 regulates pupariation by modulating ecdysone synthesis, ecdysone signaling, and nuclear translocation of EcR. Surprisingly, Nup107 is dispensable for the nuclear translocation of EcR in the target tissue, the salivary gland cells (**Figure 3**). Nup107 exerts strong control over the expression of *Halloween* genes involved in ecdysone biosynthesis, resulting in diminished 20E titer, poor EcR activation, and delayed larva-to-pupa transition (**Figures 4** and **S6**). The effect of Nup107 on *Halloween* genes adheres well with pupariation defects. These observations are in concurrence with reports of low ecdysone levels disrupting the pupariation process, leading to a halt in insect development (Christensen et al., 2020; Cruz et al., 2020).

In addition to ecdysone signaling, insect metamorphosis also has a strong contribution from receptor tyrosine kinase RTK pathway signaling. Notably, Torso signaling is essential for embryonic development and metamorphosis in *Drosophila*. The receptor expression level impacts the signaling output: high receptor levels trigger a robust and transient signal, while lower levels result in a weaker, sustained signal (Jenni et al., 2015; Konogami et al., 2016). Accordingly, the reduced ecdysone levels in the *torso* knockdown prothoracic glands induced pupariation delays (Rewitz et al., 2009). We observe a similar defect with *Nup107* knockdown, indicating a possible reduction in *torso* levels. Interestingly, we observed significantly reduced torso levels in *Nup107*-depleted organisms, suggesting an essential upstream function for Nup107 in regulating RTK pathway activation and the associated *Drosophila* metamorphosis process. In the contrasting observation, *Nup107* depleted larval tissues (salivary glands, brain complex, and prothoracic gland) are significantly smaller in size (**Figures S3** and **S7**). It is important to know how Nup107 contributes to organ size maintenance and if a crosstalk with RTK signaling is required in this context.

The developmental delays observed in *Nup107* knockdown larvae can be restored by exogenous supplementation of 20E, suggesting that Nup107 can modulate directly or indirectly the Torso receptor activity for ecdysone production and developmental regulation. Accordingly, we successfully rescued developmental delays by ubiquitous and PG-specific *torso* overexpression. Perhaps overexpression of the torso pathway mediator molecules can rescue the *Nup107* developmental delay phenotypes. The oncogenic variant of the Ras-GTPase (ras^{V12}) has been explored in a similar analysis with torso pathway mutants (Cruz et al., 2020; Rewitz et al., 2009). The alleviation of the developmental delays in the *Nup107* knockdown background upon ras^{V12} overexpression is an indication of Nup107 serving as an epistatic regulator of the torso pathway in metamorphic transitions (**Figure 6**).

In essence, our findings indicate that Nup107 influences pupariation timing by regulating the torso levels, it's signaling, and ecdysone biosynthesis (**Figure 7**). Previous research has shown that nuclear pore complexes (NPCs) are essential for maintaining global genome organization and regulating gene expression (Capelson, Doucet, et al., 2010; Capelson, Liang, et al., 2010; Iglesias et al., 2020). Particularly, Nup107 interacts with chromatin and targets active gene domains to regulate gene expression (Gozalo et al., 2020). Thus, Nup107 exerting its effects on *torso* transcription is the primary regulatory event in the *Drosophila* metamorphosis. It is important to note that the synthesis and availability of Torso ligand, PTTH, may not be affected by the Nup107 since torso or downstream effector ras^{V12} overexpression is sufficient to rescue the developmental arrest phenotype. It is thus crucial to further delineate the mechanism of Nup107-dependent regulation on *torso* pathway activation. The whole genome transcriptomics from PG can help shed more light on the regulatory roles of Nup107. This informationwill offer valuable insights into how nucleoporins regulate gene expression and serve as a model for elucidating how they govern the temporal specificity of developmental processes in organisms. These analyses will help establish a link between Nup107, the PTTH-PG axis, and the regulation of maturation timing. Our observations indicate critical roles for Nup107 in both torso-ecdysone interplay in metamorphosis and torso-independent mechanism for organ size maintenance. Together they add valuable information to hitherto unknown functions of the Nup107 in organismal development. Further investigations are required to identify the interactors of Nup107 involved in these coordination mechanisms in developmental transition.

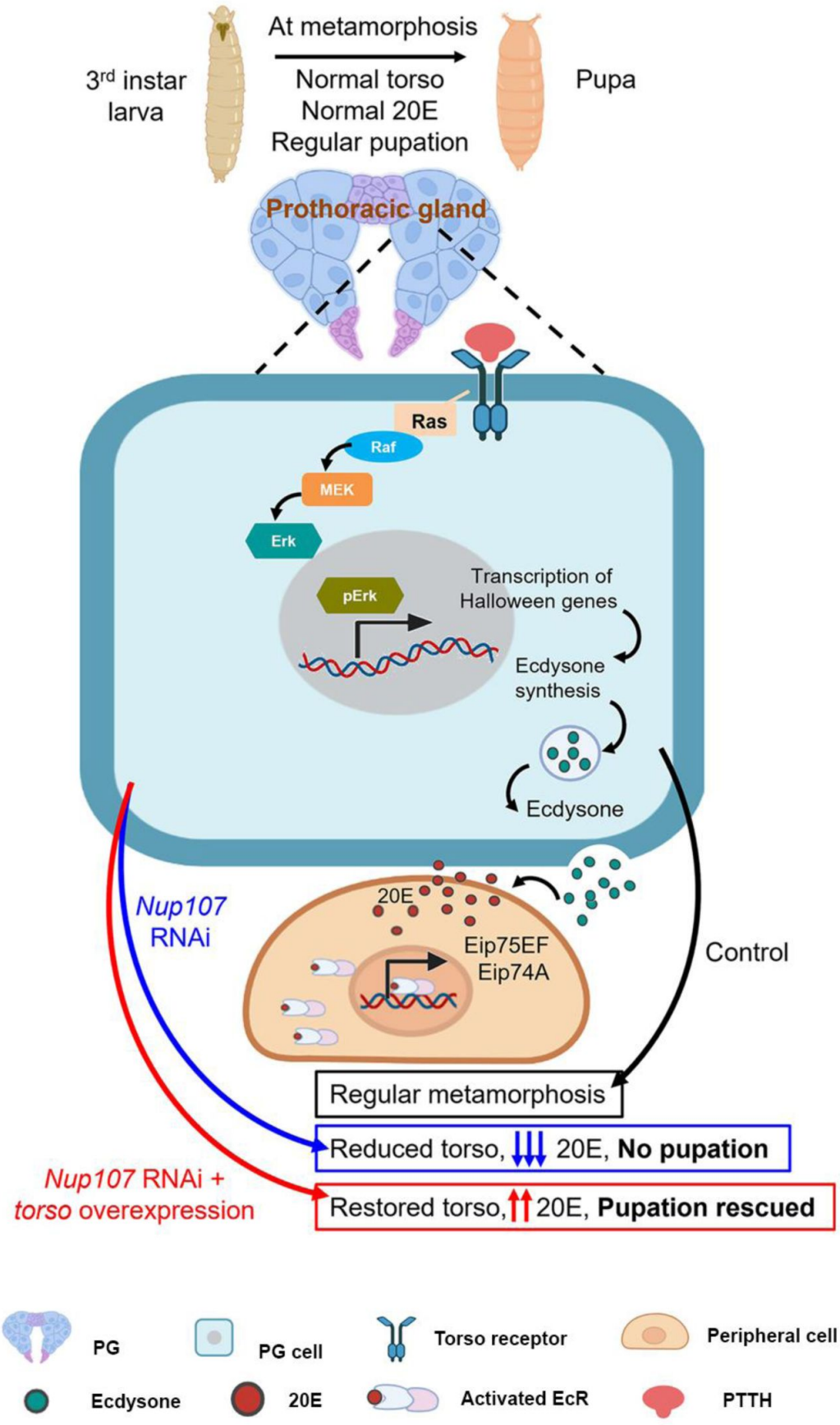
3rd instar larva
At metamorphosis
Normal torso
Normal 20E
Regular pupation
Pupa
Prothoracic gland
Ras
Raf
MEK
Erk
pErk
Transcription of Halloween genes
Ecdysone synthesis
Ecdysone
Nup107 RNAi
20E
Eip75EF
Eip74A
Control
Regular metamorphosis
Reduced torso, 20E, No pupation
Nup107 RNAi + torso overexpression
Restored torso, 20E, Pupation rescued
PG
PG cell
Torso receptor
Peripheral cell
Ecdysone
20E
Activated EcR
PTTH

Figure 7.

Theoretical Model of Nup107 functions in metamorphosis:

During metamorphosis, the prothoracic gland (PG) responds to prothoracicotropic hormone (PTTH) via Torso receptors. The MAP kinase pathway involving Raf, MEK, and ERK, initiated by Ras, leads to *Halloween* gene expression responsible for Ecdysone synthesis and release. Ecdysone is converted to active 20-hydroxyecdysone (20E) in peripheral tissues. The binding of 20E to the Ecdysone receptor (EcR) allows EcR nuclear translocation and EcR pathway activation, culminating in target gene expression facilitating the metamorphic transition. Nup107 depletion negatively impacts Torso levels and Torso pathway activation, inducing pupariation arrest, which can be rescued by autonomous activation of the Torso pathway. Image created with *BioRender.com/x18z538*.

Materials and methods

Key resources table

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
Genetic reagent (*D. melanogaster*)	w^{1118}	Bloomington *Drosophila* Stock Center	BDSC:3605; FLYB: FBst000360; RRID:BDSC_3605	w[1,118]
Genetic reagent (*D. melanogaster*)	w1118; P{GD12024}v22407	Vienna *Drosophila* Resource Center	VDRC: v22407; FLYB: FBst0454535; RRID:FlyBase_FBst0454535	P{GD12024}v22407 ($Nup107^{GD}$ RNAi)
Genetic reagent (*D. melanogaster*)	P{KK108047}VIE-260B	Vienna *Drosophila* Resource Center	VDRC: v110759; FLYB: FBst0482324; RRID:FlyBase_FBst0482324	P{KK108047}VIE-260B ($Nup107^{KK}$ RNAi)
Genetic reagent (*D. melanogaster*)	y[1] w[*]; P{w[+mW.hs]=GawB}AB1	Bloomington *Drosophila* Stock Center	BDSC:1824; FLYB: FBti0001249; RRID:BDSC_1824	AB1-Gal4
Genetic	y[1] w[*];	Bloomington	BDSC:80577;	Phm-Gal4

reagent (*D. melanogaster*)	P{w[+mC]=phtm-GAL4.O}22	*Drosophila* Stock Center	FLYB: FBti0201787; RRID:BDSC_80577	
Genetic reagent (*D. melanogaster*)	y[1] w[*]; P{Act5C-GAL4-w}E1/CyO	Bloomington *Drosophila* Stock Center	BDSC:25374; FLYB: FBti0127834; RRID:BDSC_25374	Actin5C-Gal4
Genetic reagent (*D. melanogaster*)	w[*]; wg[Sp-1]/CyO; P{w[+mC]=mRFP-Nup107.K}7.1	Bloomington *Drosophila* Stock Center	BDSC:35517; FLYB: FBti0130064; RRID:BDSC_35517	mRFP-Nup107
Genetic reagent (*D. melanogaster*)	y[1] M{w[+mC]=nanos-Cas9.P}ZH-2A w[*]	Bloomington *Drosophila* Stock Center	BDSC:54591; FLYB: FBti0159183; RRID:BDSC_54591	Nanos.Cas9
Genetic reagent (*D. melanogaster*)	w[*]; TI{w[+mW.hs]=TI}trk[Delta]/CyO; P{w[+mC]=UASp-tor.G}5.7	Bloomington *Drosophila* Stock Center	BDSC:92604; FLYB: FBti0216047; RRID:BDSC_92604	UAS-torso
Genetic reagent (*D. melanogaster*)	w[1118]; P{w[+mC]=UAS-Ras85D.V12}TL1	Bloomington *Drosophila* Stock Center	BDSC:4847; FLYB: FBti0012505; RRID:BDSC_4847	UAS-ras^{V12}
Genetic reagent (*D. melanogaster*)	Nup107^{KK};UAS-GFP	This paper	N/A	
Genetic reagent (*D. melanogaster*)	*UAS-GFP;Nup107^{GD}*	This paper	N/A	
Genetic reagent (*D. melanogaster*)	*UAS-GFP;Phm-Gal4/TM6.Tb*	This paper	N/A	
Genetic reagent (*D. melanogaster*)	*Nup107 gRNA line*	This paper	N/A	
Genetic reagent (*D. melanogaster*)	*Nup107KK;UAS-torso*	This paper	N/A	
Genetic reagent (*D. melanogaster*)	*Nup107KK;UAS-rasV12*	This paper	N/A	

Antibody	Anti-Nup107 (Rabbit polyclonal)	This paper	N/A	WB (1:500) IF (1:100)
Antibody	Anti- Nuclear Pore Complex Proteins (Mouse monoclonal)	Biolegend	Cat#902902 (MAb414)	IF (1:500)
Antibody	Anti-EcR (Mouse monoclonal)	DSHB	Cat#DDA2.7	IF (1:20)
Antibody	Anti-α tubulin (Mouse monoclonal)	DSHB	Cat#12G10	IB (1:5000)
Antibody	Goat anti-rabbit Alexa-Fluor Plus 680	Thermo Fisher Scientific	Cat#A32734	IB (1:40000)
Antibody	Goat anti-mouse Alexa-Fluor Plus 800	Thermo Fisher Scientific	Cat#A32730	IB (1:40000)
Antibody	Goat anti-rabbit Alexa Fluor 488	Thermo Fisher Scientific	Cat#A11034	IF (1:800)
Antibody	Goat anti-rabbit Alexa Fluor 568	Thermo Fisher Scientific	Cat#A11036	IF (1:800)
Antibody	Goat anti-mouse Alexa Fluor 488	Thermo Fisher Scientific	Cat#A11029	IF (1:800)
Antibody	Goat anti-mouse Alexa Fluor 568	Thermo Fisher Scientific	Cat#A11004	IF (1:800)
Antibody	Goat anti-rabbit Alexa Fluor 647	Jackson Immuno Research	Cat#111-605-045	IF (1:800)
Sequence-based reagent	Nup107_gRNA1	This paper	PCR primers (5'–3'):	TGGCCGACAGCCCGTTCCCG
Sequence-based reagent	Nup107_ gRNA2	This paper	PCR primers (5'–3'):	GGAGCTGCTCAACTCGAAACTGG
Sequence-based reagent	5'UTR Primer1_F	This paper	PCR primers (5'–3'):	GCTCCCAAATACTCGCTGCC
Sequence-	3'UTR	This paper	PCR primers	CTTCTGCCGGCGGATTTG

based reagent	Primer1_R		(5'–3'):	TT
Sequence-based reagent	5'UTR Primer2_F	This paper	PCR primers (5'–3'):	GGTACCCCATACTAATGATTC
Sequence-based reagent	3'UTR Primer2_R	This paper	PCR primers (5'–3'):	CATGTTGTTTGTCTCGCTACT
Sequence-based reagent	Nup107 (for antibody generation)	This paper	PCR primers (5'–3'):	Forward: ATCGGATCCATGGCCGACAGCCCGTTC Reverse: ATCGAATTCCTACCACGCCATCATACGATC
Sequence-based reagent	Nup107_RT	This paper	PCR primers (5'–3'):	Forward: GCAGGCTCACCGATCGGAAG Reverse: TCCATCTGCAGTAGGCGATG
Sequence-based reagent	EcR_RT	This paper	PCR primers (5'–3'):	Forward: AAGAGGATCTCAGGCGTATAA Reverse: GGCCTTTAGTAACGTGATCTG
Sequence-based reagent	Eip75A_RT	This paper	PCR primers (5'–3'):	Forward: ACCACAGCACCACCCATTT Reverse: TGTTTGGCGGTAGTTTCAGG
Sequence-based reagent	Eip74EF_RT	This paper	PCR primers (5'–3'):	Forward: CTCTGCTCCACATAAAGACG Reverse: CCGCTAAGCAGATTGTGG
Sequence-based reagent	Phm_RT	This paper	PCR primers (5'–3'):	Forward: GGATTTCTTTCGGCGCGATGTG Reverse: TGCCTCAGTATCGAAAAGCCGT
Sequence-based reagent	Spok_RT	This paper	PCR primers (5'–3'):	Forward: TATCTCTTGGGCACACTCGCTG Reverse: GCCGAGCTAAATTTCTCCGCTT
Sequence-based	Dib_RT	This paper	PCR primers (5'–3'):	Forward: TGCCCTCAATCCCTATCTG

reagent				GTC Reverse: ACAGGGTCTTCACACCCATCTC
Sequence-based reagent	Sad_RT	This paper	PCR primers (5'–3'):	Forward: CCGCATTCAGCAGTCAGTGG Reverse: ACCTGCCGTGTACAAGGAGAG
Sequence-based reagent	Shd_RT	This paper	PCR primers (5'–3'):	Forward: CGGGCTACTCGCTTAATGCAG Reverse: AGCAGCACCACCTCCATTTC
Sequence-based reagent	Torso_RT	This paper	PCR primers (5'–3'):	Forward: CAGCTACTGCGACAAGGTCATCG Reverse: CTCGGTTGCAGCTTGCAGTTG
Sequence-based reagent	Rpl49_RT	This paper	PCR primers (5'–3'):	Forward: CGTTTACTGCGGCGAGAT Reverse: GTGTATTCCGACCACGTTACA
Commercial assay or kit	RNA isolation kit	Favorgen Biotech	Cat#FATRK-001-2	
Commercial assay or kit	iScriptTM cDNA synthesis kit	BIORAD	Cat#170-8891	
Commercial assay or kit	20-hydroxyecdysone ELISA kit	Thermo Fisher Scientific	Cat#EIAHYD	
Chemical compound, drug	Fluoroshield	Sigma-Aldrich	Cat#F6057	
Chemical compound, drug	TritonX-100	Sigma-Aldrich	Cat#X100-1L	
Chemical compound, drug	Tween-20	Sigma-Aldrich	Cat#274348	
Chemical compound, drug	Paraformaldehyde	Sigma-Aldrich	Cat#158127	

Chemical compound, drug	iTaq Universal SYBR® Green Supermix	BIORAD	Cat#1725122	
Chemical compound, drug	G9 Taq Polymerase 10X buffer with MgCl2	GCC Biotech	Cat#G7115	
Chemical compound, drug	dNTPs	SBS GENETECH	Cat#EN-2	
Chemical compound, drug	20-hydroxyecdysone	Sigma-Aldrich	Cat# H5142	
Chemical compound, drug	EDTA	Sigma-Aldrich	Cat#03690	
Chemical compound, drug	SDS	HIMEDIA	Cat#GRM886	
Chemical compound, drug	Proteinase-K	MP Biomedicals	Cat#193981	
Chemical compound, drug	Tris	HIMEDIA	Cat#MB029	
Chemical compound, drug	Potassium acetate	HIMEDIA	Cat#MB042	
Chemical compound, drug	Isopropanol	MP Biomedicals	Cat#194006	
Chemical compound, drug	Ethanol	Merck	Cat#100983	
Chemical compound, drug	Sucrose	ANJ Biomedicals	Cat#100314	
Chemical compound, drug	IPTG	Sigma-Aldrich	Cat#I6758	
Chemical compound, drug	Protease inhibitor Cocktail (PIC)	Roche	Cat#04693132001	
Chemical compound, drug	NaCl	Emparta ACS	Cat#1.93206.0521	
Chemical	EGTA	Sigma-	Cat#E4378	

compound, drug		Aldrich		
Chemical compound, drug	Sodium deoxycholate	Sigma-Aldrich	Cat# 30970	
Chemical compound, drug	Sodium azide	Sigma-Aldrich	Cat#438456	
Chemical compound, drug	N-Hydroxy-succinimidyl (NHS) Sepharose	Sigma-Aldrich	Cat#H8280	
Chemical compound, drug	Urea	MP Biomedicals	Cat#194857	
Software, algorithm	ImageJ/FIJI	National Institutes of Health, USA		http://imagej.nih.gov/ij/
Software, algorithm	GraphPad Prism Software	GraphPad		https://www.graphpad.com/
Software, algorithm	Adobe Photoshop 2023	Adobe		https://www.adobe.com/in/products/photoshop.html
Software, algorithm	QuantStudio Design & Analysis Software	Applied Biosystems		https://www.thermofisher.com/in/en/home/technical-resources/software-downloads/quantstudio-3-5-real-time-pcr-systems.html

Fly stocks and genetics

Experimental *Drosophila melanogaster* stocks were reared on a standard cornmeal diet (Nutri-Fly Bloomington formulation) under controlled conditions of 25°C and 60% relative humidity unless otherwise specified. Fly lines used in this study were sourced from the Bloomington *Drosophila* Stock Centre (BDSC) at Indiana University or from the Vienna *Drosophila* Resource Center (VDRC), which are listed in the Key resources table. The UAS-GFP lines were received as a gift from Dr. Varun Chaudhary (IISER Bhopal, India). Control groups were generated through crosses between the driver line and w^{1118} flies. For RNA interference (RNAi) experiments, crosses were maintained at 29°C to optimize GAL4 expression. The $Nup107^{GD}$ line, in conjunction with *Actin5C-GAL4*, was specifically cultivated at 23°C to generate third-instar larvae for experimental purposes.

Transgenic fly generation

In the generation of transgenic flies containing gRNA, we employed a systematic approach. The online tool available at *http://targetfinder.flycrispr.neuro.brown.edu* was used for the initial sgRNA design, ensuring zero predicted off-targets. Subsequently, we utilized an additional tool available at *https://www.flyrnai.org/evaluateCrispr/* to evaluate and score the predicted efficiency of sgRNAs in the targeted region. The most efficient sgRNAs, demonstrating high specificity with no predicted off-targets, were selected and cloned into the pCFD4 vector. The Fly Facility services at the Centre for Cellular and Molecular Platforms, National Center for Biological Sciences (C-CAMP-NCBS), Bangalore, India, were utilized to clone and generate transgenic flies. Primer sequences employed in this study can be found in the Key resources table.

CRISPR –Cas9 mediated mutant Generation

Virgin nanos.Cas9 (BL-54591) flies were crossed with males of gRNA transgenic lines, and approximately 10 F1 progeny males were crossed with balancer flies specific for the gene of interest. A single-line cross was established using the F2 progeny to assess the deletion of *nup107*. Subsequently, F3 progeny were subjected to nested PCR to confirm deletions. Positive individuals were further tested for the presence of gRNA and Cas9 transgenes, and flies lacking both were propagated into stocks.

Genomic DNA Isolation

Approximately ten flies were homogenized in 250 µL of solution A, comprising 0.1 M Tris-HCl, pH 9.0, 0.1 M EDTA, and 1% SDS, supplemented with Proteinase-K. The homogenate was incubated at 70°C for 30 min. Subsequently, 35 µL of 8 M potassium acetate was added, mixed gently without vortexing, and incubated on ice for 30 min. The homogenate was then centrifuged at 13,000 rpm at 4°C for 15 min, and the supernatant was carefully collected without disturbing precipitates or the interphase. 150 µL of Isopropanol was added to the supernatant and incubated on ice for 15 min. After centrifugation at 13,000 rpm for 5 min, the supernatant was discarded, and the pellet was washed with 1 mL of 70% ethanol by centrifuging at 13,000 rpm for 5 min. The supernatant was again discarded, and the pellet was dried at 55°C until the ethanol evaporated. Finally, the pellet was resuspended in 50 µL of Tris-EDTA buffer and incubated at 37°C.

Nested PCR

Nested PCR was employed to assess the presence of deletions in flies, utilizing genomic DNA as the template. 1 µL of DNA was used for the initial PCR with an outer set of primers (Key resources table). Following this, 1 µL of the initial PCR product was employed for a subsequent PCR with an inner set of primers. The resulting PCR products were then resolved on 0.8% agarose gel to visualize the desired bands indicative of deletions.

Measurement of developmental timing and pupariation

Flies were permitted to lay eggs for 3 hours on agar plates supplemented with yeast, synchronizing the larvae. Newly hatched L1 larvae were then collected and transferred to vials. Pupariation times and dates were recorded daily during the light cycle. Data from 8-10 vials were aggregated, organized by pupariation time and cumulative percentage pupariation, and analyzed using Microsoft Excel.

Antibody generation and western blotting

To generate antibodies against *Drosophila Nup107* (CG6743), the N-terminal 210 amino acids, recognized as the most unique and antigenic region, were sub-cloned into the modified tag-less pET28a(+) vector, pET28a(+)-JK vector. The protein was expressed in *Escherichia coli* BL21 (DE3) cells, induced with 200 µM IPTG (Sigma), and incubated at 30°C for 4 hours. Following cell pelleting, the pellet was resuspended in lysis buffer (50 mM Tris pH 8.0, 1 mM EDTA, and 25% Sucrose) with 1× protease inhibitor mixture (Roche Applied Science), and lysozyme (from 50 mg/mL stock) was added to facilitate bacterial lysis. After sonication and centrifugation, the pellet was successively resuspended in inclusion body buffer I (20 mM Tris pH 8.0, 0.2 M NaCl, 1% Sodium deoxycholate, and 2 mM EGTA) and buffer II (10 mM Tris pH 8.0, 0.25% Sodium deoxycholate and 1 mM EGTA) and centrifuged. This process was repeated three times within inclusion body buffer II, and the final pellet was dissolved in 8 M Urea buffer (10 mM Tris-HCl pH 8.0, 8 M Urea, 0.1 mM NaN_3, and 1 mM EGTA), diluted to 6 M Urea, and centrifuged. The supernatant was loaded onto SDS-PAGE, and the desired band was cut for protein elution. Rabbit polyclonal antibodies against Nup107 were generated at Bio Bharati Life Sciences Pvt. Ltd., Kolkata, West Bengal, India. The antibodies obtained were subjected to affinity purification. The

purification involved the chemical cross-linking of purified antigens to N-hydroxy succinimidyl-Sepharose beads (Sigma). The elution process was carried out under low pH conditions, followed by neutralization. Subsequently, the eluted antibodies were dialyzed against phosphate-buffered saline (PBS) overnight at 4°C to remove any remaining impurities and optimize their stability and functionality for subsequent experimental use.

Larval brain complexes from third-instar larvae were dissected, lysed in Laemmli buffer, and resolved on 8% SDS-PAGE. Two equivalent head complexes were loaded per well. After transfer to a polyvinylidene difluoride membrane, blocking was performed with 5% fat-free milk. The membrane was then incubated with polyclonal anti-Nup107 antibody (1:500) and anti-α-tubulin (DSHB, 12G10) (1:5000) at 4°C overnight. Following three washes with TBS-T buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Tween-20), the membrane was incubated with secondary antibodies, anti-rabbit-Alexa-Fluor Plus 680 (Thermo Fisher Scientific #A32734) and anti-mouse-Alexa-Fluor Plus 800 (Thermo Fisher Scientific #A32730). Following incubation with secondary antibodies, the membrane was washed three times for 10 minutes each with Tris-buffered saline containing Tween-20 (TBS-T) to remove unbound antibodies. The washed membrane was then subjected to imaging using the Li-Cor IR system (Model: 9120), allowing for the visualization and analysis of the protein bands on the membrane.

Immunostaining

For immunostaining *Drosophila* salivary glands, the previously reported protocol was followed (Mehta et al., 2021). The larvae were dissected in cold phosphate-buffered saline (PBS) to isolate salivary glands. Glands underwent pre-extraction with 1% PBST (PBS + 1% Triton X-100), then fixation in freshly prepared 4% paraformaldehyde for 30 minutes at room temperature. Subsequently, the glands were thoroughly washed with 0.3% PBST (0.3% Triton X-100 containing PBS). Blocking was performed for 1 h with 5% normal goat serum (#005-000-001, The Jackson Laboratory, USA). The glands were stained with the following primary antibodies: anti-Nup107 (1:100), mAb414 (1:500, Bio-legend) and anti-EcR (1:20, DDA2.7, DSHB) overnight at 4°C. Tissues were washed three times with 0.3% PBST (PBS + 0.3% Triton X-100), followed by incubation with secondary antibodies: anti-rabbit Alexa Fluor 488 (1:800, #A11034, Thermo Fisher Scientific), anti-rabbit Alexa Fluor 568 (1:800, #A11036, Thermo Fisher Scientific), anti-mouse Alexa Fluor 488 (1:800, #A11029, Thermo Fisher Scientific), anti-mouse Alexa Fluor 568 (1:800, #A11004, Thermo Fisher Scientific), and anti-rabbit Alexa Fluor 647 (1:800, Jackson ImmunoResearch). Following secondary antibody incubation, tissues were washed three times with 0.3% PBST (PBS + 0.2% Triton X-100) and mounted with DAPI-containing Fluoroshield (#F6057, Sigma). The same protocol was followed for staining of the Brain complex and prothoracic gland.

Fluorescence Intensity quantification

Images were acquired using an Olympus Confocal Laser Scanning Microscope FV3000. Subsequent image processing was conducted using the Fiji software, and signal intensities were averaged using GraphPad software (Prism). To quantify the EcR nuclear to cytoplasmic ratio, three distinct regions of interest (ROI) were designated per nucleus and its surrounding cytoplasm. Fiji software measured the average intensity in each ROI, and the mean of these intensities was determined per nucleus and its adjacent cytoplasm. The final graph depicts the ratio of the mean intensities observed in the nucleus to that in the cytoplasm. All experiments were conducted with a minimum of three independent replicates.

Quantitative RT-PCR

Total RNA was extracted from various genotypes (control, ubiquitously depleted *Nup107*, and prothoracic gland-specific depletion of *Nup107*) of whole late third instar larvae utilizing the total tissue RNA isolation kit (Favorgen Biotech). One microgram of total RNA was employed for cDNA synthesis with the iScript cDNA synthesis kit (Bio-Rad). The resulting cDNA was diluted fivefold,

and 1 µL from each genotype was utilized as a template. RT-PCR was conducted using SYBR Green PCR master mix on an Applied Biosystems QuantStudio 3 Real-Time PCR System. The Rpl69 gene served as the control, and relative transcript levels were determined using the CT value ($2^{-\Delta\Delta CT}$). Differences in gene expression were analyzed using Student's t-test, with a p-value < 0.05 considered significant. The primers used are detailed in the Key resources table. The resultant graph illustrated the fold change in gene expression, plotted using GraphPad software (Prism).

20-hydroxyecdysone (20E) level measurements

Three biological replicates of 25 mg larvae from each genotype were collected at the specified times after egg laying (AEL). The larvae were washed, dried, and weighed before being flash-frozen on dry ice and stored at −80°C. Ecdysone extraction was done by thoroughly homogenizing the frozen samples in 300 µL ice-cold methanol with a plastic pestle. After centrifugation at 17,000 x g for 10 minutes, the supernatant was divided into two Eppendorf tubes containing approximately 150 µL supernatant. Methanol from both tubes was evaporated in a vacuum centrifuge for 60 minutes, and the resulting pellets were redissolved by adding 200 µL Enzyme Immunoassay (EIA) buffer to one of the tubes. After vortexing, the same 200 µL EIA buffer was transferred to the second tube, followed by another round of vortexing. The Enzyme-Linked Immunosorbent Assay (ELISA) was performed using a 20-hydroxyecdysone ELISA kit (#EIAHYD) from Thermo Fisher Scientific.

20E Rescue Experiment

20-hydroxyecdysone (#H5142, Sigma) was dissolved in ethanol to achieve a 5 mg/mL concentration. Salivary glands from third instar larvae of different genotypes were extracted and then incubated for 6 hours in Schneider's S2 media with 50 µM 20E. After the incubation, the glands underwent procedures for immunostaining and RNA extraction.

To facilitate the rescue of the larvae through feeding, 30-third instar larvae, *Nup107* depleted larvae cultured at 29°C incubator, were first washed with water. They were then transferred to vials containing either 20E (at a final concentration of 0.2 mg/mL) or 95% ethanol (in the same amount as the 20E). Once the larvae were added to the vials, these were returned to the 29°C incubator and observed for pupariation, recording the time of this developmental stage.

Data and Resource Availability

All relevant data and resource can be found within the article and its supplementary information.

Supplementary Information

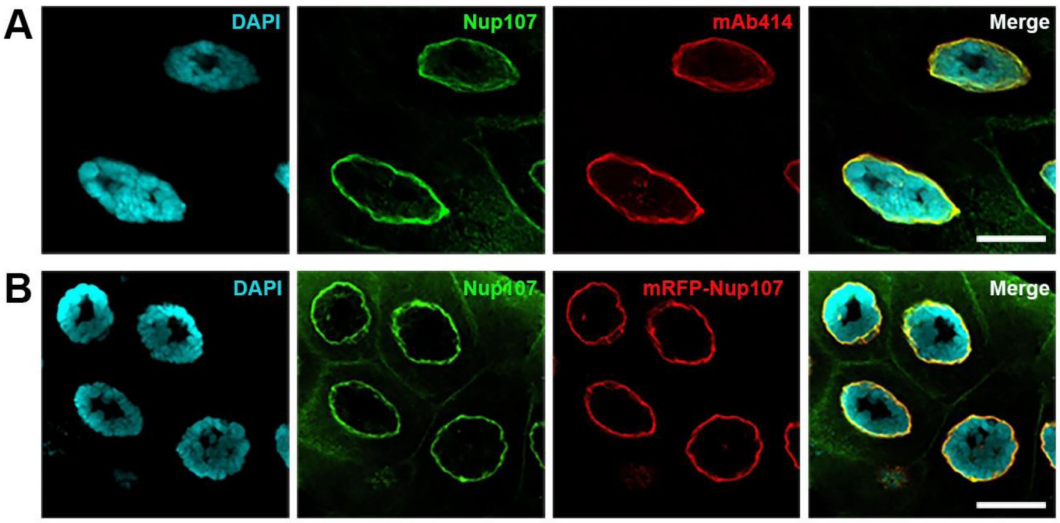

Figure S1.

Nup107 staining in salivary glands:

(A-B) Custom-generated polyclonal anti-Nup107 antibody colocalizes with pan-FG-Nup antibody, mAb414 (A), and mRFP-tagged Nup107 (B) at the nuclear rim of the third instar salivary gland. DNA stained with DAPI. Scale bars, 20 μm.

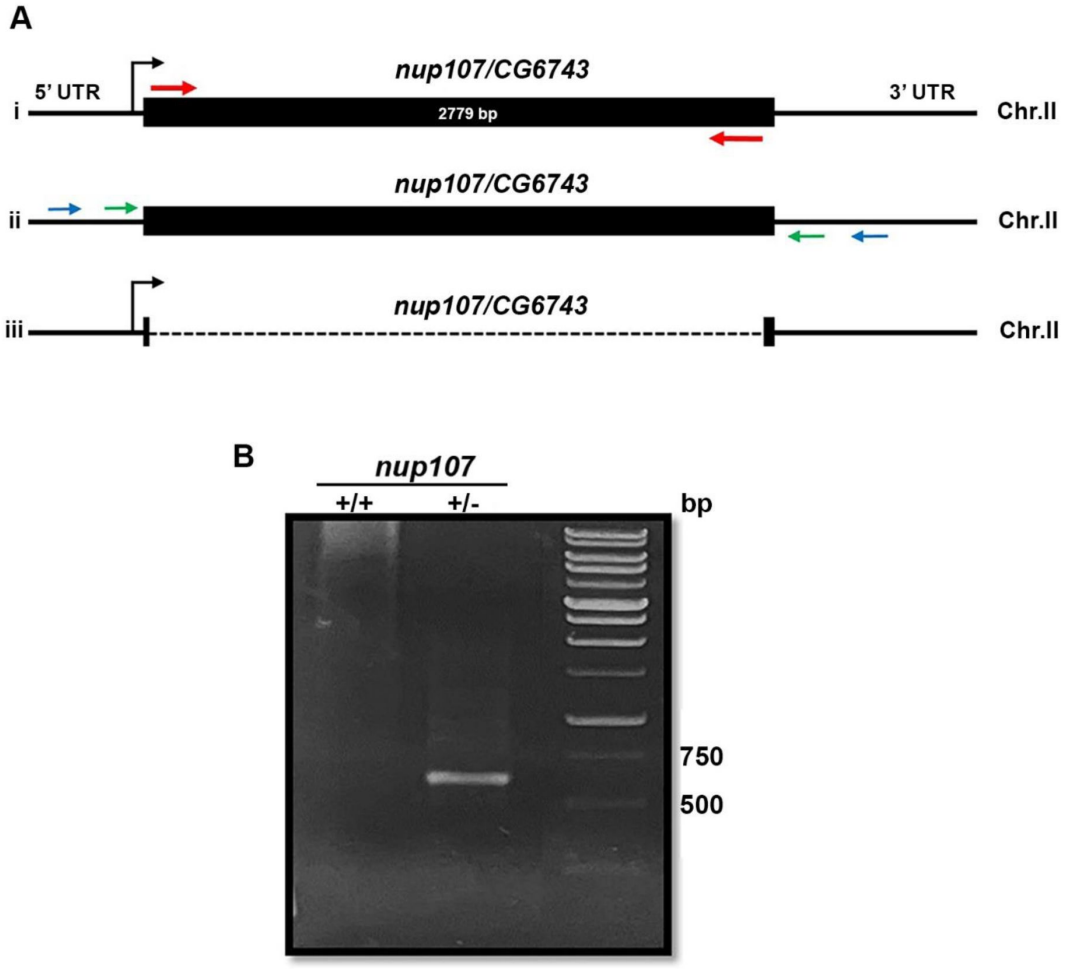

Figure S2.

***Nup107* CRISPR mutant generation:**

(A) Schematic representation of *Nup107*^{KO} generation. (Ai) The genomic locus of *nup107* on chromosome-II (2L). The filled black box corresponds to the *nup107* ORF (2779 bp) with gRNA(s) positions indicated by red arrows. The first and second gRNAs were designed near start and stop codons, respectively, of the *nup107* locus. (Aii) Blue and green arrows indicate two sets of primers located in the 5′-UTR and 3′-UTR regions used for screening of *Nup107* mutant. (Aiii) The black discontinuous line represents the *nup107* (2752 bp) deletion allele.
(B) Confirmation of *Nup107* deletion mutant (heterozygous) line assessed by the presence of a ~630 bp band amplified from isolated genomic DNA.

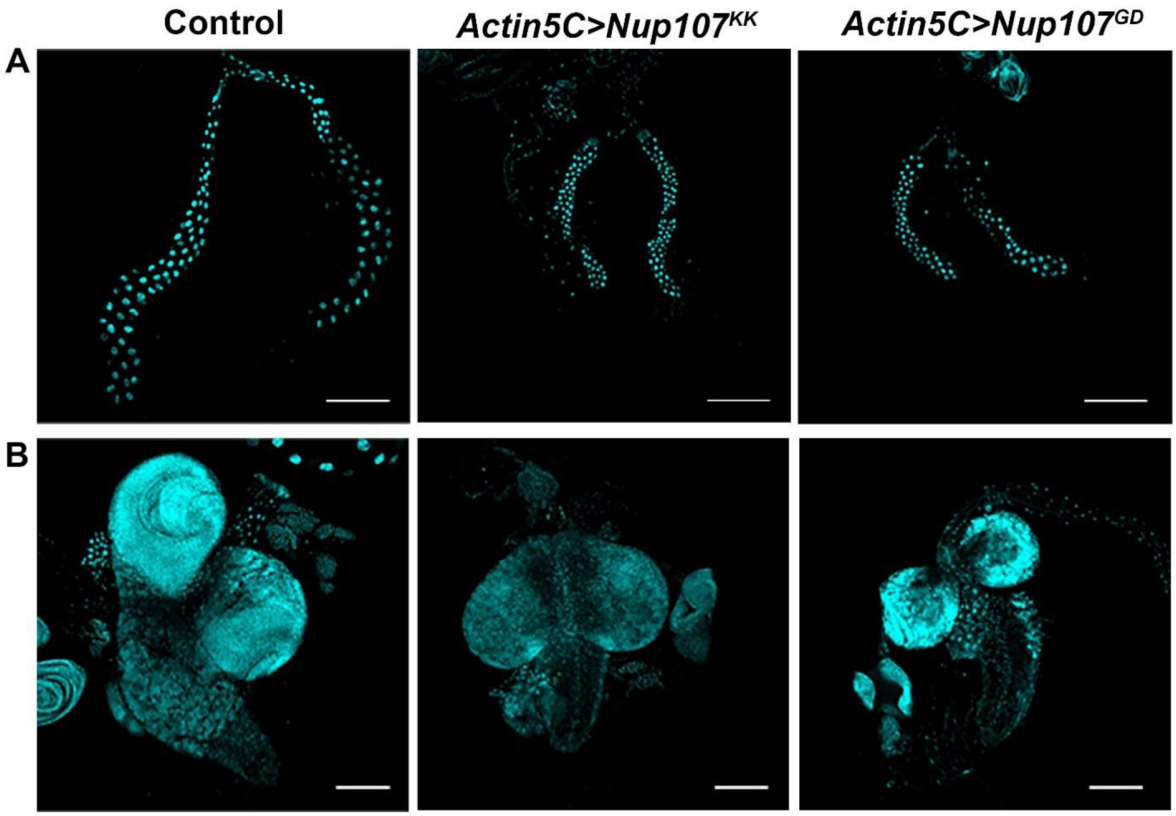

Figure S3.

Compromised organ size due to ubiquitous depletion of *Nup107*:

Actin5C-Gal4 was used as a ubiquitous driver.
(A-B) Third instar larval salivary gland (A), and brain complex (B) images of Control, *Nup107*^{KK} RNAi and *Nup107*^{GD} RNAi. DNA stained with DAPI. Scale bars are 200 µm and 100 µm in (A) and (B), respectively.

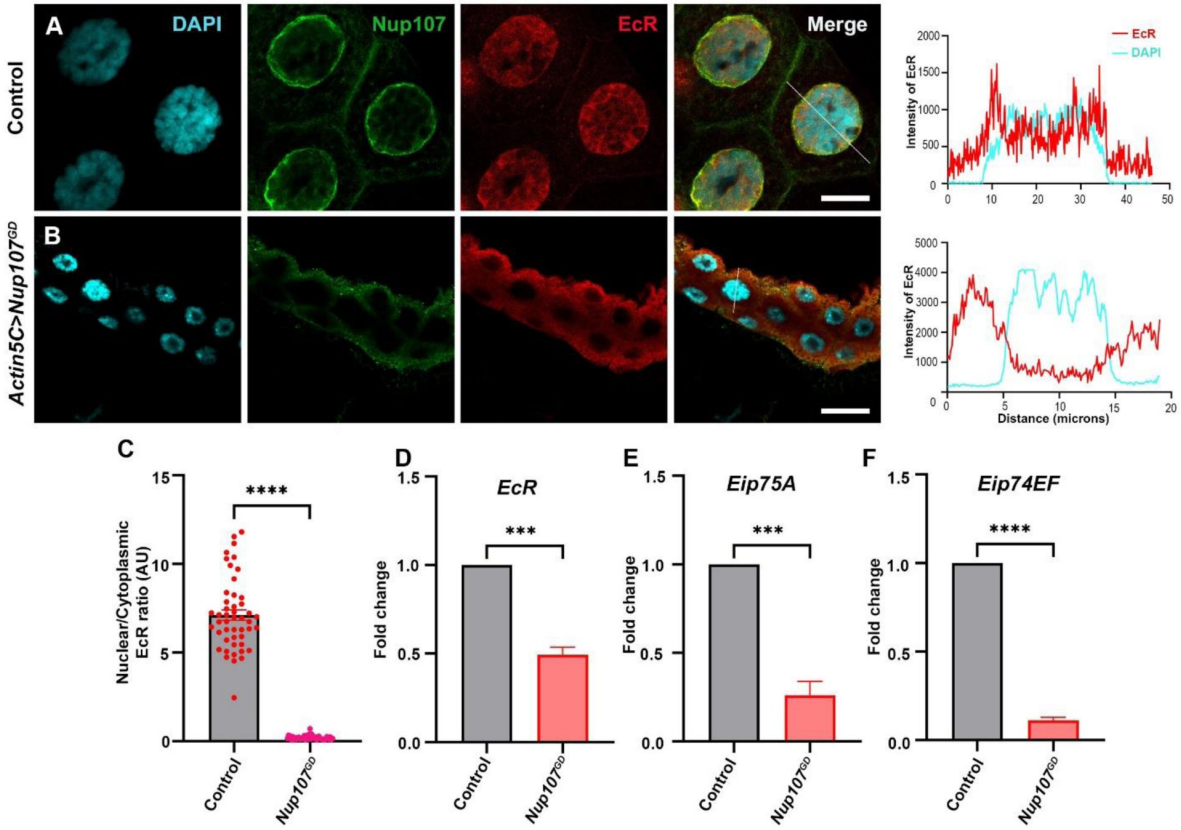

Figure S4.

Ubiquitous knockdown of *Nup107* using *Nup107^{GD}* RNAi disrupts ecdysone signaling.

(A-B) Staining of third instar larval salivary glands from control (A) and ubiquitous *Nup107^{GD}* knockdown (B) with Ecdysone receptor (anti-EcR antibody, red) and Nup107 (anti-Nup107 antibody, green). DNA is stained with DAPI, and Scale bars, 20 μm. Charts represent the line scan intensity profile of EcR (Red) and DAPI (Cyan) in the salivary gland nucleus region.
(C) Quantification of nucleo-cytoplasmic ratio of EcR under control and *Nup107* knockdown conditions. At least 45 nuclei were analyzed from 7 to 8 pairs of salivary glands. Statistical significance was derived from the Student's t-test. Error bars represent SEM. ****p = < 0.0001.
(D-F) Analysis of ecdysone-inducible genes, *EcR* (D), *Eip75A* (E) and *Eip74EF* (F) expression. Data are represented from at least three independent experiments.
Statistical significance was derived from the Student's t-test. The error bars represent the SEM. ***p = < 0.0007 and ****p = < 0.0001.

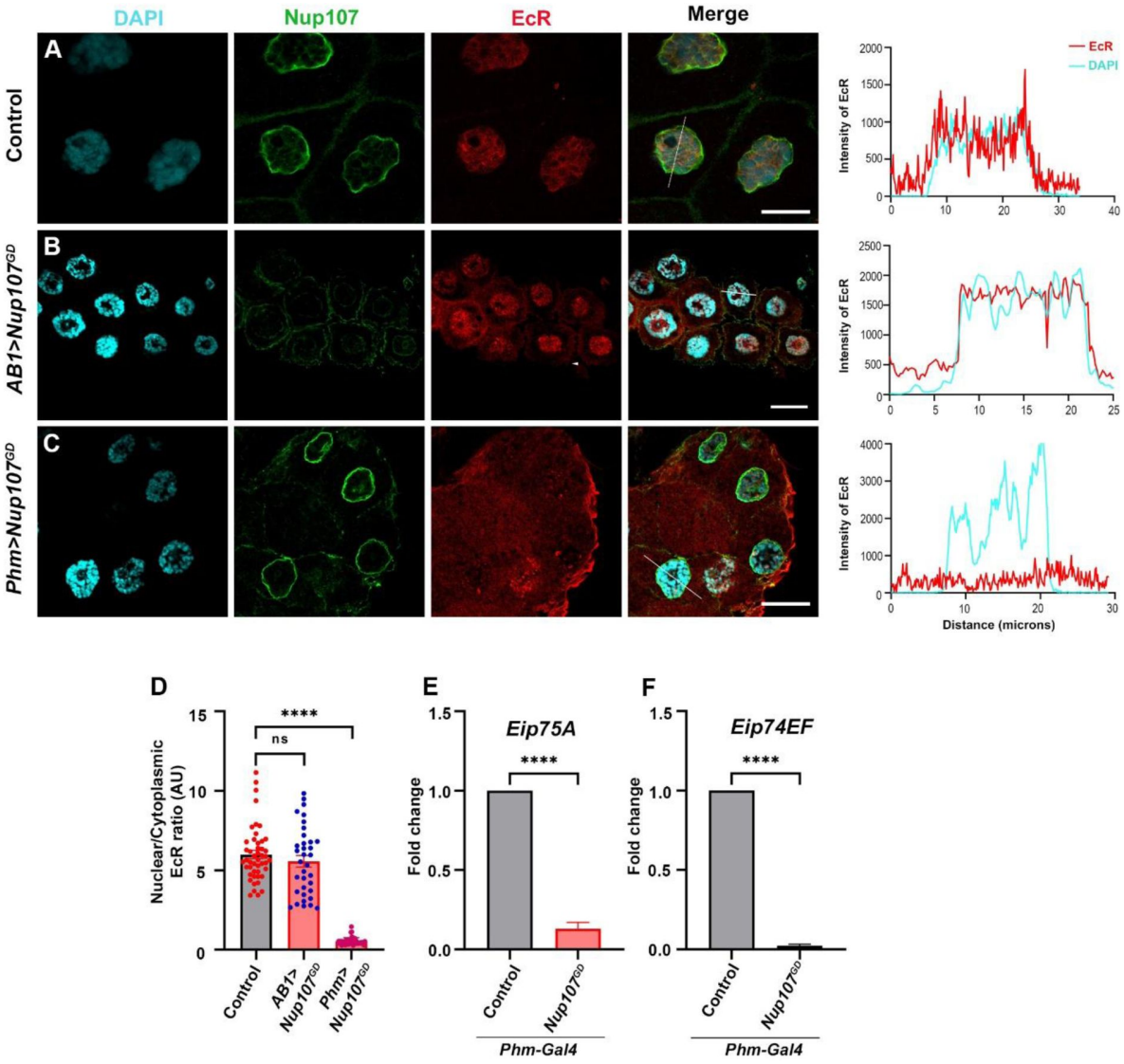

Figure S5.

Nup107^{GD} RNAi mediated Nup107 depletion regulates Ecdysone receptor-dependent signaling.

(A-C) Detection of and quantitation of nucleocytoplasmic distribution of EcR (anti-EcR antibody, red) and Nup107 (anti-Nup107 antibody, green) in control (A), salivary gland-specific *Nup107^{GD}* depletion (B), and prothoracic gland-specific *Nup107^{GD}* depletion (C) from third instar larval salivary gland nuclei. DNA is stained with DAPI. Scale bars, 20 µm. Charts represent the line scan intensity profile of EcR (Red) and DAPI (Cyan) in the salivary gland nucleus region.
(D) EcR nucleo-cytoplasmic quantification ratio from salivary gland, and prothoracic gland-specific *Nup107* knockdown. At least 45 nuclei were analyzed from 7 to 8 pairs of salivary glands. Statistical significance was derived from the Student's t-test. Error bars represent SEM. ****p = < 0.0001 and ns is non-significant.
(E-F) Quantitation of expression of *Eip75A* (E), and *Eip74EF* (F) ecdysone-inducible genes at the onset of metamorphosis (RNA isolated from late third instar larval stage). Data are represented from at least three independent experiments. Statistical significance was derived from the Student's t-test. Error bars represent SEM. ****p = < 0.0001.

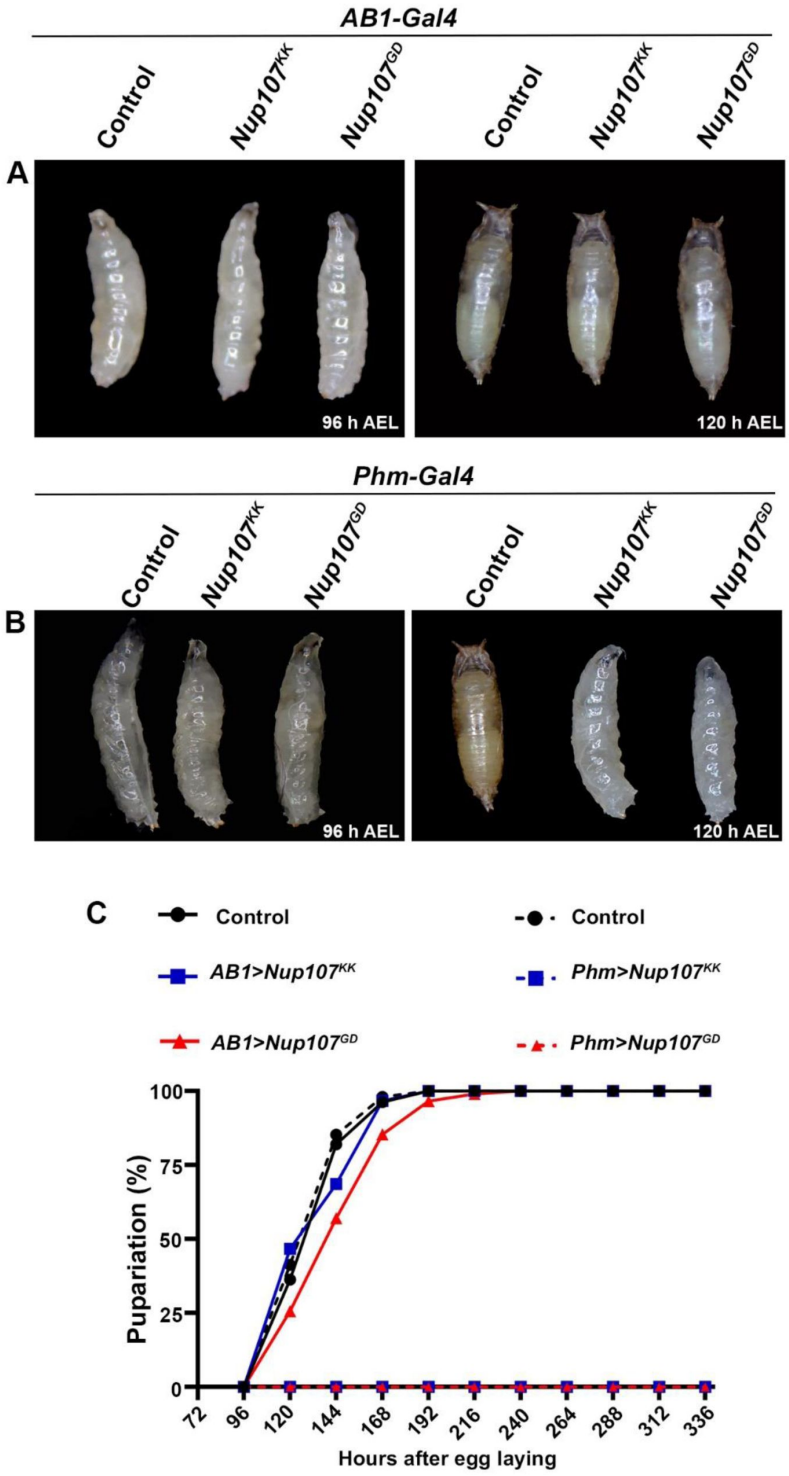

Figure S6.

Nup107 regulates metamorphosis via Ecdysone synthesis.

(A) Growth profile of third instar larvae from *AB1-Gal4* driven control and *Nup107* knockdowns (*Nup107*^{KK} and *Nup107*^{GD} RNAi lines) at 96 h AEL (hours after egg laying) and 120 h AEL.
(B) Growth profile of third instar larvae from *Phm-Gal4* driven control and *Nup107* knockdowns (*Nup107*^{KK} and *Nup107*^{GD} RNAi lines) at 96 h AEL and 120 h AEL.
(C) Comparison of pupariation profiles of control, and *Nup107* knockdown organisms.

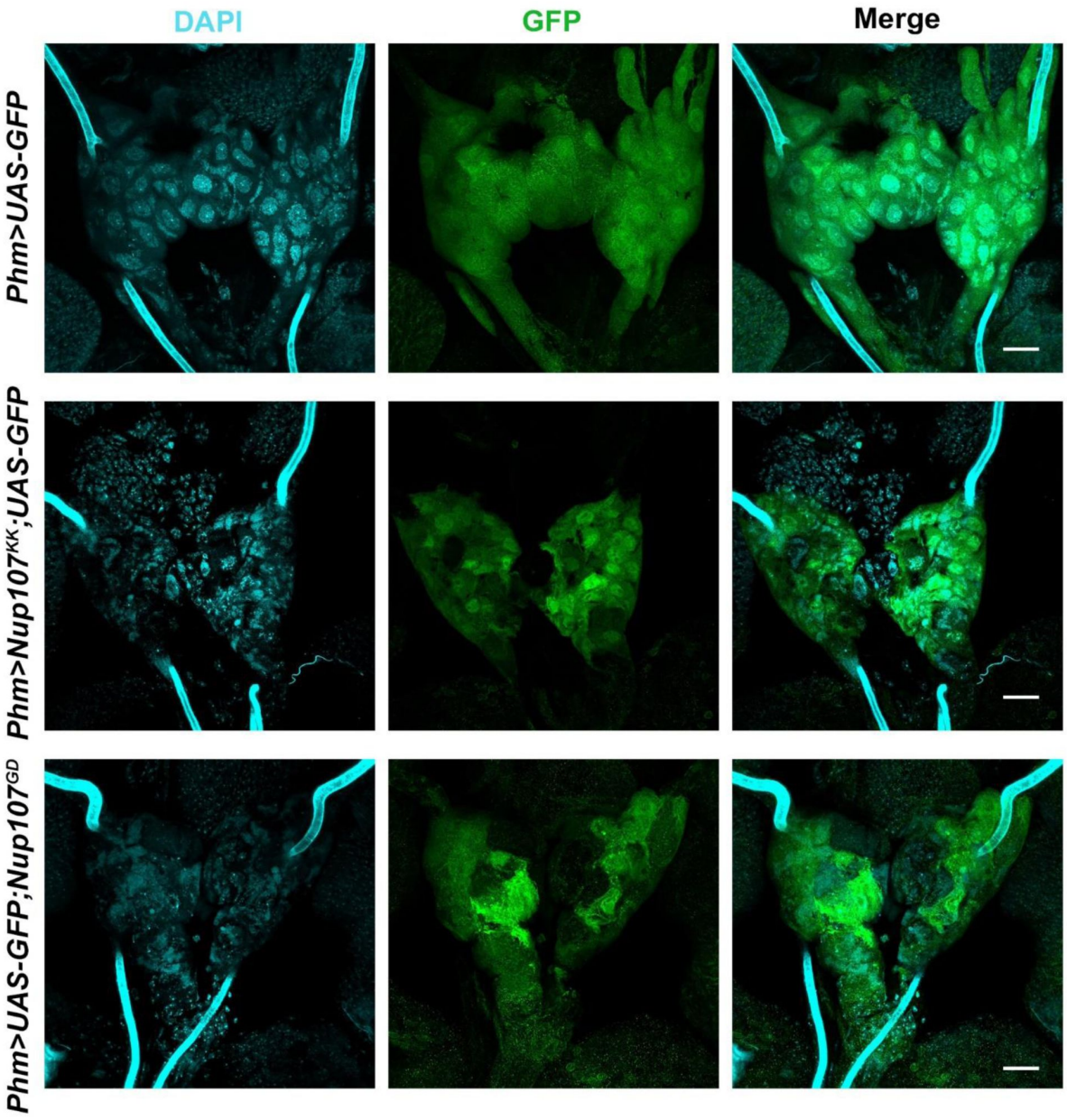

Figure S7.

***Nup107* depletion compromises the PG size.**

(A-C) Prothoracic gland specific driver *Phm-Gal4* driven expression of GFP in the prothoracic glands of the third instar larva image of Control (A), $Nup107^{KK}$ RNAi (B) and $Nup107^{GD}$ RNAi (C). DNA stained with DAPI. Scale bars, 20 μm.

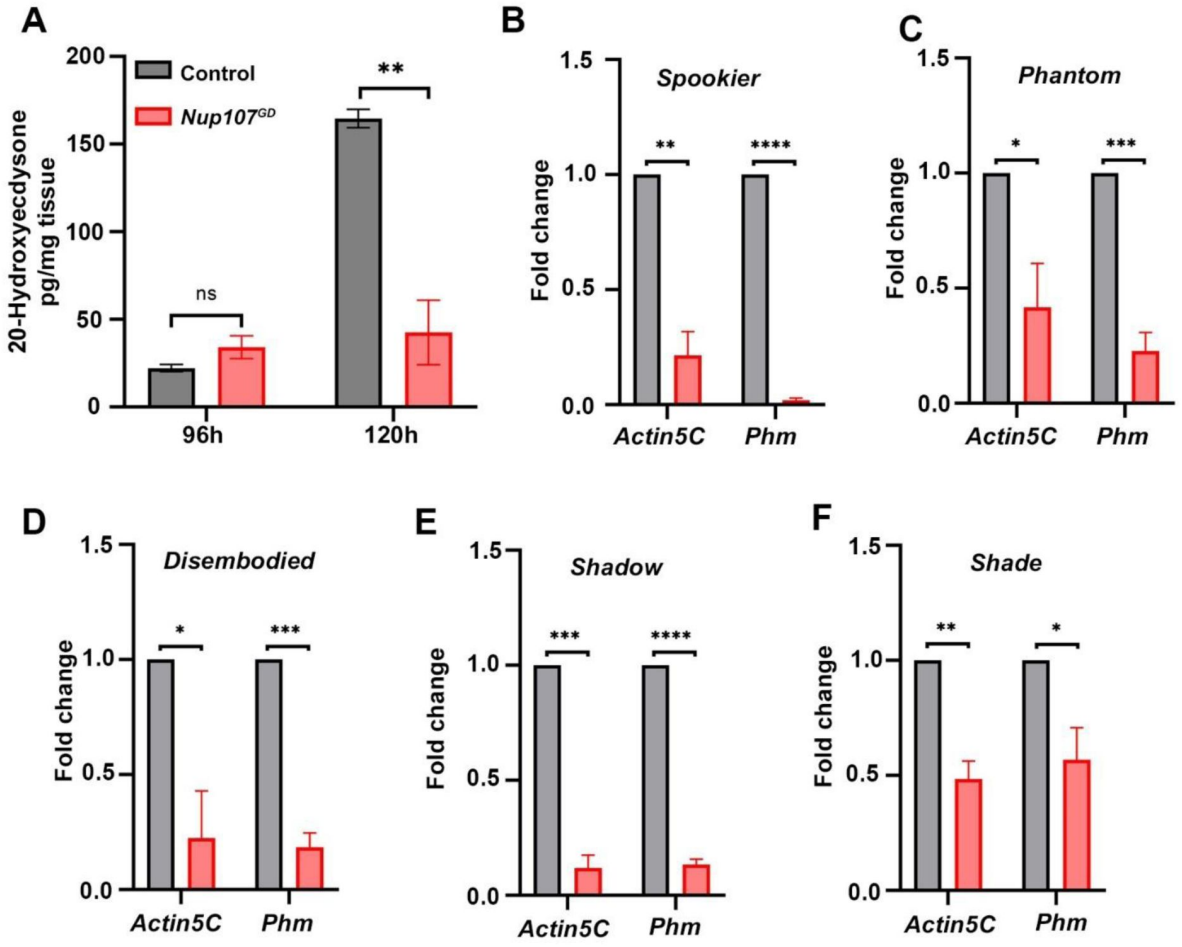

Figure S8.

Nup107^{GD} RNAi mediated depletion of *Nup107* critically regulates expression of ecdysone-biosynthetic genes:

(A) ELISA measurements of whole-body 20-hydroxyecdysone (20E) levels in control and prothoracic gland-specific (*Phm-Gal4*) *Nup107*^{GD} depletion at 96 and 120 h AEL. Data are represented from at least three independent experiments. Statistical significance was derived from the Student's t-test. Error bars represent SEM. ***p* = < 0.0025 and 'ns' is non-significant. (B-F) Quantification of ecdysone-biosynthetic gene expression levels of *Spookier* (B), *Phantom* (C), *Disembodied* (D), *Shadow* (E), and *Shade* (F) in cDNA isolated from control, and *Nup107* knockdown late third instar larvae. Data are represented from at least three independent experiments. Statistical significance was derived from the Student's t-test. Error bars represent SEM. *****p* = < 0.0001, ****p* = <0.0005, ***p* = <0.001 and **p* = <0.03.

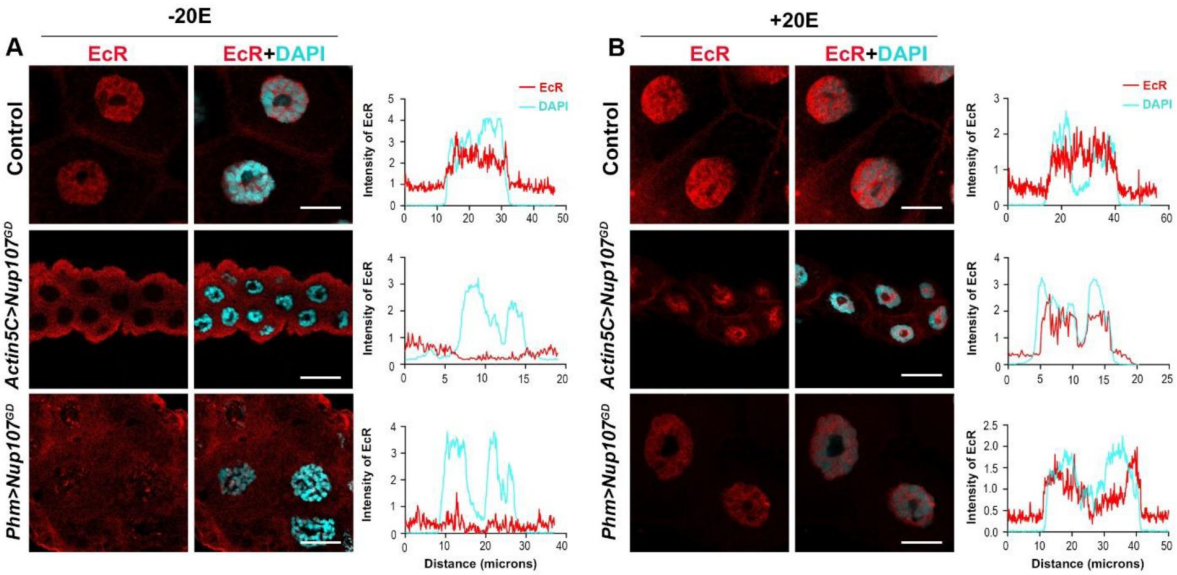

Figure S9.

Ecdysone (20E) supplementation rescues *Nup107^{GD}* *dependent Nup107* depletion specific EcR signaling defects.

(A-B) Visualization of the nucleocytoplasmic distribution of EcR (anti-EcR antibody, red) without 20E (A) and with 20E (B) treatment in larval salivary glands of control, ubiquitous (*Actin5C-Gal4*) *Nup107^{GD}* knockdown, and prothoracic gland-specific (*Phm-Gal4*) *Nup107^{GD}* knockdown. DNA is stained with DAPI. Scale bar, 20 μm. Charts represent the line scan intensity profile of EcR (Red) and DAPI (Cyan) in the salivary gland nucleus region.

Figure S10.

Torso rescues metamorphic defects of *Nup107* knockdown:

(A) Growth profile of third instar larvae from different genotypes (Control, *Nup107^{KK}*, *Nup107^{KK};UAS-torso,* and *Nup107^{KK};UAS-ras^{V12}*) at 96 hours AEL (after egg laying).
(B-E) Quantification of ecdysone-biosynthetic gene expression levels of *Spookier* (B), *Phantom* (C), *Disembodied* (D) and *Shadow* (E) in cDNA isolated from control and *Nup107* depleted *torso* overexpressing larvae (by using *Actin5C-Gal4* and *Phm-Gal4*). Data are represented from at least three independent experiments. Statistical significance was derived from the Student's t-test. Error bars represent SEM. (*) represents p <0.03 and 'ns' is non-significant.

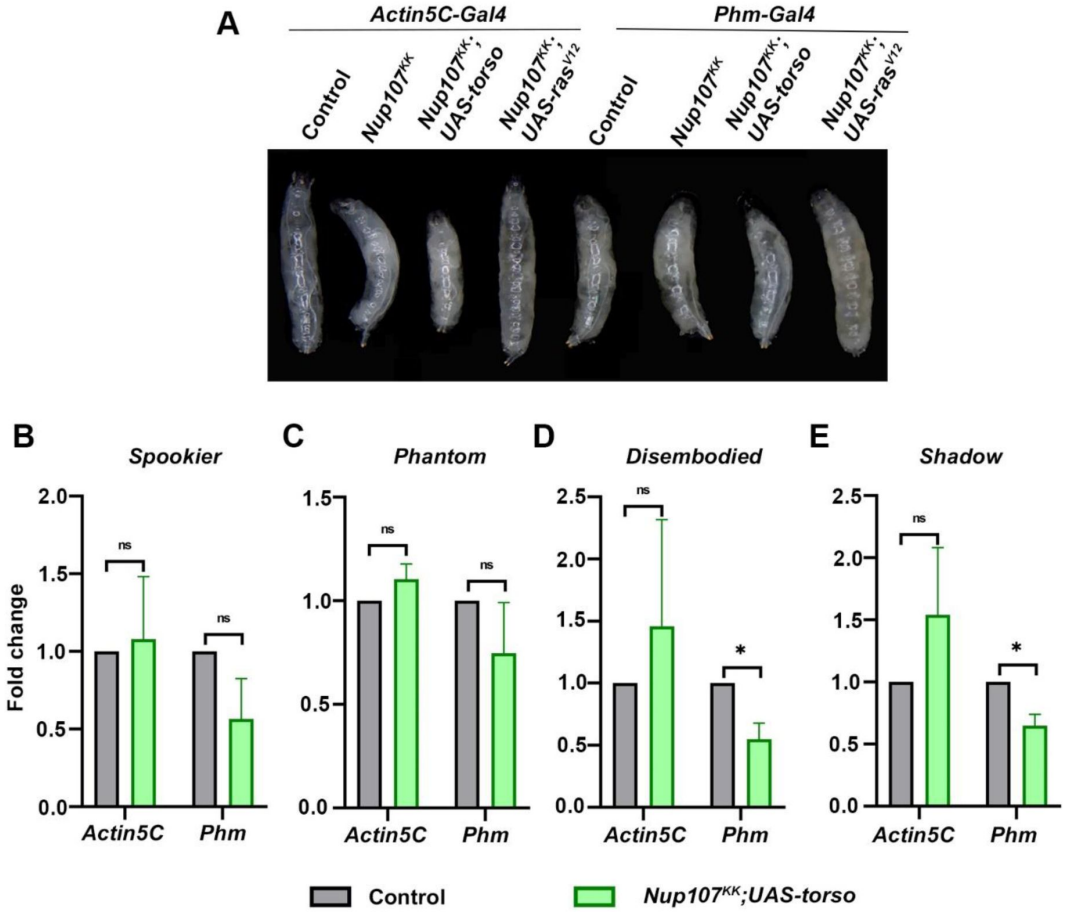

Table S1.

Exogenous 20E supplementation analysis.

Genotype	-20E	+20E
Control	100%	100%
Phm>Nup107^{KK}	0%	70-80%

Acknowledgements

We thank the Bloomington *Drosophila* Stock Centre (BDSC) and Vienna *Drosophila* Resource Centre (VDRC) for generously providing the fly lines crucial for this study. Special recognition is given to Bio Bharati Life Sciences Pvt. Ltd., Kolkata, India, for their significant contribution to antibody generation and the Developmental Studies Hybridoma Bank (DSHB) for supplying antibodies. Our sincere thanks go to C-CAMP Bengaluru for their instrumental role in generating the transgenic fly. The Indian Institute of Science Education and Research Bhopal Central Instrumentation Facility is acknowledged for its valuable support in DNA sequencing and facilitating access to confocal microscopes. This work is supported by the Science and Engineering Research Board grant no. CRG/2020/000496 provided to RKM.

Additional information

Author Contribution

JK and RKM conceptualized the project and the experimental plan and wrote the manuscript. JK performed all experiments and analyzed data. PAJ made initial observations with Nup107 RNAi and analyzed and discussed data. RKM provided intellectual support in data analysis and secured funding for the project.

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Reviewer #1 (Public review):

This study provides a thorough analysis of Nup107's role in Drosophila metamorphosis, demonstrating that its depletion leads to developmental arrest at the third larval instar stage due to disruptions in ecdysone biosynthesis and EcR signaling. Importantly, the authors establish a novel connection between Nup107 and Torso receptor expression, linking it to the hormonal cascade regulating pupariation.

However, some contradictory results weaken the conclusions of the study. The authors claim that Nup107 is involved in the translocation of EcR from the cytoplasm to the nucleus. However, the evidence provided in the paper suggests it more likely regulates EcR expression positively, as EcR is undetectable in Nup107-depleted animals, even below background levels. Additionally, the link between Nup107 and Torso is not fully substantiated. While overexpression of Torso appears to rescue the lack of 20E production in the prothoracic gland, the distinct phenotypes of Torso and Nup107 depletion-developmental delay in the former versus complete larval arrest in the latter complicate understanding of Nup107's precise role.

To clarify these discrepancies, further investigation into whether Nup107 interacts with other critical signaling pathways related to the regulation of ecdysone biosynthesis, such as EGFR or TGF-β, would be beneficial and could strengthen the findings.

In summary, although the study presents some intriguing observations, several conclusions are not well-supported by the experimental data.

https://doi.org/10.7554/eLife.105165.1.sa3

Reviewer #2 (Public review):

Summary:

The manuscript by Kawadkar et al investigates the role of Nup107 in developmental progression via the regulation of ecdysone signaling. The authors identify an interesting phenotype of Nup107 whole-body RNAi depletion in Drosophila development - developmental arrest at the late larval stage. Nup107-depleted larvae exhibit mislocalization of the Ecdysone receptor (EcR) from the nucleus to the cytoplasm and reduced expression of EcR target genes in salivary glands, indicative of compromised ecdysone signaling. This mis-localization of EcR in salivary glands was phenocopied when Nup107 was depleted only in the prothoracic gland (PG), suggesting that it is not nuclear transport of EcR but the presence of ecdysone (normally secreted from PG) that is affected. Consistently, whole-body levels of ecdysone were shown to be reduced in Nup107 KD, particularly at the late third instar stage when a spike in ecdysone normally occurs. Importantly, the authors could rescue the developmental arrest and EcR mislocalization phenotypes of Nup107 KD by adding exogenous ecdysone, supporting the notion that Nup107 depletion disrupts biosynthesis of ecdysone, which arrests normal development. Additionally, they found that rescue of the Nup107 KD phenotype can also be achieved by over-expression of the receptor tyrosine kinase torso, which is thought to be the upstream regulator of ecdysone synthesis in the PG. Transcript levels of the torso are also shown to be downregulated in the Nup107KD, as are transcript levels of multiple ecdysone biosynthesis genes. Together, these experiments reveal a new role of Nup107 or nuclear pore levels in hormone-driven developmental progression, likely via regulation of levels of torso and torso-stimulated ecdysone biosynthesis.

Strengths:

The developmental phenotypes of an NPC component presented in the manuscript are striking and novel, and the data appears to be of high quality. The rescue experiments are particularly significant, providing strong evidence that Nup107 functions upstream of torso and ecdysone levels in the regulation of developmental timing and progression.

Weaknesses:

The underlying mechanism is however not clear, and any insight into how Nup107 may regulate these pathways would greatly strengthen the manuscript. Some suggestions to address this are detailed below.

Major questions:

(1) Determining how specific this phenotype is to Nup107 vs. to reduced NPC levels overall would give some mechanistic insight. Does knocking down other components of the Nup107 subcomplex (the Y-complex) lead to similar phenotypes? Given the published gene regulatory function of Nup107, do other gene regulatory Nups such as Nup98 or Nup153 produce these phenotypes?

(2) In a related issue, does this level of Nup107 KD produce lower NPC levels? It is expected to, but actual quantification of nuclear pores in Nup107-depleted tissues should be added. These and the above experiments would help address a key mechanistic question - is this phenotype the result of lower numbers of nuclear pores or specifically of Nup107?

(3) Additional experiments on how Nup107 regulates the torso would provide further insight. Does Nup107 regulate transcription of the torso or perhaps its mRNA export? Looking at nascent levels of the torso transcript and the localization of its mRNA can help answer this question. Or alternatively, does Nup107 physically bind the torso?

(4) The depletion level of Nup107 RNAi specifically in the salivary gland vs. the prothoracic gland should be compared by RT-qPCR or western blotting.

(5) The UAS-torso rescue experiment should also include the control of an additional UAS construct - so Nup107; UAS-control vs Nup107; UAS-torso should be compared in the context of rescue to make sure the Gal4 driver is functioning at similar levels in the rescue experiment.

Minor:

(6) Figures and figure legends can stand to be more explicit and detailed, respectively.

https://doi.org/10.7554/eLife.105165.1.sa2

Reviewer #3 (Public review):

Summary:

In this study by Kawadkar et al, the authors investigate the developmental role of Nup107, a nucleoporin, in regulating the larval-to-pupal transition in Drosophila through RNAi knockdown and CRISPR-Cas9-mediated gene editing. They demonstrate that Nup107, an essential component of the nuclear pore complex (NPC), is crucial for regulating ecdysone signaling during developmental transitions. The authors show that the depletion of Nup107 disrupts these processes, offering valuable insights into its role in development.

Specifically, they find that:

(1) Nup107 depletion impairs pupariation during the larval-to-pupal transition.
(2) RNAi knockdown of Nup107 results in defects in EcR nuclear translocation, a key regulator of ecdysone signaling.
(3) Exogenous 20-hydroxyecdysone (20E) rescues pupariation blocks, but rescued pupae fail to close.
(4) Nup107 RNAi-induced defects can be rescued by activation of the MAP kinase pathway.

Strengths:

The manuscript provides strong evidence that Nup107, a component of the nuclear pore complex (NPC), plays a crucial role in regulating the larval-to-pupal transition in Drosophila, particularly in ecdysone signaling.

The authors employ a combination of RNAi knockdown, CRISPR-Cas9 gene editing, and rescue experiments, offering a comprehensive approach to studying Nup107's developmental function.

The study effectively connects Nup107 to ecdysone signaling, a key regulator of developmental transitions, offering novel insights into the molecular mechanisms controlling metamorphosis.

The use of exogenous 20-hydroxyecdysone (20E) and activation of the MAP kinase pathway provides a strong mechanistic perspective, suggesting that Nup107 may influence EcR signaling and ecdysone biosynthesis.

Weaknesses:

The authors do not sufficiently address the potential off-target effects of RNAi, which could impact the validity of their findings. Alternative approaches, such as heterozygous or clonal studies, could help confirm the specificity of the observed phenotypes.

NPC Complex Specificity: While the authors focus on Nup107, it remains unclear whether the observed defects are specific to this nucleoporin or if other NPC components also contribute to similar defects. Demonstrating similar results with other NPC components would strengthen their claims.

Although the authors show that Nup107 depletion disrupts EcR signaling, the precise molecular mechanism by which Nup107 influences this process is not fully explored. Further investigation into how Nup107 regulates EcR nuclear translocation or ecdysone biosynthesis would improve the clarity of the findings.

There are some typographical errors and overly strong phrases, such as "unequivocally demonstrate," which could be softened. Additionally, the presentation of redundant data in different tissues could be streamlined to enhance clarity and flow.

https://doi.org/10.7554/eLife.105165.1.sa1

Author response:

> ***Public Reviews:***
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> ***Reviewer #1 (Public review):***
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> *This study provides a thorough analysis of Nup107's role in* Drosophila *metamorphosis, demonstrating that its depletion leads to developmental arrest at the third larval instar stage due to disruptions in ecdysone biosynthesis and EcR signaling. Importantly, the authors establish a novel connection between Nup107 and Torso receptor expression, linking it to the hormonal cascade regulating pupariation.*
>
> *However, some contradictory results weaken the conclusions of the study. The authors claim that Nup107 is involved in the translocation of EcR from the cytoplasm to the nucleus. However, the evidence provided in the paper suggests it more likely regulates EcR expression positively, as EcR is undetectable in Nup107-depleted animals, even below background levels.*

We appreciate the concern raised in this public review. However, we must clarify that we do not claim that Nup107 regulates the translocation of EcR from the cytoplasm. It is important to note that we posited this hypothesis if Nup107 will regulate EcR nuclear translocation (9th line of 2nd paragraph on page 6). We have spelled this out more clearly as the 3rd sub-section title of the Results section, and in the discussion (8th line of 2nd paragraph on page 11). Overall, we have expressed surprise that Nup107 is not directly involved in the nuclear translocation of EcR.

Ecdysone hormone acts through the EcR to induce the transcription of EcR also and creates a positive autoregulatory loop that enhances the EcR level through ecdysone signaling (1). Since Nup107 depletion leads to a reduction in ecdysone levels, it disrupts the transcription autoregulatory EcR expression loop. This can contribute to the reduced EcR levels seen in Nup107-depleted animals.

> *Additionally, the link between Nup107 and Torso is not fully substantiated. While overexpression of Torso appears to rescue the lack of 20E production in the prothoracic gland, the distinct phenotypes of Torso and Nup107 depletion-developmental delay in the former versus complete larval arrest in the latter complicate understanding of Nup107's precise role.*

We understand that there are differences in the developmental delay when Tosro and Nup107 depletion is analyzed. However, the two molecules being compared here are very different, and the extent of Torso depletion is not evident in other studies (2). Even if the extent of depletion of Torso and Nup107 is similar, we believe that Nup107, being a more widely expressed protein, induces stronger defects owing to its importance in cellular physiology. We think that RNAi-mediated depletion of Nup107 causes a defect in 20E biosynthesis through the *Halloween* genes, inducing a developmental arrest.

> *To clarify these discrepancies, further investigation into whether Nup107 interacts with other critical signaling pathways related to the regulation of ecdysone biosynthesis, such as EGFR or TGF-β, would be beneficial and could strengthen the findings.*
>
> *In summary, although the study presents some intriguing observations, several conclusions are not well-supported by the experimental data.*

We agree with the reviewer's suggestion. As noted in the literature, five RTKs-torso, InR, EGFR, Alk, and Pvr-stimulate the PI3K/Akt pathway, which plays a crucial role in the PG functioning and controlling pupariation and body size (3). We have checked the torso and EGFR signaling. We rescued Nup107 defects with the torso overexpression, however, constitutively active EGFR (BL-59843) did not rescue the phenotype (data was not shown). Nonetheless, we plan to examine the EGFR pathway activation by measuring the pERK levels in Nup107-depleted PGs.

> ***Reviewer #2 (Public review):***
>
> *Summary:*
>
> *The manuscript by Kawadkar et al investigates the role of Nup107 in developmental progression via the regulation of ecdysone signaling. The authors identify an interesting phenotype of Nup107 whole-body RNAi depletion in* Drosophila *development - developmental arrest at the late larval stage. Nup107-depleted larvae exhibit mis-localization of the Ecdysone receptor (EcR) from the nucleus to the cytoplasm and reduced expression of EcR target genes in salivary glands, indicative of compromised ecdysone signaling. This mis-localization of EcR in salivary glands was phenocopied when Nup107 was depleted only in the prothoracic gland (PG), suggesting that it is not nuclear transport of EcR but the presence of ecdysone (normally secreted from PG) that is affected. Consistently, whole-body levels of ecdysone were shown to be reduced in Nup107 KD, particularly at the late third instar stage when a spike in ecdysone normally occurs. Importantly, the authors could rescue the developmental arrest and EcR mislocalization phenotypes of Nup107 KD by adding exogenous ecdysone, supporting the notion that Nup107 depletion disrupts biosynthesis of ecdysone, which arrests normal development. Additionally, they found that rescue of the Nup107 KD phenotype can also be achieved by over-expression of the receptor tyrosine kinase torso, which is thought to be the upstream regulator of ecdysone synthesis in the PG. Transcript levels of the torso are also shown to be downregulated in the Nup107KD, as are transcript levels of multiple ecdysone biosynthesis genes. Together, these experiments reveal a new role of Nup107 or nuclear pore levels in hormone-driven developmental progression, likely via regulation of levels of torso and torso-stimulated ecdysone biosynthesis.*

> *Strengths:*
>
> *The developmental phenotypes of an NPC component presented in the manuscript are striking and novel, and the data appears to be of high quality. The rescue experiments are particularly significant, providing strong evidence that Nup107 functions upstream of torso and ecdysone levels in the regulation of developmental timing and progression.*
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> *Weaknesses:*
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> *The underlying mechanism is however not clear, and any insight into how Nup107 may regulate these pathways would greatly strengthen the manuscript. Some suggestions to address this are detailed below.*
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> *Major questions:*
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> *(1) Determining how specific this phenotype is to Nup107 vs. to reduced NPC levels overall would give some mechanistic insight. Does knocking down other components of the Nup107 subcomplex (the Y-complex) lead to similar phenotypes? Given the published gene regulatory function of Nup107, do other gene regulatory Nups such as Nup98 or Nup153 produce these phenotypes?*

We thank this public review to raise this concern. Working with a Nup-complex like the Nup107 complex, this concern is anticipated but difficult to address as many Nups function beyond their complex identity. Our observations with all other members of the Nup107-complex, including dELYS, suggest that except Nup107, none of the other Nup107-complex members could induce larval developmental arrest.

In this study, we primarily focused on the Nup107 complex (outer ring complex) of the NPC. We have not examined other nucleoporins outside of this complex, such as Nup98 and Nup153. However, previous studies have reported that Nup98 and Nup153 interact with chromatin, with these investigations conducted in *Drosophila* S2 cells (4, 5, 6). In the future, we may check whether Nup98 and Nup153 depletion can produce the arrest phenotype.

> *(2) In a related issue, does this level of Nup107 KD produce lower NPC levels? It is expected to, but actual quantification of nuclear pores in Nup107-depleted tissues should be added. These and the above experiments would help address a key mechanistic question - is this phenotype the result of lower numbers of nuclear pores or specifically of Nup107?*

We agree with the concern raised here, and we plan to assess nucleoporin intensity using mAb414 antibody (exclusively FG-repeat Nup recognizing antibody) in the Nup107 depletion background. Our past observations suggest that Nup107-depletion does not affect the overall nuclear pore complex assembly in *Drosophila* salivary glands (Data is not shown).

> *(3) Additional experiments on how Nup107 regulates the torso would provide further insight. Does Nup107 regulate transcription of the torso or perhaps its mRNA export? Looking at nascent levels of the torso transcript and the localization of its mRNA can help answer this question. Or alternatively, does Nup107 physically bind the torso?*

While the concern regarding torso transcript level is genuine, we have already reported in the manuscript that Nup107 levels directly regulate torso expression. When Nup107 is depleted torso levels go down, which in turn controls ecdysone production and subsequent EcR signaling (Figure 6B of the manuscript). However, the exact nature of Nup107 regulation on torso expression is still unclear. Since the Nup107 is known to interact with chromatin (7), it may affect torso transcription. The possibility of a physiologically relevant interaction

between Nup107 and the torso in a cellular context is unlikely due to their distinct sub-cellular localizations. If we investigate this further, it will require a significant amount of time for having reagents and experimentation, and currently stands beyond the scope of this manuscript.

> *(4) The depletion level of Nup107 RNAi specifically in the salivary gland vs. the prothoracic gland should be compared by RT-qPCR or western blotting.*

Although we know that the Nup107 protein signal is reduced in SG upon knockdown (Figure 3B), we have not compared the Nup107 transcript level in these two tissues (SG and PG). As suggested here, we will knock down Nup107 using SG and PG-specific drivers and quantify the Nup107 depletion level by RT-qPCR.

> *(5) The UAS-torso rescue experiment should also include the control of an additional UAS construct - so Nup107; UAS-control vs Nup107; UAS-torso should be compared in the context of rescue to make sure the Gal4 driver is functioning at similar levels in the rescue experiment.*

This is a very valid point, and we took this into account while planning the experiment. To maintain the GAL4 function, we used the $Nup107^{KK}$;UAS-GFP as control alongside the $Nup107^{KK}$;UAS-torso. This approach ensures that GAL4 dilution does not affect observations made in the experiments. It can be noticed in Figure S7 that the presence of GFP signal in prothoracic glands and their reduced size indicates genes downstream to both UAS sequences are transcribed, and GAL4 dilution does not play a role here.

> *Minor:*
>
> *(6) Figures and figure legends can stand to be more explicit and detailed, respectively.*

We will revisit all figures and their corresponding legends to ensure appropriate and explicit details are provided.

> ***Reviewer #3 (Public review):***
>
> *Summary:*
>
> *In this study by Kawadkar et al, the authors investigate the developmental role of Nup107, a nucleoporin, in regulating the larval-to-pupal transition in* Drosophila *through RNAi knockdown and CRISPR-Cas9-mediated gene editing. They demonstrate that Nup107, an essential component of the nuclear pore complex (NPC), is crucial for regulating ecdysone signaling during developmental transitions. The authors show that the depletion of Nup107 disrupts these processes, offering valuable insights into its role in development.*
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>
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> *Strengths:*
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> *The manuscript provides strong evidence that Nup107, a component of the nuclear pore complex (NPC), plays a crucial role in regulating the larval-to-pupal transition in* Drosophila*, particularly in ecdysone signaling.*
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> *The authors employ a combination of RNAi knockdown, CRISPR-Cas9 gene editing, and rescue experiments, offering a comprehensive approach to studying Nup107's developmental function.*
>
> *The study effectively connects Nup107 to ecdysone signaling, a key regulator of developmental transitions, offering novel insights into the molecular mechanisms controlling metamorphosis.*
>
> *The use of exogenous 20-hydroxyecdysone (20E) and activation of the MAP kinase pathway provides a strong mechanistic perspective, suggesting that Nup107 may influence EcR signaling and ecdysone biosynthesis.*
>
> *Weaknesses:*
>
> *The authors do not sufficiently address the potential off-target effects of RNAi, which could impact the validity of their findings. Alternative approaches, such as heterozygous or clonal studies, could help confirm the specificity of the observed phenotypes.*

This is a very valid point raised, and we are aware of the consequences of the off-target effects of RNAi. To assert the effects of authentic RNAi and reduce the off-target effects, we have used two RNAi lines ($Nup107^{GD}$ and $Nup107^{KK}$) against Nup107. Both RNAi induced comparable levels of Nup107 reduction, and using these lines, ubiquitous and PG specific knockdown produced similar phenotypes. Although the $Nup107^{GD}$ line exhibited a relatively stronger knockdown compared to the $Nup107^{KK}$ line, we preferentially used the $Nup107^{KK}$ line because the $Nup107^{GD}$ line is based on the P-element insertion, and the exact landing site is unknown. Furthermore, there is an off-target predicted for the $Nup107^{GD}$ line, where a 19bp sequence aligns with the bifocal (bif) sequence. The bif-encoded protein is involved in axon guidance and regulation of axon extension. However, the $Nup107^{KK}$ line does not have a predicted off-target molecule, and we know its precise landing site on the second chromosome. Thus, the $Nup107^{KK}$ line was ultimately used in experimentation for its clearer and more reliable genetic background.

We are also investigating Nup107 knockdown in the prothoracic gland, which exhibits polyteny. Additionally, the number of cells in the prothoracic gland is quite limited, approximately 50-60 cells (8). Given this, there is a possibility that a clonal study may not yield the phenotype. However, we will consider moving forward with this approach also.

> *NPC Complex Specificity: While the authors focus on Nup107, it remains unclear whether the observed defects are specific to this nucleoporin or if other NPC components also contribute to similar defects. Demonstrating similar results with other NPC components would strengthen their claims.*

We thank this public review to raise this concern. Working with a Nup-complex like the Nup107 complex, this concern is anticipated but difficult to address as many Nups function beyond their complex identity. Our observations with all other members of the Nup107-complex, including dELYS, suggest that except Nup107, none of the other Nup107-complex members could induce larval developmental arrest. Since the study is primarily focused on the Nup107 complex (outer ring complex) of the NPC, we have not examined other nucleoporins outside of this complex.

> *Although the authors show that Nup107 depletion disrupts EcR signaling, the precise molecular mechanism by which Nup107 influences this process is not fully explored. Further investigation into how Nup107 regulates EcR nuclear translocation or ecdysone biosynthesis would improve the clarity of the findings.*

We appreciate the concern raised. Through our observation, we have proposed the upstream effect of Nup107 on the PTTH-torso-20E-EcR axis regulating developmental transitions. We know that Nup107 regulates torso levels, but we do not know if Nup107 directly interacts with torso. We would like to address whether Nup107 exerts control on PTTH levels also.

We must emphasize that Nup107 does not directly regulate the translocation of EcR. On the contrary, we have demonstrated that EcR translocation is 20E dependent and Nup107 independent. Through our observations, we have argued that Nup107 regulates the expression of *Halloween* genes required for ecdysone biosynthesis. We are interested in identifying if Nup107 associates directly or through some protein to chromatin to bring about the changes in gene expression required for normal development.

> *There are some typographical errors and overly strong phrases, such as "unequivocally demonstrate," which could be softened. Additionally, the presentation of redundant data in different tissues could be streamlined to enhance clarity and flow.*

We thank the reviewer for this observation. We will remove all typographical errors and make reasonable statements based on our conclusions.

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https://doi.org/10.7554/eLife.105165.1.sa0