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Investigation

Su(var)2-10- and *Su(var)205*-dependent upregulation of the heterochromatic gene *neverland* is required for developmental transition in *Drosophila*

Yuya Ohhara ,[1,2,*] Yuki Kato,[2] Takumi Kamiyama,[3,4] Kimiko Yamakawa-Kobayashi[1,2]

[1]School of Food and Nutritional Sciences, University of Shizuoka, Shizuoka, Shizuoka 422-8526, Japan,
[2]Graduate School of Integrated Pharmaceutical and Nutritional Sciences, University of Shizuoka, Shizuoka, Shizuoka 422-8526, Japan,
[3]College of Biological Sciences, Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Ibaraki 305-8572, Japan,
[4]Life Science Center for Survival Dynamics, Tsukuba Advanced Research Alliance (TARA), University of Tsukuba, Tsukuba, Ibaraki 305-8577, Japan

*Corresponding author: School of Food and Nutritional Sciences, University of Shizuoka, 52-1, Yada, Suruga-ku, Shizuoka, Shizuoka 422-8526, Japan.
Email: y-ohhara@u-shizuoka-ken.ac.jp

Abstract

Animals develop from juveniles to sexually mature adults through the action of steroid hormones. In insect metamorphosis, a surge of the steroid hormone ecdysone prompts the transition from the larval to the adult stage. Ecdysone is synthesized by a series of biosynthetic enzymes that are specifically expressed in an endocrine organ, the prothoracic gland. At the late larval stage, the expression levels of ecdysone biosynthetic enzymes are upregulated through the action of numerous transcription factors, thus initiating metamorphosis. In contrast, the mechanism by which chromatin regulators support the expression of ecdysone biosynthetic genes is largely unknown. Here, we demonstrate that *Su(var)2-10* and *Su(var)205*, *suppressor of variegation* [*Su(var)*] genes encoding a chromatin regulator Su(var)2-10 and nonhistone heterochromatic protein 1a, respectively, regulate the transcription of one of the heterochromatic ecdysone biosynthetic genes, *neverland*, in *Drosophila melanogaster*. Knockdown of *Su(var)2-10* and *Su(var)205* in the prothoracic gland caused a decrease in *neverland* expression, resulting in a defect in larval-to-prepupal transition. Furthermore, overexpression of *neverland* and administration of 7-dehydrocholesterol, a biosynthetic precursor of ecdysone produced by Neverland, rescued developmental defects in *Su(var)2-10* and *Su(var)205* knockdown animals. These results indicate that *Su(var)2-10*- and *Su(var)205*-mediated proper expression of *neverland* is required for the initiation of metamorphosis. Given that Su(var)2-10-positive puncta are juxtaposed with the pericentromeric heterochromatic region, we propose that *Su(var)2-10*- and *Su(var)205*-dependent regulation of inherent heterochromatin structure at the *neverland* gene locus is essential for its transcriptional activation.

Keywords: *Drosophila*; heterochromatin; Su(var)2-10; HP1a; ecdysone; *neverland*; prothoracic gland

Introduction

Animals develop from juveniles to sexually mature adults during postembryonic development. Similar to puberty in mammals, the developmental transition in arthropods from juveniles to adults is triggered by steroid hormones. In holometabolous insect larvae, metamorphosis is triggered by the action of steroid hormones called ecdysteroids. The best-characterized bioactive ecdysteroid is 20-hydroxyecdysone (20E). 20E activates its specific receptor, a heterodimer of ecdysone receptor (EcR) and ultraspiracle (USP), to induce downstream gene expression cascades and subsequent metamorphic events, such as tissue remodeling and cell death in larval tissues.

A precursor of 20E, ecdysone, is produced from the prothoracic gland (PG), a polyploid endocrine organ. The initial step of ecdysone biosynthesis, the conversion of dietary cholesterol to 7-dehydrocholesterol (7-DHC), is catalyzed by Neverland (Nvd) (Fig. 1a) (Yoshiyama *et al.* 2006; Yoshiyama-Yanagawa *et al.* 2011). 7-DHC is then metabolized to 5β-ketodiol by at least 3 enzymes, including Spookier/Spook (Spok/Spo) (Namiki *et al.* 2005; Ono *et al.* 2006), Shroud (Sro) (Niwa *et al.* 2010), and CYP6T3 (Ou *et al.* 2011) (Fig. 1a). 5β-ketodiol is further converted into ecdysone through sequential enzymatic reactions mediated by Phantom (Phm) (Niwa *et al.* 2004; Warren *et al.* 2004), Disembodied (Dib) (Warren *et al.* 2002; Niwa *et al.* 2005), and Shadow (Sad) (Warren *et al.* 2002) (Fig. 1a). Ecdysone is secreted from the PG through a vesicular trafficking mechanism (Yamanaka *et al.* 2015) and then converted into 20E by Shade (Shd) in peripheral organs (Petryk *et al.* 2003) (Fig. 1a).

Transcription factors and nuclear receptors that regulate the transcription of ecdysone biosynthetic enzymes have been identified in *Drosophila* (Niwa and Niwa 2016; Kamiyama and Niwa 2022). Examples of these are EcR, βFtz-F1, Broad, ventral veins lacking, knirps, snail, forkhead box protein O–USP heterodimer, and cap-n-collar and Keap1 complex, which are involved in the transcription of multiple ecdysone biosynthetic enzymes (Parvy *et al.* 2005, 2014; Xiang *et al.* 2010; Deng and Kerppola 2013;

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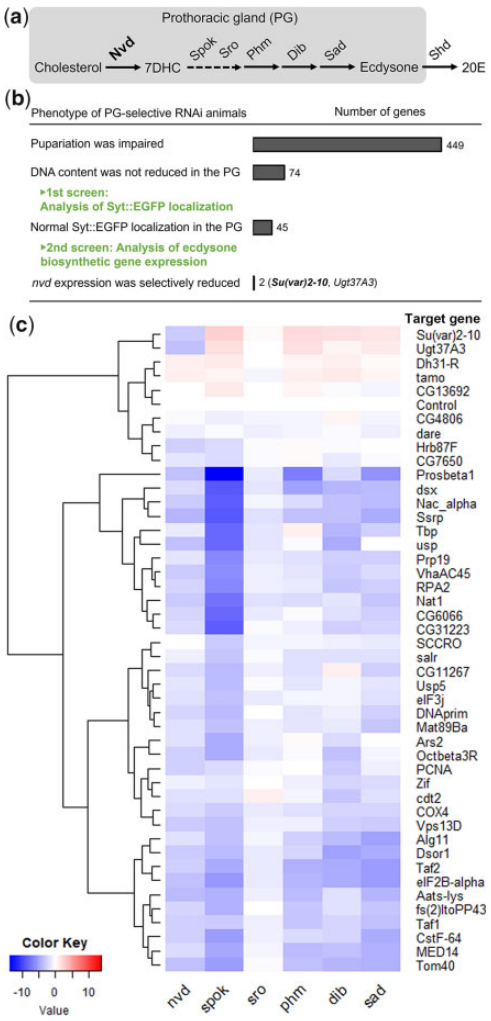

Fig. 1. PG-selective RNAi screen to identify novel transcriptional regulators of ecdysone biosynthetic genes. a) Schematic diagram of the ecdysone biosynthetic pathway in the PG. b) Scheme of PG-selective RNAi screens. Candidate genes and results of the first and second screens are summarized in Supplementary Tables 3 and 5, respectively. c) A heat map representing the relative expression levels of ecdysone biosynthetic genes in control (*phm>+*) and knockdown animals (*phm>gene-of-interest-RNAi*). The gene expression levels were measured using qPCR at 96 h after hatching.

Moeller *et al.* 2013; Cheng *et al.* 2014; Danielsen *et al.* 2014; Koyama *et al.* 2014; Zeng *et al.* 2020). Furthermore, recent studies have revealed that the genes *nvd* and *spok*, located in the pericentromeric heterochromatic region (Ono *et al.* 2006; Yoshiyama *et al.* 2006; Uryu *et al.* 2018), are specifically and directly activated by transcription factors Séance and Ouija Board (Ouib), respectively, in cooperation with Molting defective (Mld) (Komura-Kawa *et al.* 2015; Uryu *et al.* 2018). In addition, a poly(A) deadenylation complex named CCR4-NOT selectively regulates the expression of *spok* (Zeng *et al.* 2018), and a poly(A) binding protein, Pabp, regulates *spok* expression through the regulation of nuclear translocation of Mld (Kamiyama *et al.* 2020).

In addition to transcription regulators and poly(A)-binding proteins, chromatin regulators are also involved in the regulation of ecdysone biosynthetic gene expression. Ada2a-containing complex histone acetyltransferase (Pankotai *et al.* 2010; Borsos *et al.* 2015), the polycomb repressive complex-2 histone methyltransferase (Yang *et al.* 2021), and the insulator-associated protein CCCTC-binding factor (Fresán *et al.* 2015) have been identified as essential for the upregulation of multiple ecdysone biosynthetic genes. Furthermore, given that certain heterochromatin-residing genes require a heterochromatic environment for normal expression (Wakimoto and Hearn 1990; Eberl *et al.* 1993; Lu *et al.* 1996, 2000; Yasuhara and Wakimoto 2006), it is possible that the transcription of the heterochromatic ecdysone biosynthetic genes *nvd* and *spok* is under the control of inherent epigenetic factors, such as heterochromatin-associated chromatin regulators. However, chromatin regulators that control the transcription of a specific ecdysone biosynthetic enzyme, including heterochromatic *nvd* and *spok*, have not been identified.

In the present study, we performed a PG-selective RNAi screen to identify novel regulators of ecdysone biosynthetic genes. We identified *Su(var)2-10*, which belongs to the *suppressor of variegation* [*Su(var)*] genes (Hari *et al.* 2001; Elgin and Reuter 2013), as a novel regulator of *nvd* gene expression. *Su(var)* genes including *Su(var)2-10* have been originally isolated as essential genes for heterochromatin formation in the *white* (*w*) locus adjacent to pericentric heterochromatin due to chromosomal inversion [$In(1)w^{m4}$]: $In(1)w^{m4}$ animals show variegated eyes (red and white patches) because of partial silencing of the inverted *white* locus, while loss of *Su(var)* genes in the $In(1)w^{m4}$ background leads to a red-eye phenotype due to less heterochromatin formation in the inverted *white* locus (Elgin and Reuter 2013). *Su(var)2-10* encodes a nuclear protein called Su(var)2-10 [a.k.a. protein inhibitor of activated STAT (PIAS)] possessing small ubiquitin-related modifier (SUMO) E3 ligase activity (Hari *et al.* 2001; Rytinki *et al.* 2009; Rabellino *et al.* 2017). Recent studies have been elucidated that Su(var)2-10 deposits SUMO on chromosome to recruit the histone methyltransferase, leading to suppression of spurious transcription and ensuring normal gene expression in the heterochromatin (Ninova, Chen, *et al.*, 2020; Ninova, Godneeva, *et al.*, 2020). Our genetic evidence shows that knockdown of *Su(var)2-10* in the PG resulted in the third instar arrest phenotype due to downregulation of *nvd* expression. Furthermore, we found that *Su(var)205*, encoding heterochromatic protein 1a (HP1a), a major component of heterochromatic chromosomes (Eissenberg *et al.* 1992; Eissenberg and Elgin 2014; Schoelz and Riddle 2022), also controls pupariation via *nvd* upregulation. Considering that *Su(var)* genes are essential for heterochromatin formation and normal expression of heterochromatin-residing genes (Elgin and Reuter 2013), we propose that *Su(var)2-10*- and *Su(var)205*-dependent regulation of inherent heterochromatin structure at the *nvd* gene locus is essential for its transcriptional activation.

Materials and methods

Drosophila stocks and medium

Fly stocks and their genotypes used in this study are listed in Supplementary Tables 1 and 2. Stocks used for the RNAi screen are summarized in Supplementary Table 3. w^{1118} served as a wild-type strain. Fly stocks were reared on a glucose/cornmeal/yeast medium (1 g glucose, 0.7 g cornmeal, 0.4 g yeast extract, 50 mg agar in 10 ml water) supplemented with 30 µl propionic acid and 35 µl butylparaben (167 mg/ml in 70% ethanol). Fly stocks placed in vials (MKC-20, Hi-tech) with the culture medium were kept in the 25°C culture room at relative humidity levels between 40% and 60% under a 12-h light/dark cycle (Fluorescent light was turn on from 8:00 AM to 8:00 PM JST). The glucose/cornmeal/yeast medium was used for the first RNAi screen (see *PG-Selective RNAi Screening*).

German food, a nutrient-rich semi-defined medium, was used for all experiments except for the first RNAi screen. A total of 22.5 g German food powder (Genesee Scientific, San Diego, CA, USA, 66–115) was added to 100 ml water, and the mixture was boiled using a microwave. After stirring for 30 min at room temperature (18–25°C), 600 µl propionic acid was added to the mixture to obtain a German food medium.

Staging and analysis of developmental progression

Parent flies were maintained in plastic bottles (Genesee Scientific, San Diego, CA, USA, 32–310) and allowed to lay eggs for 24 h on grape juice agar plates (2 g agar in 100 ml grape juice, poured into a 4.5 cm × 1.6 cm plastic dish) supplemented with dry yeast powder (Oriental Yeast, Japan). Newly hatched 10–35 larvae were transferred to vials (MKC-30, Hi-tech) containing 1–3.5 g of the German food medium. Larvae were cultured in the 25°C incubator (LTI-400E, EYELA) at relative humidity levels between 40% and 60% under a 12-h light/dark cycle (Fluorescent light was turn on from 8:00 AM to 8:00 PM JST), and developmental stages and lethality were scored every 24 h. *TM6B/TM6B Gal80* balancer-possessing animals were excluded at 48 h after hatching, and *CyO-GFP* balancer-possessing animals were excluded at 0 h after hatching using a fluorescent stereomicroscope.

PG-selective RNAi screening

PG-selective RNAi screening was performed using the Gal4/UAS system to identify novel regulators of ecdysone biosynthetic genes. In the first screen, candidate genes were knocked down in the PG and the membrane localization of EGFP-fused Synaptotagmin (Syt) (Syt::EGFP) (Zhang *et al.* 2002) was observed to exclude genes involved in the regulation of Syt-mediated vesicle trafficking. Ten virgin females carrying PG-selective *phantom-Gal4* (*phm-Gal4*) (Rewitz *et al.* 2009) and *UAS-Syt::EGFP* were crossed with 5 *UAS-RNAi* males to obtain the offspring in which gene of interest was knocked down in the PG (*phm>Syt::EGFP gene-of-interest-RNAi*). To obtain control animals (*phm>Syt::EGFP*), females carrying *phm-Gal4* and *UAS-Syt::EGFP* were crossed with w^{1118} males. Parent flies were cultured on the glucose/cornmeal/yeast medium in a plastic vial for 2 days. At day 6 after crossing, in which most of control larvae were in the wandering stage, Syt::EGFP expression in control and knockdown animals were observed as described in *Immunohistochemistry*.

In the second screen, newly hatched control (*phm>+*) and knockdown larvae (*phm>gene-of-interest-RNAi*) were collected and reared on the German food as described in *Staging and Analysis of Developmental Progression*. At 96 h after hatching, control and knockdown larvae were sampled, and the expression levels of ecdysone biosynthetic genes were analyzed using qPCR as described in *Quantitative RT-PCR*.

Immunohistochemistry

Immunohistochemistry was performed to investigate the expression levels/patterns of Syt::EGFP, Nvd, Spok, Sro, and Dib proteins. Larvae were dissected in PBS and fixed for 25 min with 4% paraformaldehyde in 0.01% PBT (0.01% Triton X-100 in PBS). Tissues were washed with 0.1% PBT 3 times for 10 min each and washed with 1% PBT for 5 min to increase antibody permeability. Tissues were then blocked with 1% goat serum (Sigma, G9023) in 0.1% PBT for 30 min, and incubated at 4°C overnight with a primary antibody against GFP (mouse IgG, monoclonal, 3E6) (Thermo Fisher Scientific, A-11120) at a 1:1,000 dilution, Nvd (guinea pig IgG, polyclonal) (Ohhara *et al.* 2015) at a 1:200 dilution, Spok (guinea pig IgG, polyclonal) (Gibbens *et al.* 2011) at a 1:500 dilution, Sro (guinea pig IgG, polyclonal) (Shimada-Niwa and Niwa 2014) at a 1:1,000 dilution, or Dib (rabbit pig IgG, polyclonal) (Parvy *et al.* 2005) at a 1:500 dilution in a blocking solution. Note that anti-Nvd antibody solution was incubated with fixed w^{1118} larval tissues at 4°C overnight before the experiment to reduce the background signal. Tissues were washed with 0.1% PBT 3 times for 10 min each and incubated at 4°C overnight with Alexa 488-conjugated goat IgG against mouse/rabbit/guinea pig (Thermo Fisher Scientific, A-11001/A-11008/A-11073) at 1:1,000 dilution and Hoechst 33342 (Thermo Fisher Scientific, 62249) at 1:1,500 dilution in 0.1% PBT. After washing with 0.1% PBT for 10 min each, brain-ring gland complexes were dissected and mounted in a mounting medium (2.4 g Mowiol 4-88 [Sigma-Aldrich, 81381], 6 g glycerol, 6 ml distilled water, and 12 ml 0.2 M Tris–HCl [pH 8.5]). Images were taken with a Zeiss LSM700 and a Zeiss LSM800, and image analyses were performed using Image J/Fiji software (Schindelin *et al.* 2012).

For visualization of GFP-fused Su(var)2-10 proteins, tissues were incubated at 4°C overnight with primary antibodies against GFP (rabbit IgG, polyclonal) (Medical and Biological Laboratories, #598; see the related information including specificity of this antibody at https://ruo.mbl.co.jp/bio/dtl/A/?pcd=598) at a 1:1,000 dilution. We also used anti-HP1a antibody (mouse IgG, monoclonal) (Developmental Studies Hybridoma Bank, C1A9), which can specifically recognize *Drosophila* HP1a protein (James and Elgin 1986) at a 1:50 dilution in a blocking solution. Alexa 488-conjugated goat IgG against rabbit IgG (Thermo Fisher Scientific, A-11008) and Alexa 647-conjugated goat IgG against mouse IgG (Thermo Fisher Scientific, A-21235) were used as secondary antibodies at 1:1,000 dilution, with Hoechst 33342 (Thermo Fisher Scientific, 62249) at 1:1,500 dilution. Washing, blocking, mounting, and image acquisition were performed in the same procedure as described above.

Quantitative RT-PCR

Quantitative RT-PCR (qPCR) was performed to measure the expression levels of ecdysone biosynthetic genes. Total RNA was extracted from larvae using TRIzol (Thermo Fisher Scientific, 15596026). A total of 0.1–0.5 µg of RNA samples were reverse-transcribed using the SuperScript III kit (Thermo Fisher Scientific, 18080051). The obtained cDNA samples were used as a template for qPCR using the Quantifast SYBR Green PCR kit (QIAGEN, 204056) and Rotor-Gene Q (QIAGEN) with a primer concentration of 1 µM. All reactions were performed at 95°C for 10 min, followed by 50 cycles of 95°C for 10 s and 60°C for 30 s. Dissociation curve analysis was applied to all reactions to ensure the presence of a single PCR product. The expression levels of the target genes were calculated using the relative standard curve method. Stock cDNA used for the relative standard curves was synthesized from pooled RNA derived from larvae raised under the same conditions and diluted serially. The expression levels of the target genes were normalized to an endogenous reference gene, *ribosomal protein 49* (*rp49*) (also known as *ribosomal protein L32*). The mean expression level of the control was set to 1. The primer sets used for qPCR are listed in Supplementary Table 4.

7-DHC and 20E supplementation experiments

About 50 µl of sterol solution (10 mg/ml 7-DHC [Sigma-Aldrich, 30800] or 20E [Tokyo chemical industry, H1480] in ethanol) was added to 2 g of the German food to obtain a 7-DHC or 20E-containing medium (final sterol concentration: 0.5 mg/g). Two grams of the German food with 50 µl of ethanol served as a control

medium. A total of 15–30 larvae reared on the German food were transferred to the control or sterol-containing media at 48 h after hatching. Developmental stages were scored every 24 h.

Image analysis

To quantify DNA signal intensity in the PG, a series of images obtained by immunostaining/histochemistry were processed using Image J/Fiji as follows. First, the PG area was segmented: In transgenic animals expressing mCherry.nls in the PG, the mCherry.nls signal was binarized using a function named "Make binary"; In case of the PGs of wild type and mutant animals stained with anti-Dib antibody, the Dib signal was binarized and then the nuclei of PG cells were filled using "Make binary" and "Fill holes." Next, DNA signal overlapped with the PG area was calculated using "Image calculator." The processed images were z-stacked, and PG area in a z-stacked image was selected using "Freehand selection" tool. Integrated density within the selected region was measured and then adjusted using average DNA staining intensity obtained from a z-stacked image of the brain lobe. Adjusted DNA staining intensity was then divided by the PG cell number to calculate mean DNA intensity per PG cell. In accordance with the result of ploidy measurement in the previous study, the mean chromatin values (C values) in the PG of wild type (+/+) and transgenic control animals (*phm>mCherry.nls*) at 96 h after hatching were set to 58 and 53, respectively (Ohhara *et al.* 2019).

For quantification of Nvd, Spok, and Sro immunostaining signal in the PG, a series of images were also processed using Image J/Fiji as follows. PG area in a z-stacked image was selected using "Freehand selection," and average immunostaining signal intensity was measured within the region. The immunostaining signal intensity was adjusted using average immunostaining intensity obtained from a z-stacked image of the brain lobe. The mean value of adjusted signal intensity in control animals was set to 1.

For GFP-fused Su(var)2-10 observations, Su(var)2-10 punctum was defined as a GFP-positive nuclear punctum showing GFP signal intensity 1.5 – 2 times higher than surrounding GFP-positive nuclear region. The number of Su(var)2-10 puncta-positive PG cells was counted manually using the cell counter plugin in Image J/Fiji software (https://imagej.nih.gov/ij/plugins/cell-counter.html).

Statistical analysis

Statistical analyses were performed using R software (version 4.1.0) (Ihaka and Gentleman 1996). Data were analyzed using Fisher's exact test, Student's *t*-test, Mann–Whitney *U*-test, Dunnett's test, Tukey's test, or Steel's multiple comparison test.

Results

A PG-selective RNAi screen to identify novel transcriptional regulators of ecdysone biosynthetic genes

Previously, we performed a PG-selective RNAi screen to identify novel regulators of ecdysone biosynthesis and identified 449 genes that regulate larval-to-prepupal transition (Ohhara *et al.* 2019) (Fig. 1b and Supplementary Fig. 1 and Supplementary Table 3). We further analyzed the PG cells of knockdown animals to screen the genes that are involved in the regulation of the cell cycle in the PG (Ohhara *et al.* 2019), given that proper progression of the endocycle, a variant of the cell cycle that is characterized by the absence of mitosis (Lilly and Duronio, 2005), is essential for ecdysone biosynthesis in the PG (Ohhara *et al.* 2017, 2019; Zeng *et al.* 2020). Once the C value in PG cells reaches 32 (which corresponds to 3 rounds of endocycling), the expression levels of ecdysone biosynthetic enzymes are significantly upregulated in the late third instar, between 72 and 96 h after hatching (Ohhara *et al.* 2017, 2019). Of the 449 genes, 74 were potentially dispensable for endocycle progression in PG cells, given that knockdown of these genes did not cause a decrease in DNA content in PG cells compared to control animals at 72 h after hatching, at which point the C value in PG cells reached 32 (Fig. 1b and Supplementary Fig. 1 and Supplementary Table 3). In addition, knockdown of these genes did not cause morphological defects in the PG (Ohhara *et al.* 2019). Here, we focused on these genes as candidates for novel transcriptional regulators of ecdysone biosynthetic enzymes and performed the following screening.

Using immunohistochemistry, we determined Syt::EGFP localization, thus excluding genes that are involved in the trafficking of vesicle-mediated ecdysone secretory granules (Yamanaka *et al.* 2015). A vesicle-localizing protein, Syt::EGFP (Zhang *et al.* 2002), was expressed in the PG using the PG-selective *phm-Gal4* (Rewitz *et al.* 2009) in control (*phm>Syt::EGFP*) and knockdown animals (*phm>Syt::EGFP gene-of-interest-RNAi*). Syt::EGFP was localized to the plasma membrane in the PG of control animals, whereas knockdown of membrane trafficking regulators, such as *Sec8* and *Sec15*, resulted in loss of Syt::EGFP membrane localization, as previously reported (Yamanaka *et al.* 2015) (Figure S1 in File S2). Furthermore, knockdown of *amx*, *CG10333*, *CG32069*, *dwg*, *Him*, *not*, *Nup214*, *ps*, *SF2*, *Trs23*, and *Vha44* caused a decrease in Syt::EGFP membrane localization (Supplementary Fig. 1), suggesting that these genes are required for membrane localization of Syt::EGFP in the PG. In addition, knockdown of *CG32104*, *MED17*, *Nurf-38*, *Rtf1*, *ubl*, and *Wdr3* caused a reduction in Syt::EGFP protein levels in the PG (Supplementary Fig. 1). Thus, these genes may be involved in the post-transcriptional regulation of Syt. Therefore, these 19 genes were excluded from the candidate list. In addition, we excluded 10 genes whose knockdown did not cause developmental arrest or delayed pupariation (Supplementary Table 3). The remaining 45 genes were then tested in the second screen (Fig. 1b).

Next, we examined the expression levels of ecdysone biosynthetic genes, *nvd*, *spok*, *sro*, *phm*, *dib*, and *sad*, in control (*phm>+*) and knockdown animals (*phm>gene-of-interest-RNAi*) using qPCR at 96 h after hatching. Compared to the control, many knockdown animals showed a decrease in expression of multiple ecdysone biosynthetic genes (Fig. 1c and Supplementary Table 5). The expression levels of ecdysone biosynthetic genes were not significantly reduced in *CG4806*, *CG13692*, *dare*, *Dh31-R*, and *tamo* knockdown animals (Fig. 1c and Supplementary Table 5), which suggests that these genes are not involved in the transcriptional regulation of ecdysone biosynthetic enzymes, but rather involved in the post-transcriptional regulation of ecdysone biosynthetic enzymes or steroidogenesis-related biochemical reactions such as the mitochondrial electron transfer system. Actually, *dare*, which encodes a flavoenzyme that carries electrons for the steroidogenesis in mitochondria, has been identified as essential for ecdysone production in the PG (Freeman *et al.* 1999). In contrast, the expression level of *nvd*, but not other ecdysteroidogenic genes, was significantly reduced in *Su(var)2-10* and *Ugt37A3* knockdown animals (Fig. 1c and Supplementary Table 5). This suggests that *Su(var)2-10* and *Ugt37A3* selectively regulate *nvd* transcription. Because *Su(var)2-10* serves as a transcriptional coregulator and a chromatin regulator (Rytinki *et al.* 2009; Rabellino *et al.* 2017; Ninova, Chen, *et al.* 2020; Ninova, Godneeva, *et al.*,

2020), we focused on *Su(var)2-10* hereafter and performed further genetic analyses to reveal its function.

Su(var)2-10 and *Su(var)205* knockdown animals exhibit larval stage arrest phenotype due to a reduction of *nvd* expression

To elucidate the importance of *Su(var)2-10* in ecdysone biosynthesis, we investigated the developmental phenotype and expression levels of ecdysone biosynthetic genes in 3 control animals, possessing only *phm-Gal4* (*phm>+*) or *UAS-Su(var)2-10 RNAi-1/2* construct [*+>Su(var)2-10-RNAi-1/2*], and 2 independent *Su(var)2-10* RNAi animals [*phm>Su(var)2-10-RNAi-1/2*]. We confirmed that knockdown of *Su(var)2-10* led to third instar arrest phenotype (Fig. 2, a and b) and that the expression levels of *nvd* were significantly reduced in *Su(var)2-10* knockdown animals compared to control animals (Fig. 2c). We also confirmed that Nvd protein levels were clearly reduced in the PG of *Su(var)2-10* knockdown animals (Supplementary Fig. 2). These results indicate that *Su(var)2-10* is required for *nvd* upregulation. Expression levels of other ecdysone biosynthetic gene were not significantly reduced in *Su(var)2-10* knockdown animals (Fig. 3c), while *sro* expression was slightly reduced in *phm>Su(var)2-10-RNAi-1* animals (Fig. 2c), suggesting the possibility that *Su(var)2-10* also regulates *sro* expression. However, immunostaining revealed that there was no significant difference in Sro protein levels between control and *Su(var)2-10* knockdown animals (Supplementary Fig. 2). In addition, *spok* transcription and Spok protein levels were not reduced in *Su(var)2-10* knockdown animals (Fig. 2c and Supplementary Fig. 2). These results suggest that *Su(var)2-10* is dispensable for upregulation of *spok*, *sro*, and other ecdysone biosynthetic genes.

We next sought to determine whether developmental arrest in *Su(var)2-10* knockdown animals was due to downregulation of *nvd* expression. Wild-type *Bombyx nvd* (*Bm-nvd*^{WT}) or a mutated type of *Bombyx nvd* possessing H190A mutation (*Bm-nvd*^{H190A}), which causes a loss of the Nvd enzymatic activity (Yoshiyama-Yanagawa *et al.* 2011), were expressed under the control of UAS in the PG of *Su(var)2-10* knockdown animals. The larval-to-prepupal transition was blocked in *phm>Su(var)2-10-RNAi-1* and *RNAi-2* animals, as well as in *Bm-nvd*^{H190A}-expressing *Su(var)2-10* knockdown animals [*phm>Su(var)2-10-RNAi-1/2 Bm-nvd*^{H190A}], whereas pupariation was induced in *Bm-nvd*^{WT}-expressing *Su(var)2-10* knockdown animals [*phm>Su(var)2-10-RNAi-1/2 Bm-nvd*^{WT}] (Fig. 2d). Furthermore, administration of 7-DHC, the first ecdysone intermediate produced by Nvd, or 20E rescued developmental arrest in *phm>Su(var)2-10-RNAi-1* and *RNAi-2* animals (Supplementary Fig. 3). These results indicate that developmental arrest in *Su(var)2-10* knockdown animals is caused by the downregulation of *nvd*.

Su(var)2-10 knockdown animals showed an increase in DNA content in PG cells and PG tissue size (Supplementary Fig. 4). This hypertrophic PG phenotype has been observed in mutant *Su(var)205* animals (Spierer *et al.* 2005), which encodes a major component of heterochromatin called HP1a (Eissenberg *et al.* 1992; Eissenberg and Elgin 2014; Schoelz and Riddle 2022). Because both *Su(var)205* and *Su(var)2-10* belong to the *Su(var)* genes (Eissenberg *et al.* 1992; Hari *et al.* 2001), which are essential for heterochromatin formation and normal expression of heterochromatin-residing genes (Elgin and Reuter 2013; Ninova, Godneeva, *et al.*, 2020; Schoelz and Riddle 2022), we hypothesized that *Su(var)205* is also involved in the regulation of *nvd*. First, we confirmed that knockdown of *Su(var)205* in the PG caused an increase in DNA content and tissue size (Supplementary Fig. 4).

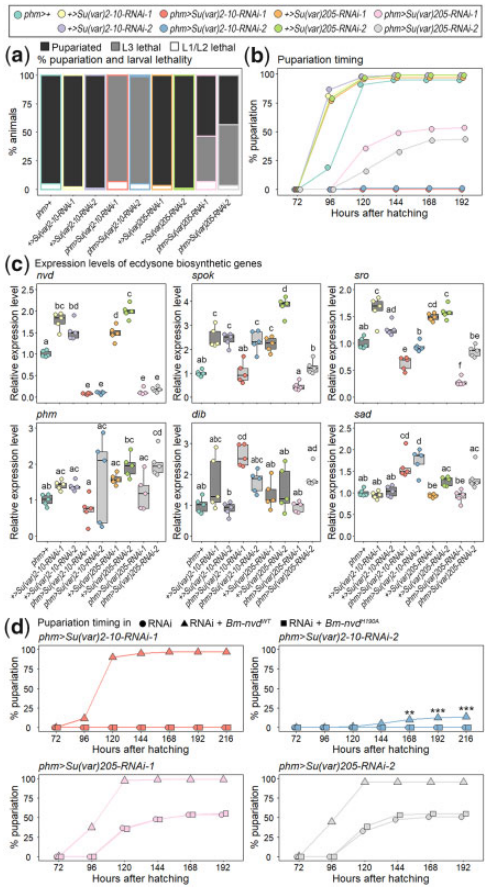

Fig. 2. Knockdown of *Su(var)2-10* and *Su(var)205* causes impaired pupariation due to reduced *nvd* expression. a and b) Knockdown of *Su(var)2-10* and *Su(var)205* in the PG causes developmental resulted in third instar arrest. Percentages of pupariated animals and larvae died at first/second and third instar (shown as "L1/L2 lethal" and "L3 lethal," respectively) (a) and percentages of pupariated animals at indicated time points (b) in control animals possessing only *phm-Gal4* (*phm>+*) or *UAS-RNAi* construct (*+>gene-of-interest-RNAi*), *Su(var)2-10* knockdown [*phm>Su(var)2-10-RNAi-1* and *phm>Su(var)2-10-RNAi-2*], and *Su(var)205* knockdown animals [*phm>Su(var)205-RNAi-1* and *phm>Su(var)205-RNAi-2*] are shown. Sample sizes are 90–100 for each genotype. c) *nvd* expression levels are reduced in *Su(var)2-10* and *Su(var)205* knockdown animals. Expression levels of ecdysone biosynthetic genes (*nvd*, *spok*, *sro*, *phm*, *dib*, and *sad*) were measured using qPCR at 96 h after hatching. Box and scatter plots of 5 biological replicates are shown in each experimental group. Statistically significant differences between groups are indicated by different lowercase letters ($P < 0.05$; Tukey's test), while there is no statistically significant difference between groups with the same lowercase letter ($P > 0.05$). d) Overexpression of *nvd* rescues developmental arrest in *Su(var)2-10* and *Su(var)205* knockdown animals. Percentages of pupariated animals among knockdown animals (*phm>gene-of-interest-RNAi*) (circle), *Bm-nvd*^{WT}-expressing knockdown animals (*phm>gene-of-interest-RNAi Bm-nvd*^{WT}) (triangle), and *Bm-nvd*^{H190A}-expressing knockdown animals (*phm>gene-of-interest-RNAi Bm-nvd*^{H190A}) (square) are shown at indicated time points. Sample sizes are 55–80 for each group. Asterisks indicate a statistically significant difference between *phm>Su(var)2-10-RNAi-2* and *phm>Su(var)2-10-RNAi-2 Bm-nvd*^{WT} (** $P < 0.01$, *** $P < 0.001$; Fisher's exact test).

Furthermore, approximately half of the *Su(var)205* knockdown animals [*phm>Su(var)205-RNAi-1/2*] were arrested at the third instar, and those animals that did develop further showed a significant delay in pupariation (Fig. 2, a and b). In addition, the expression levels of *nvd* mRNA and Nvd protein levels were reduced in *Su(var)205* knockdown animals (Fig. 2c and

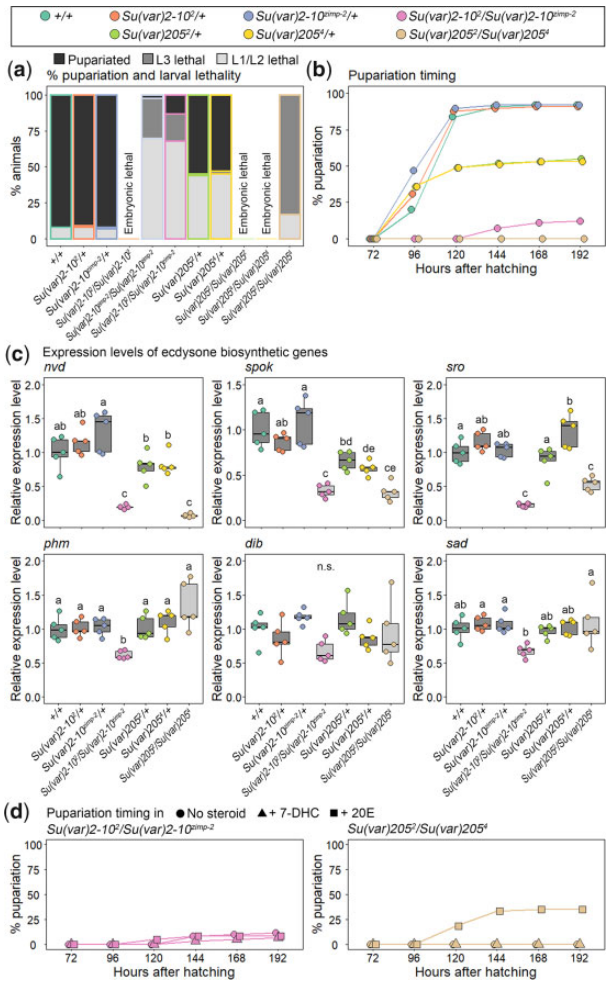

Fig. 3. nvd expression is reduced in *Su(var)2-10* and *Su(var)205* mutants. a) *Su(var)2-10* and *Su(var)205* mutants show developmental defects. Percentages of pupariated animals and larvae died at 1st/2nd and 3rd instar (shown as "L1/L2 lethal" and "L3 lethal," respectively) in wild-type animals (+/+), heterozygous *Su(var)2-10* mutants [$Su(var)2\text{-}10^{2}$/+ and $Su(var)2\text{-}10^{zimp-2}$/+], homozygous *Su(var)2-10* mutants [$Su(var)2\text{-}10^{2}$/$Su(var)2\text{-}10^{2}$ and $Su(var)2\text{-}10^{zimp-2}$/$Su(var)2\text{-}10^{zimp-2}$], trans-heterozygous *Su(var)2-10* mutants [$Su(var)2\text{-}10^{2}$/$Su(var)2\text{-}10^{zimp-2}$], heterozygous *Su(var)205* mutants [$Su(var)205^{2}$/+ and $Su(var)205^{4}$/+], homozygous *Su(var)205* mutants [$Su(var)205^{2}$/$Su(var)205^{2}$ and $Su(var)205^{4}$/$Su(var)205^{4}$], and trans-heterozygous *Su(var)205* mutants [$Su(var)205^{2}$/$Su(var)205^{4}$] are shown. Sample sizes are 100 for each genotype. b) Percentages of pupariated animals among wild-type and mutants are shown at indicated time points. c) *nvd* expression levels are reduced in *Su(var)2-10* and *Su(var)205* trans-heterozygous mutants. Expression levels of ecdysone biosynthetic genes (*nvd*, *spok*, *sro*, *phm*, *dib*, and *sad*) were measured using qPCR at 96 h after hatching. Box and scatter plots of 5 biological replicates are shown in each experimental group. Statistically significant differences between groups are indicated by different lowercase letters ($P < 0.05$; Tukey's test), while there is no statistically significant difference between groups with the same lowercase letter ($P > 0.05$). n. s., not significant. d) Administration of 20E rescues developmental arrest in *Su(var)205* mutant animals. Percentages of pupariated trans-heterozygous *Su(var)2-10* and *Su(var)205* mutants cultured on a control medium (circle) and 7-DHC- (triangle) or 20E-supplemented media (square) are shown at indicated time points. Sample sizes are 60 for each group.

Supplementary Fig. 2), and their developmental defects were rescued by forced expression of $Bm\text{-}nvd^{WT}$ and the administration of 7-DHC and 20E (Fig. 2d and Supplementary Fig. 3). These results indicate that *Su(var)205*-mediated *nvd* upregulation is required for proper onset of larval-to-prepupal transition.

nvd expression is reduced in *Su(var)2-10* and *Su(var)205* mutants

To confirm the importance of *Su(var)2-10* and *Su(var)205* in ecdysone biosynthesis, we observed the phenotype of animals carrying mutant alleles of *Su(var)2-10*, $Su(var)2\text{-}10^{2}$, and $Su(var)2\text{-}10^{zimp-2}$, which possess a point mutation in the coding region (W260Stop) (Hari *et al.* 2001) and the insertion of a transposon in the *Su(var)2-10* gene region (Mohr and Boswell 1999), respectively. Most of the wild type (+/+) and heterozygous *Su(var)2-10* mutants [$Su(var)2\text{-}10^{2}$/+ and $Su(var)2\text{-}10^{zimp-2}$/+] underwent pupariation by 120 h after hatching, whereas homozygous $Su(var)2\text{-}10^{2}$ mutants [$Su(var)2\text{-}10^{2}$/$Su(var)2\text{-}10^{2}$] did not hatch, and homozygous $Su(var)2\text{-}10^{zimp-2}$ mutants [$Su(var)2\text{-}10^{zimp-2}$/$Su(var)2\text{-}10^{zimp-2}$] died at the first (41%), second (29%), and third instar (28%) (Fig. 3a). These results indicate that *Su(var)2-10* is required for embryonic and larval development. Trans-heterozygous *Su(var)2-10* mutants [$Su(var)2\text{-}10^{2}$/$Su(var)2\text{-}10^{zimp-2}$], on the other hand, showed milder phenotypes: 28%, 40%, and 19% of $Su(var)2\text{-}10^{2}$/$Su(var)2\text{-}10^{zimp-2}$ animals died at the first, second, and third instar, respectively. The other 13% of the animals underwent pupariation (Fig. 3a), although they showed a significant delay in pupariation (Fig. 3b). These results suggest that ecdysone biosynthesis is downregulated in $Su(var)2\text{-}10^{2}$/$Su(var)2\text{-}10^{zimp-2}$ animals. Indeed, *nvd* mRNA expression levels were reduced in $Su(var)2\text{-}10^{2}$/$Su(var)2\text{-}10^{zimp-2}$ animals compared to wild type and heterozygous *Su(var)2-10* mutants at 96 h after hatching (Fig. 3c). In addition, the expression levels of *spok*, *sro*, *phm*, and *sad* were reduced in $Su(var)2\text{-}10^{2}$/$Su(var)2\text{-}10^{zimp-2}$ animals (Fig. 3c). Considering that *phm>Su(var)2-10-RNAi-1* animals showed a slight decrease in *sro* expression, it is likely that *Su(var)2-10* is also involved in the transcriptional regulation of *sro* in the PG. However, because neither 7-DHC nor 20E administration rescued developmental defects in $Su(var)2\text{-}10^{2}$/$Su(var)2\text{-}10^{zimp-2}$ (Fig. 3d), developmental delay in $Su(var)2\text{-}10^{2}$/$Su(var)2\text{-}10^{zimp-2}$ animals is probably caused by multiple mechanisms.

We further investigated the phenotypes of animals carrying *Su(var)205* mutant alleles, $Su(var)205^{2}$ and $Su(var)205^{4}$, possessing point mutations that cause amino acid substitutions (V26M and K169Stop, respectively) (Eissenberg *et al.* 1992; Shaffer *et al.* 1993). Approximately half of the heterozygous *Su(var)205* mutants [$Su(var)205^{2}$/+ and $Su(var)205^{4}$/+] underwent larval-to-prepupal transition at the same time as wild-type animals, whereas the other half died at the first or second instar (Fig. 3, a and b). Homozygous *Su(var)205* mutants [$Su(var)205^{2}$/$Su(var)205^{2}$ and $Su(var)205^{4}$/$Su(var)205^{4}$] did not hatch (Fig. 3a). These results indicate that *Su(var)205* is required for embryonic and early larval development. In contrast, most of the trans-heterozygous *Su(var)205* mutant animals [$Su(var)205^{2}$/$Su(var)205^{4}$] developed into the third instar, but they could not undergo pupariation (Fig. 3, a and b). This suggests the possibility that $Su(var)205^{2}$/$Su(var)205^{4}$ animals were arrested at the third instar because of a decrease in ecdysone biosynthetic activities. This possibility is supported by the fact that expression levels of *nvd*, *spok*, and *sro* were low in $Su(var)205^{2}$/$Su(var)205^{4}$ animals (Fig. 3c), and administration of 20E, but not 7-DHC, partially rescued their developmental defects (Fig. 3d). These results suggest that $Su(var)205^{2}$/$Su(var)205^{4}$ animals died at the third instar due to reduced ecdysone biosynthetic activities. Considering that *nvd* and *sro* expression levels were reduced in *phm>Su(var)205-RNAi-1* (Fig. 2c), it is probable that *Su(var)205* is involved in not only *nvd* but also *sro* transcriptional upregulation in the PG. However, another possibility is that *Su(var)205* expressed in other tissues is involved in

sro expression indirectly. Altogether, the above results serve as evidence that *Su(var)2-10* and *Su(var)205* are required for *nvd* upregulation.

In addition, we found that *Su(var)2-10* and *Su(var)205* mutants did not show defects in DNA content and cell number in the PG (Supplementary Fig. 4). This result indicates that *Su(var)2-10* and *Su(var)205* are dispensable for mitosis and endocycle progression in the PG. However, we could not confirm the hypertrophic PG phenotype in *Su(var)2-10* and *Su(var)205* mutants (Supplementary Fig. 4), which suggests that *Su(var)2-10* and *Su(var)205* mutations might affect systemic growth signaling, such as the insulin signaling pathway.

Su(var)2-10 protein is localized near the pericentromeric heterochromatin

We investigated the localization patterns of *Su(var)2-10* and *Su(var)205* gene products, Su(var)2-10 and HP1a proteins, respectively, in PG cells using immunohistochemistry. We utilized 2 transgenic lines carrying GFP-fused Su(var)2-10 to visualize Su(var)2-10 protein distribution: *Su(var)2-10-GFP*, in which Su(var)2-10 carrying C-terminal GFP tag is expressed under the control of endogenous upstream sequences of *Su(var)2-10* gene [generated by the model organism Encyclopedia of Regulatory Network (modERN) Project; related information is available in FlyBase (http://flybase.org/reports/FBtp0111904.html)], and *Su(var)2-10*^{CC02013}, in which GFP-coding sequence franked by splicing acceptor and donor sites was inserted within the first intron between 2 coding exons of *Su(var)2-10* gene (Buszczak *et al.*, 2007) (https://flybase.org/reports/FBal0211741.html). In these strains, GFP-fused Su(var)2-10 was localized in the nucleus, whereas HP1a was localized in a part of the nucleus that represents the heterochromatic region (Supplementary Fig. 5). Interestingly, GFP-fused Su(var)2-10 puncta were detected near HP1a-positive and DNA-dense core heterochromatic regions in PG cells (Supplementary Fig. 5). These results suggest that Su(var)2-10 is juxtaposed with the heterochromatic region.

Su(var)2-10-GFP was then introduced in control [*phm>mCherry.nls, Su(var)2-10-GFP*] and *Su(var)2-10* knockdown animals [*phm>mCherry.nls Su(var)2-10-RNAi-1/2, Su(var)2-10-GFP*], as well as *Su(var)205* knockdown animals [*phm>mCherry.nls Su(var)205-RNAi-1/2, Su(var)2-10-GFP*]. We confirmed that Su(var)2-10-GFP expression was diminished in PG cells of *Su(var)2-10* knockdown animals (Fig. 4, a and b). In contrast, the HP1a protein localization pattern was unchanged in PG cells of *Su(var)2-10* knockdown animals (Fig. 4, a and b), suggesting that *Su(var)2-10* does not control HP1a localization within PG cells. Likewise, HP1a protein levels were significantly reduced in PG cells of *Su(var)205* knockdown animals; however, Su(var)2-10-GFP expression level and the percentage of Su(var)2-10-GFP-puncta-positive cells in the PG of *Su(var)205* knockdown animals were comparable to those of control animals (Fig. 4, a and b). These results suggest that Su(var)2-10 and HP1a proteins do not mutually regulate their protein levels or their subcellular localization.

Insulin and PTTH signal-mediated *nvd* upregulation and developmental acceleration is abrogated by *Su(var)2-10* and *Su(var)205* knockdown

The PG receives various neuropeptides, such as prothoracicotropic hormone (PTTH) and insulin-like peptides (ILPs), which leads to the activation of ecdysone biosynthesis (McBrayer *et al.* 2007; Walkiewicz and Stern 2009; Yamanaka *et al.* 2013; Niwa and Niwa 2016; Pan *et al.* 2021). We investigated whether *Su(var)2-10* and *Su(var)205* act downstream of PTTH and insulin signaling in the PG. As shown in previous studies, forced expression of an active form of insulin receptor (InR.A1325D; indicated as InR^{CA}) in the PG (*phm>InR*^{CA}) accelerates the timing of pupariation (Ohhara *et al.* 2017) (Fig. 5a). In contrast, *Su(var)2-10* knockdown in InR^{CA}-expressing animals [*phm>InR*^{CA} *Su(var)2-10-RNAi-1*] completely blocked this acceleration, and the animals were arrested at the larval stage, similar to what occurred in the case of the *Su(var)2-10* knockdown animals (Fig. 5, a and b). Furthermore, knockdown of *Su(var)205* in InR^{CA}-expressing animals [*phm>InR*^{CA} *Su(var)205-RNAi-1*] also abrogated the acceleration of pupariation, and the percentage of pupariated animals among InR^{CA}-expressing *Su(var)205* knockdown animals was comparable with that among *Su(var)205* knockdown animals (Fig. 5, a and c). These results suggest that *Su(var)2-10* and *Su(var)205* are required for insulin signaling-mediated acceleration of pupariation.

In contrast, forced expression of a constitutively active form of Ras (Ras^{V12}), a downstream effector of PTTH (Rewitz *et al.* 2009), partially rescued developmental arrest in *Su(var)2-10* knockdown animals [*phm>Ras*^{V12} *Su(var)2-10-RNAi-1*] (Fig. 5b), suggesting that *Su(var)2-10* is independent of PTTH signaling in the PG. However, the percentage of pupariated animals was low among Ras^{V12}-expressing *Su(var)2-10* knockdown animals (34%) compared to the corresponding percentage among control (95%) and Ras^{V12}-overexpressing animals (*phm>Ras*^{V12}) (82%) (Fig. 5, a and b). Thus, we could not exclude the possibility that *Su(var)2-10* acts downstream of PTTH signaling. In contrast, pupariation in Ras^{V12}-expressing *Su(var)205* knockdown animals [*phm>Ras*^{V12} *Su(var)205-RNAi-1*] was significantly delayed compared with that in Ras^{V12}-expressing animals (Fig. 5, a and c), suggesting that *Su(var)205* is required for PTTH signal-mediated acceleration of larval-to-prepupal transition.

The above results raise the possibility that *Su(var)2-10* and *Su(var)205* are required for insulin and PTTH signal-mediated upregulation of *nvd* transcription. To test this possibility, we examined the expression levels of ecdysone biosynthetic enzymes in InR^{CA}- and Ras^{V12}-expressing *Su(var)2-10* and *Su(var)205* knockdown animals at 72 h after hatching. InR^{CA} overexpression led to transcriptional enhancement of *nvd* and other ecdysone biosynthetic genes (Fig. 5d), indicating that insulin signaling accelerates the transcription of ecdysone biosynthetic genes. In contrast, *nvd* upregulation caused by InR^{CA} overexpression was cancelled in *Su(var)2-10* and *Su(var)205* knockdown animals (Fig. 5d). These results suggest that insulin and PTTH signal-mediated enhancement of *nvd* expression requires *Su(var)2-10* and *Su(var)205*. In addition, *spok*, *sro*, *phm*, and *sad* upregulation were blocked in InR^{CA}-expressing *Su(var)2-10* and *Su(var)205* knockdown animals (Fig. 5d), suggesting that *Su(var)2-10* and *Su(var)205* were also involved in insulin signal-mediated upregulation of these ecdysone biosynthetic genes. However, considering that *Su(var)2-10* and *Su(var)205* knockdown caused selective downregulation of *nvd* expression, *Su(var)2-10* and *Su(var)205* are not essential for transcription of *spok*, *sro*, *phm*, and *sad* but required for InR^{CA}-induced ectopic upregulation of these genes.

Ras^{V12} overexpression also caused transcriptional upregulation of *nvd*, while Ras^{V12}-induced enhancement of *nvd* expression was cancelled by *Su(var)2-10* and *Su(var)205* knockdown (Fig. 5d), suggesting that *Su(var)2-10* and *Su(var)205* act downstream of PTTH signal to induce *nvd* expression. The expression levels of other ecdysone biosynthetic genes were enhanced in Ras^{V12}-expressing animals (Fig. 5d), but this upregulation was not abrogated by *Su(var)2-10* and *Su(var)205* knockdown (Fig. 5d). These

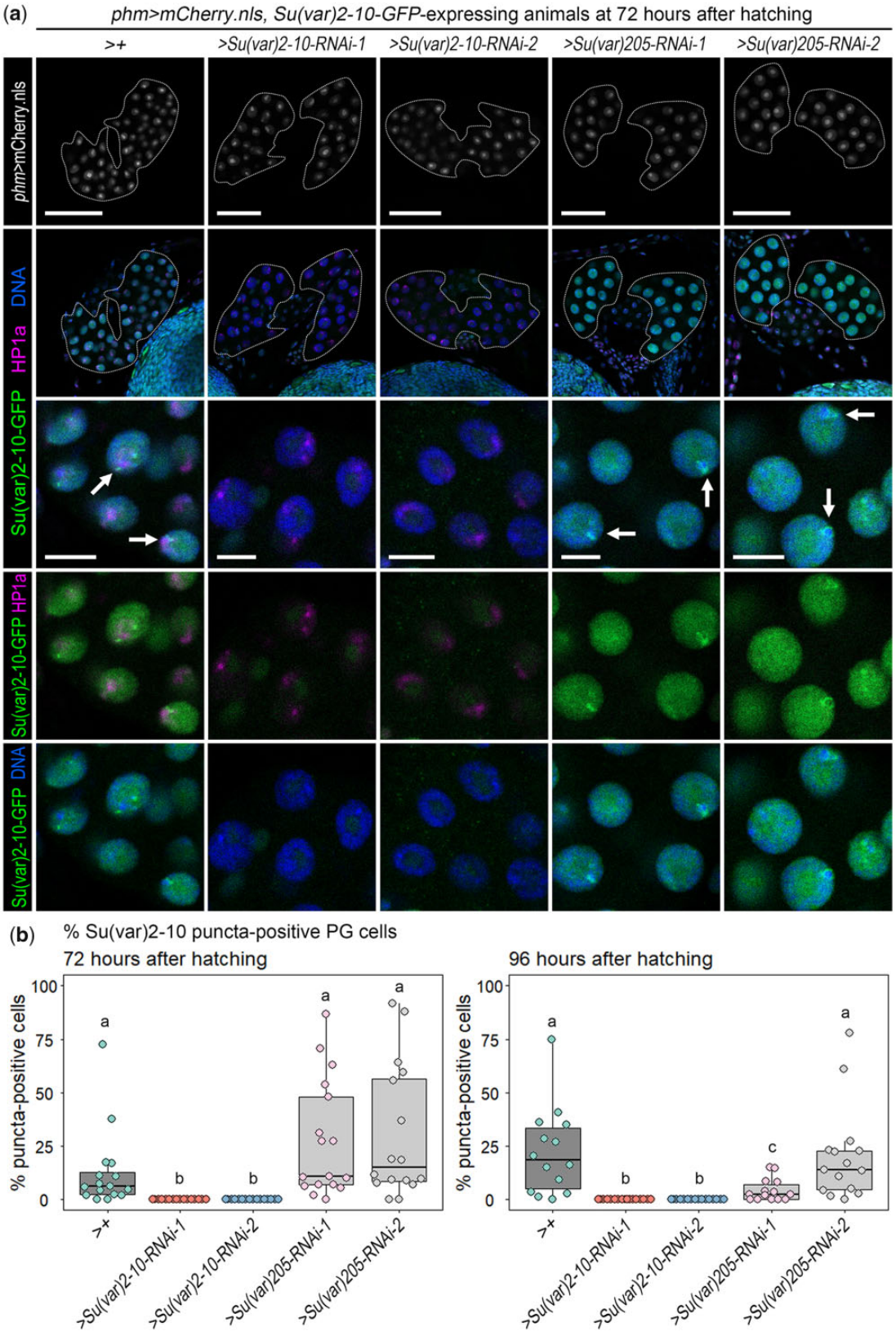

Fig. 4. Su(var)2-10 and HP1a protein localization patterns in PG cells. a) Confocal sections of the PGs (upper 2 panels) and PG cells in higher magnification (lower 3 panels) of control [*phm>mCherry.nls, Su(var)2-10-GFP*], *Su(var)2-10* knockdown [*phm>mCherry.nls Su(var)2-10-RNAi-1/2, Su(var)2-10-GFP*], and *Su(var)205* knockdown animals [*phm>mCherry.nls Su(var)205-RNAi-1/2, Su(var)2-10-GFP*] at 72 h after hatching. Su(var)2-10-GFP and HP1a proteins were visualized by antibodies against GFP (green) and HP1a (magenta), respectively. DNA was detected by Hoechst (blue), and the nuclei of PG cells were labeled by mCherry.nls (white). The PGs are indicated by dotted lines. Arrows indicate Su(var)2-10-GFP-positive puncta juxtaposed to the heterochromatic region. Scale bars: 50 μm (upper panels) and 10 μm (lower panels). b) Scatter and box plots showing the percentages of Su(var)2-10-GFP puncta-positive PG cells in control and knockdown animals at 72 (left) and 96 h after hatching (right). Statistically significant differences between groups are indicated by different lowercase letters ($P < 0.05$; Steel–Dwass test), while there is no statistically significant difference between groups with the same lowercase letter ($P > 0.05$). Sample sizes are 14–17 for each group.

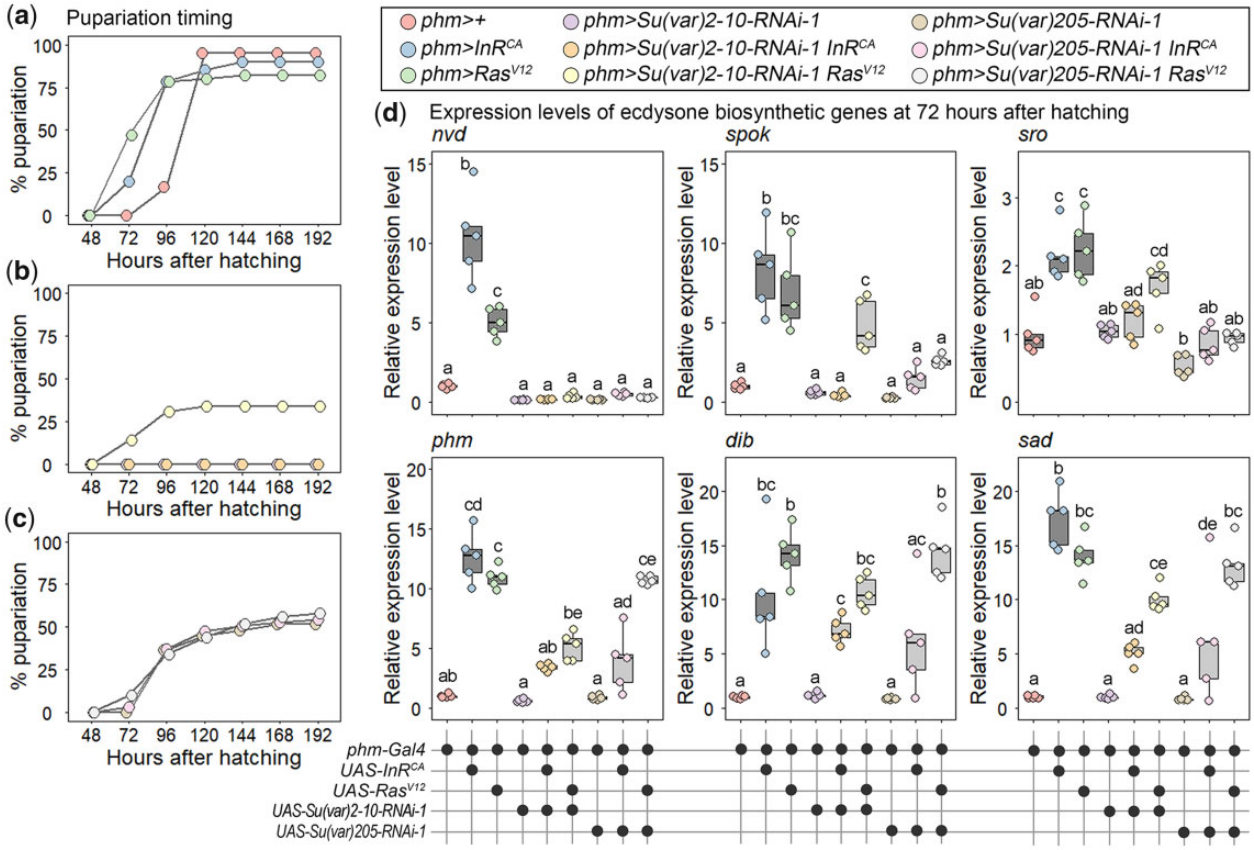

Fig. 5. Insulin and PTTH signal-mediated developmental acceleration and transcriptional upregulation of *nvd* are abrogated by *Su(var)2-10* and *Su(var)205* knockdown. a–c) Percentages of pupariated animals among control (*phm>+*) (a), *Su(var)2-10* knockdown [*phm>Su(var)2-10-RNAi-1*] (b), and *Su(var)205* knockdown animals [*phm>Su(var)205-RNAi-1*] (c) with *UAS-InR*^{CA} and *UAS-Ras*^{V12} (triangle and square, respectively) or without any additional transgenes (circle) are shown at indicated time points. Sample sizes are 50–65 for each genotype. d) Upregulation of *nvd* transcription caused by InR^{CA}- and Ras^{V12}-overexpression is cancelled by *Su(var)2-10* and *Su(var)205* knockdown. Expression levels of ecdysone biosynthetic genes (*nvd*, *spok*, *sro*, *phm*, *dib*, and *sad*) were measured using qPCR at 72 h after hatching. Box and scatter plots of 5 biological replicates are shown in each experimental group. Statistically significant differences between groups are indicated by different lowercase letters ($P < 0.05$; Tukey's test), while there is no statistically significant difference between groups with the same lowercase letter ($P > 0.05$).

results suggest that *Su(var)2-10* and *Su(var)205* are dispensable for PTTH signal-mediated upregulation of other ecdysone biosynthetic genes.

Discussion

In *Drosophila*, ecdysone biosynthetic enzymes show a significant increase in expression levels in the late third instar (Ou *et al.* 2016; Ohhara *et al.* 2017), and this surge is essential for larval-to-prepupal transition. The transcriptional regulatory mechanisms of ecdysone biosynthetic enzymes by transcription factors have been investigated over the last 20 years. In contrast, comparatively little is known about the chromatin regulators supporting the gene expression of these enzymes. In the present study, we demonstrated that *Su(var)2-10* and *Su(var)205* are required for upregulation of *nvd*. Knockdown of *Su(var)2-10* and *Su(var)205* in the PG caused a defect in larval-to-prepupal transition and a decrease in *nvd* expression (Figs. 1 and 2), and *nvd* overexpression or administration of 7-DHC, a biosynthetic precursor of ecdysone produced by Nvd, rescued developmental defects in *Su(var)2-10* and *Su(var)205* knockdown animals (Fig. 2 and Supplementary Fig. 2). The expression level of *nvd* was also reduced in *Su(var)2-10* and *Su(var)205* mutants (Fig. 3). These results indicate that *Su(var)2-10* and *Su(var)205* promote pupariation through the regulation of *nvd* expression.

An important question that is yet to be resolved is how *Su(var)2-10* and *Su(var)205* regulate the expression of heterochromatic *nvd* gene. Both *Su(var)2-10* and *Su(var)205* belong to the *Su(var)* gene group, a set of genes required for heterochromatin formation (Eissenberg *et al.* 1992; Hari *et al.* 2001; Elgin and Reuter 2013). At the molecular level, *Su(var)2-10* encodes a SUMO E3 ligase to modify chromatin state and gene expression via SUMOylation of target proteins (Rytinki *et al.* 2009; Ninova, Godneeva, *et al.* 2020), whereas HP1a, the product of *Su(var)205* gene, is a nonhistone chromosomal protein deposited on heterochromatin to ensure normal heterochromatin formation (Eissenberg and Elgin 2014; Schoelz and Riddle 2022). Several studies have shown that heterochromatin-residing genes, such as *rolled* and *light*, require a heterochromatin environment and *Su(var)205* for their normal expression in *Drosophila* (Wakimoto and Hearn 1990; Eberl *et al.* 1993; Lu *et al.* 1996, 2000; Yasuhara and Wakimoto 2006). Furthermore, a recent study showed that Su(var)2-10 supports proper expression of heterochromatic genes through the regulation of histone H3 lysine 9 trimethylation (H3K9me3) (Ninova, Godneeva, *et al.* 2020). Given that binding of HP1a to H3K9me3 is responsible for structural properties of the heterochromatic region (Canzio *et al.* 2011; Eissenberg and Elgin 2014; Larson *et al.* 2017; Strom *et al.* 2017; Schoelz and Riddle 2022), one possibility is that Su(var)2-10-mediated histone modification and subsequent HP1a recruitment in the *nvd* gene locus

is required for the establishment of an inherent chromatin structure that is suitable for its normal expression. In contrast, SUMOylation promotes the targeting of HP1a to pericentromeric heterochromatin in mammals (Maison *et al.* 2011). Hence, another possible mechanism is that Su(var)2-10 SUMOylates HP1a in the PG to confer a proper heterochromatic structure that is required for *nvd* transcriptional activation, although it remains unclear whether HP1a is SUMOylated in a Su(var)2-10-dependent manner. It would be interesting to investigate whether H3K9me3 level and HP1a accumulation in the *nvd* locus is dependent on Su(var)2-10 activity.

Although we could not obtain the data showing the direct interaction between Su(var)2-10/HP1a and the *nvd* gene locus, we observed that HP1a was localized to the heterochromatic region in PG cells and that Su(var)2-10 protein is juxtaposed with the pericentromeric heterochromatin region in PG cells (Fig. 4 and Supplementary Fig. 5). These results support the hypothesis that Su(var)2-10 and HP1a interact with the *nvd* gene locus to allow its normal expression. Importantly, heterochromatin-juxtaposed Su(var)2-10 puncta were observed in some, but not all, PG cells and the percentages of Su(var)2-10-pisitive puncta were divergent among PGs (Fig. 4), raising the possibility that Su(var)2-10 localization near the heterochromatic region is affected by heterogeneous cellular physiologies, such as cell cycle. Given that endocycle progression is asynchronous among PG cells (Ohhara *et al.*, 2017; Ohhara *et al.*, 2019) and that the distribution of chromatin-associated proteins including histone H1 and proliferating cell nuclear antigen in the polytene chromosome is dynamically changed in accordance with the endocycle progression (Andreyeva *et al.*, 2017), we put forward a hypothesis that Su(var)2-10 localization near the heterochromatic regions is coupled with endocycle progression in PG cells. For example, a possible mechanism is that Su(var)2-10 protein is recruited near the heterochromatic region when heterochromatic structure is loosed before or after DNA replication.

Su(var)2-10 belongs to a conserved PIAS protein family. PIAS proteins were originally identified as suppressors of transcription factors called signal transducer and activator of transcription (STAT) proteins (Rytinki *et al.* 2009). Importantly, the newest study demonstrated that Su(var)2-10 promotes larval-to-prepupal transition through the negative regulation of STAT92E (a sole STAT protein in *Drosophila*) in the PG (Cao *et al.* 2022). The study also showed that the autocrine Unpaired3 (Upd3)-induced JAK (Janus kinase)/STAT signaling in the PG negatively controls the timing of pupariation (Cao *et al.* 2022). Thus, one possible mechanism is that Su(var)2-10 upregulates *nvd* expression via the negative regulation of Upd3-induced JAK/STAT signaling. In addition, Su(var)2-10 proteins positively or negatively regulate various proteins, including transcription factors other than STATs (Rytinki *et al.* 2009; Rabellino *et al.* 2017). In *Drosophila* PG, βFtz-F1, a nuclear receptor/transcription factor regulating ecdysone biosynthesis, is the substrate of SUMOylation (Talamillo *et al.* 2013). Thus, βFtz-F1 is likely to be a prominent candidate for the Su(var)2-10 target protein. However, because inhibition of βFtz-F1 causes a decrease in Phm and Dib expression (Parvy *et al.* 2005), other SUMO E3 ligases are likely involved in the regulation of βFtz-F1. Alternatively, we speculate that Su(var)2-10 modulates the activity of other transcription factors, such as Séance and Mld, to promote *nvd* expression (Uryu *et al.* 2018). Further studies are needed to elucidate the target proteins and pathways of Su(var)2-10 in the PG.

In addition to *nvd*, the expression levels of *spok* and *sro* were also reduced in $Su(var)2\text{-}10^{2}/Su(var)2\text{-}10^{zimp-2}$ and $Su(var)205^{2}/Su(var)205^{4}$ mutants (Fig. 3c). This suggests that *Su(var)2-10* and *Su(var)205* are also involved in the regulation of *spok* and *sro* expression. Knockdown of *Heterogeneous nuclear ribonucleoprotein at 87F* (*Hrb87F*), which encodes an RNA-binding protein and belongs to the *Su(var)* gene group (Piacentini *et al.* 2009), resulted in a decrease in *nvd* and *spok* expression (Fig. 1). Considering that Hrb87F promotes gene expression through interaction with HP1a (Piacentini *et al.* 2009), one possible mechanism is that Hrb87F acts in concert with HP1a to promote *nvd* and *spok* expression.

Ecdysone biosynthesis in the PG is controlled by neuropeptides, such as ILPs and PTTH (McBrayer *et al.* 2007; Walkiewicz and Stern 2009; Yamanaka *et al.* 2013; Niwa and Niwa 2016; Pan *et al.* 2021). ILPs and PTTH stimulate insulin-mTOR and Ras/MAPK signaling pathways in the PG, respectively, which in turn activate ecdysone biosynthesis and subsequent larval-to-prepupal transition through the regulation of the endocycle (Ohhara *et al.* 2017; Shimell *et al.* 2018). Because the acceleration of larval-to-prepupal transition and *nvd* transcriptional upregulation by forced activation of insulin and Ras/MAPK signaling in the PG was abrogated by *Su(var)2-10* and *Su(var)205* knockdown (Fig. 5), it can be surmised that *Su(var)2-10* and *Su(var)205* allow the ILP/PTTH-endocycle pathway to stimulate *nvd* expression. In addition, considering that DNA content and tissue size did not decrease but, rather, increased in the PGs of *Su(var)2-10* and *Su(var)205* knockdown animals (Supplementary Fig. 3), *Su(var)2-10* and *Su(var)205* seem to negatively modulate the activity of endocycling in the PG. One possible mechanism is that *Su(var)2-10* and *Su(var)205* permit *nvd* transcriptional upregulation while acting in a negative feedback loop to downregulate endocycle progression to switch from endocycling to the ecdysteroidogenic phase in the PG.

In summary, our results indicate that Su(var)2-10 and HP1a are novel regulators of *nvd* expression and larval-to-prepupal transition (Fig. 6). The genetic evidence in our study shows that Su(var)2-10 and HP1a positively regulate the transcription of the heterochromatic gene *nvd*, supporting the idea that a heterochromatic environment is required for certain heterochromatin-residing genes to be normally expressed. This study provides the basis for understanding the transcriptional upregulation mechanisms of heterochromatic genes and their significance in steroidogenesis and development.

Data availability

All numerical data (except for the data obtained in RNAi screens) and R scripts for figures and statistical analysis are available in Figshare (https://doi.org/10.6084/m9.figshare.20626095). Confocal images are also shared in Figshare (https://doi.org/10.6084/m9.figshare.20646858). The data obtained in the first and second RNAi screens are shown in Supplementary Tables 3 and 5, respectively. The numerical data visualized in Supplementary Fig. 1a are available in Ohhara *et al.*, 2019.

Supplemental material is available at GENETICS online.

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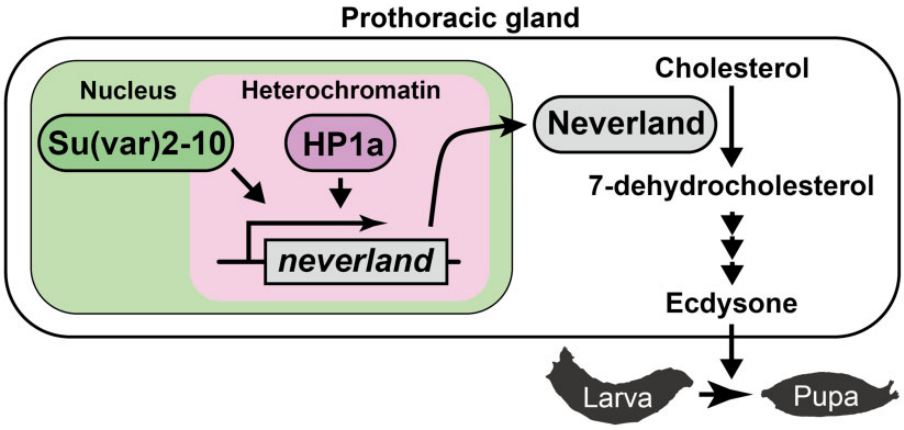

Fig. 6. A model for *Su(var)2-10*- and *Su(var)205*-mediated regulation of *nvd* transcription and developmental transition. *Drosophila* PG expresses a heterochromatin-residing gene, *neverland* (*nvd*), which encodes an enzyme that catalyzes the initial step of ecdysteroid biosynthesis. Our genetic evidence shows that *Su(var)2-10* and *Su(var)205*, encoding Su(var)2-10 and HP1a proteins, respectively, positively regulate the transcription of *neverland* and that *Su(var)2-10*- and *Su(var)205*-mediated upregulation of *neverland* is required for ecdysone biosynthesis and subsequent larval-to-prepupal transition. We propose that Su(var)2-10- and HP1a-dependent regulation of inherent heterochromatin structure is essential for appropriate expression of the *neverland* gene.

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Conflicts of interest

None declared.

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