Ecdysone receptor isoform specific regulation of secretory granule acidification in the larval *Drosophila* salivary gland

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ABSTRACT

Bulk production and release of glue containing secretory granules takes place in the larval salivary gland during *Drosophila* development in order to attach the metamorphosing animal to a dry surface. These granules undergo a maturation process to prepare glue for exocytosis, which includes homotypic fusions to increase the size of granules, vesicle acidification and ion uptake. The steroid hormone 20-hydroxyecdysone is known to be required for the first and last steps of this process: glue synthesis and secretion, respectively. Here we show that the B1 isoform of Ecdysone receptor (EcR), together with its binding partner Ultraspiracle, are also necessary for the maturation of glue granules by promoting their acidification via regulation of Vha55 expression, which encodes an essential subunit of the V-ATPase proton pump. This is antagonized by the EcR-A isoform, overexpression of which decreases EcR-B1 and Vha55 expression and glue granule acidification. Our data shed light on a previously unknown, ecdysone receptor isoform-specific regulation of glue granule maturation.

1. Introduction

The larval salivary gland of Drosophila L3 wandering larvae is a well-established model system to investigate secretory granule biogenesis, maturation and secretion (Biyasheva et al., 2001; Burgess et al., 2011; Costantino et al., 2008; Farkas and Sutáková, 1999; Kamalesh et al., 2021; Ma et al., 2020; Neuman et al., 2021). These glands produce a mucous protein mixture called glue, which contains several Sgs (Salivary gland secretion) glycoproteins. The glue is stored in secretory granules before being secreted (Beckendorf and Kafatos, 1976; Costantino et al., 2008; Lane et al., 1972). During maturation, immature granules fuse with each other, leading to the enlargement of granule size and the decrease of granule number (Farkas and Sutáková, 1999; Lane et al., 1972; Niemeyer and Schwarz, 2000). It was reported that this reduction in granule number can be initiated with 20-hydroxyecdysone treatment (Farkas and Sutáková, 1999). Later, acidification and increase in calcium and chloride ion concentrations take place in the lumen of the granule that eventually leads to the restructuring of the glue content (Syed et al., 2022). Interestingly, a similar process (acidification and chloride ion uptake) was reported to be required for insulin granule

maturation in the pancreas (Barg et al., 2001). An endosomal contribution is needed during the maturation process of granules before secretion (Ma and Brill, 2021; Ma et al., 2020). Just before pupariation, glue is secreted and released from the gland to attach the (pre)pupa to a solid surface upon drying (Beckendorf and Kafatos, 1976). After exocytosis is completed, a few remaining granules can be found in the salivary gland cells, which eventually fuse with lysosomes and are subsequently degraded during a process called crinophagy (Boda et al., 2019; Csizmadia et al., 2018; Weckman et al., 2014).

The periodic change in the titer of the 20-hydroxyecdysone (ecdysone) steroid hormone plays an important role in the development of the *Drosophila melanogaster:* it regulates molting and metamorphosis of the larvae (Baehrecke, 1996; Borst et al., 1974; Riddiford, 1993). Furthermore, it is required for the transcription and assembly of the V-ATPase complex in the fat body of *Bombyx mori* (Dai et al., 2020). The expression of five V-ATPase subunits was reported to decrease in response to the *miR-8–3p* microRNA in *Drosophila melanogaster*. Increased ecdysone signaling downregulated the expression of *miR-8–3p*; thus, the amount of assembled V-ATPase increased, and acidification took place (Lim et al., 2020). Ecdysone response elements were identified in the

promoter of the lysosomal hydrolase cathepsin D in *Bombyx mori*, and 20-hydroxyecdysone was found also to be required for the induction of cathepsin B gene (Gui et al., 2006; Lee et al., 2009; Yu et al., 2012). Taken together, these data raise the possibility that ecdysone signaling may also be involved in secretory granule maturation.

Ecdysone binds to its receptor (EcR) and Ultraspiracle (USP), which together form a heterodimer nuclear receptor (Koelle et al., 1991; Thomas et al., 1993; Yao et al., 1993). EcR and Usp can bind to regulatory sequences of genes that have an ecdysone response element to regulate their expression (Antoniewski et al., 1996; Cherbas et al., 1991; Riddihough and Pelham, 1987; Thomas et al., 1993; Yao et al., 1992). The ecdysone receptor has three different isoforms encoded by the same gene: EcR-A, EcR-B1 and EcR-B2, which are tissue and function specific (Cherbas et al., 2003; Davis et al., 2005; Mouillet et al., 2001; Schubiger et al., 1998; Talbot et al., 1993). These isoforms differ in their N-terminal A/B domains, which regulate isoform specific processes, but all of them have the same DNA-binding and hormone-binding domains (Mouillet et al., 2001; Talbot et al., 1993). It is known that EcR-A is mainly expressed in the imaginal discs, while EcR-B1 is specific for larval tissues, and in midgut, diploid cell islands and histoblast nests (Talbot et al., 1993). In some cases, insect EcR-A and EcR-B1 were shown to function in an antagonistic way (Mouillet et al., 2001; Truman et al., 1994).

Different ecdysone titers play important roles in the salivary gland development (Biyasheva et al., 2001; Ou and King-Jones, 2013). The first ecdysone peak in mid-L3 larval stage induces the expression of the *sgs* genes (Biyasheva et al., 2001; Costantino et al., 2008; Ou and King-Jones, 2013). The next ecdysone peak is larger and is responsible for glue secretion via the EcR/USP heterodimer (Biyasheva et al., 2001; Ou and King-Jones, 2013). After pupation, another ecdysone peak leads to the larval salivary gland histolysis (Biyasheva et al., 2001; Jiang et al., 2000; Ou and King-Jones, 2013). Whether and how ecdysone signaling affects glue granule maturation is still incompletely understood.

2. Materials and methods

2.1. Fly work

The flies were kept in glass tubes on a standard medium that contained cornmeal, sucrose and yeast. The RNA interference stocks for EcR (29374) (FlyBase ID: FBst0029374), Usp (27258) (FlyBase ID: FBst0027258), EcR-B1 (9329) (FlyBase ID: FBst0009329) and EcR-A (9328) (FlyBase ID: FBst0009328), the overexpression lines for EcR-B1 (6469) (FlyBase ID: FBst0006469) (Lee et al., 2000) and EcR-A (6470) (FlyBase ID: FBst0006470) (Lee et al., 2000), the w^{1118} line (3605) (FlyBase ID: FBst0003605) and the Sgs3-GFP reporter (5884) (FlyBase ID: FBst0005884) (Biyasheva et al., 2001) were obtained from the Bloomington Drosophila Stock Center (BDSC), Bloomington, IN, USA. The RNA interference line for EcR (v37058) (FlyBase ID: FBst0461817) was obtained from Vienna Drosophila Resource Center (VDRC), Vienna, Austria. *Sgs3-GFP, Sgs3-dsRed; fkh-Gal4*, the *UAS-GFP-Lamp1, Sgs3-dsRed*; *fkh-Gal4* and the *Sgs3-GFP; fkhGal4* flies were already in our hands (Csizmadia et al., 2018). *Sgs3-dsRed* reporter was originally provided by Andrew Andres, University of Nevada, Las Vegas, NV (Costantino et al., 2008), and the *fkh-Gal4* by Eric Baehrecke, University of Massachusetts Medical School, Worcester, MA (Berry and Baehrecke, 2007).

We examined three different stages of the fruit fly: L3 wandering larvae (−6 h), larvae before puparium formation (BPF, −2 h) and (white) prepupa (PP, 0 h). Late L3 staged larvae that wandered out from the food without returning, while still showed active movements were considered as − 6 h (wandering) staged. This was confirmed during microscopy as glands at this stage do not secrete glue, and granules fill the cytoplasm of the cells. Larvae that already stopped moving were considered as − 2 h BPF. This was confirmed during microscopy as glands at this stage already start secreting glue, thus the lumen expands

and is filled with secreted material. Animals were considered to be 0 h (also known as white) prepupae when puparium formation was complete and the whole animal was still completely white (note that the prepupal case turns brown within 1 h of puparium formation).

2.2. Lysotracker staining, immunhistochemistry and imaging

For imaging of salivary glands, samples were dissected in a 1:9 mixture of PBS and glycerol containing DAPI (4′,6-diamidino-2-phenylindole) and imaged immediately.

For Lysotracker Red staining we dissected the prepupal salivary glands in phosphate-buffered saline (PBS) and incubated the samples in PBTX-DOC (0.05% Triton X-100% and 0.025% sodium deoxycholate in PBS) for a few seconds. Samples were then washed with PBS four times and incubated in Lysotracker Red (Invitrogen) diluted in PBS (1:2000) for two minutes. Then samples were washed 5 times in PBS and covered in a 1:9 mixture of PBS and glycerol containing DAPI.

For immunohistochemistry, salivary glands were dissected in PBS. Samples were then incubated for 30 min in 4% paraformaldehyde in PBS at room temperature. After that salivary glands were washed four times in PBS and incubated 2 × 15 min in PBS. Then the samples were incubated in 0,1% PBTX solution for 10 min and in 10% goat serum-containing PBTX for 30 min. Salivary glands were transferred to the primary antibody solution diluted in 10% goat serum–containing PBTX and incubated at 4 °C for 2 days. We next washed the samples in PBTX and incubated them 2 × 15 min in PBTX. Next, salivary glands were incubated in 10% goat serum-containing PBTX for 30 min, followed by the secondary antibody solution diluted in 10% goat serum-containing PBTX for 4 h. Samples were then rinsed 3 times in PBTX and incubated in DAPI containing PBTX. The samples were rinsed in PBTX and washed for 2 × 15 min in PBTX. The salivary glands were then rinsed 3 times in PBS and incubated for 2 × 15 min in PBS. Finally, samples were mounted in Vectashield (Vector Laboratories).

The following antibodies were used: monoclonal mouse anti-EcR-B1 (1:10) (DSHB, AD4.4), monoclonal mouse anti-EcR-A (1:5) (DSHB, 15G1a), polyclonal rabbit anti-Vha55 (1:100) (A322, (Pyza et al., 2004)) Alexa Fluor 568-conjugated anti-mouse (1:1000) (Invitrogen) and Alexa Fluor 488-conjugated anti-rabbit (1:1000) (Invitrogen).

Fluorescent images were captured with an AxioImager M2 microscope (Carl Zeiss), equipped with an Apotome2 grid confocal unit (Carl Zeiss). We used EC Plan-Neofluar 10x/0.3 Air, EC Plan-Neofluar 20x/0.5 Air and EC Plan-Apochromat 40x/0.95 Air objectives, an Orca Flash 4.0 LT sCMOS camera (Hamamatsu Photonics) and ZEN 2.3 software (Carl Zeiss). Images were processed in ZEN 2.3 (Carl Zeiss), Microsoft PowerPoint and Photoshop CS and CS5 (Adobe) were used for creating the graphical abstract and the figures.

2.3. Electron microscopy and acid phosphatase cytochemistry

For electron microscopy, dissected prepupal salivary glands were fixed in 3.2% paraformaldehyde, 1% glutaraldehyde, 1% sucrose and 0.003 M CaCl$_2$ containing 0.1 M sodium cacodylate buffer (pH 7.4) (overnight at 4 °C). The samples were postfixed in 0.5% osmium tetroxide (1 h) and incubated in half-saturated aqueous uranyl acetate (30 mins). Next, specimens were dehydrated in a graded series of ethanol and embedded into TAAB 812 Resin Kit (T024) (Taab), according to the manufacturer's recommendations. The 70 nm sections of the specimens were stained in Reynold's lead citrate.

For acid phosphatase cytochemistry, salivary glands of larvae shortly before puparium formation were fixed with 2% paraformaldehyde, 2% glutaraldehyde, 1% sucrose and 0.003 M CaCl$_2$ containing 0.1 N sodium cacodylate buffer (pH 7.4) overnight at 4 °C. Next, acid phosphatase cytochemistry was performed as described before (Lőrincz et al., 2014).

Electron micrographs were taken with a JEOL JEM-1011 transmission electron microscope equipped with a Morada camera (Olympus) and iTEM software (Olympus).

2.4. Statistics

Every light microscopy experiment was repeated at least 2 times and a minimum of 5 independent salivary glands were examined.

Fluorescence images were evaluated by the same person using either ImageJ software (National Institutes of Health, Bethesda, MD, USA) or the structures were counted manually. For the experiments in Figs. 1 and 4, 30 cells from 5 independent salivary glands (6 cells/ salivary gland) were examined. For experiments shown in Figs. 2 and 5, 10 granules/ cells were counted from 5 independent salivary glands (2 cells/ salivary gland). Both the cells and granules were selected randomly from original single focal planes of images. The colocalization of Sgs3-dsRed and GFP-Lamp1 was manually quantified. We defined colocalization of Sgs3-dsRed and Sgs3-GFP by calculating Pearson's coefficients using the Coloc 2 plugin of ImageJ (1 means perfect colocalization, 0 means incidental or no colocalization, and −1 means mutually exclusive localization).

We imported the data to SPSS Statistics 28 software (IBM) for testing the normality of data distribution. The p values were calculated with the proper statistical tests: for pairwise comparison of datasets where at least one showed non-normal distribution, or two normal distribution datasets, Mann-Whitney (U) test (Fig. 4), or T-test (Fig. 7) was used, respectively. For comparison of multiple samples that included at least one variable with non-Gaussian data distribution, Kruskal-Wallis test (Figs. 1 and 4) was used. For generating plots, we used Excel (Microsoft) and Photoshop CS5 (Adobe).

3. Results

3.1. Ecdysone receptor and Ultraspiracle are required for acidification of secretory granules

The *Drosophila* Ecdysone receptor (EcR) and its binding partner Ultraspiracle (Usp) are necessary for glue granule secretion (Biyasheva et al., 2001). It is also known that the number of the granules decreases and the granule size increases in ecdysone treated salivary gland cells (Farkas and Sutáková, 1999). These data led us to investigate the possible role of EcR and Usp in secretory granule maturation. We used *Sgs3-GFP, Sgs3-dsRed; fkhGal4* flies (Csizmadia et al., 2018) to investigate glue granule acidification, which is an important step of their maturation (Syed et al., 2022). These flies express both a GFP- and a dsRed-tagged form of Sgs3 glue protein controlled by the genomic promoter of Sgs3 (Biyasheva et al., 2001; Costantino et al., 2008; Csizmadia et al., 2018). The genome of these flies also contains a forkhead-Gal4 (fkhGal4) transcriptional activator transgene, which allows us to express UAS-driven transgenes such as RNAi constructs in the salivary glands (Csizmadia et al., 2018). Most of the GFP signal is quenched due to secretory granule acidification in control prepupal
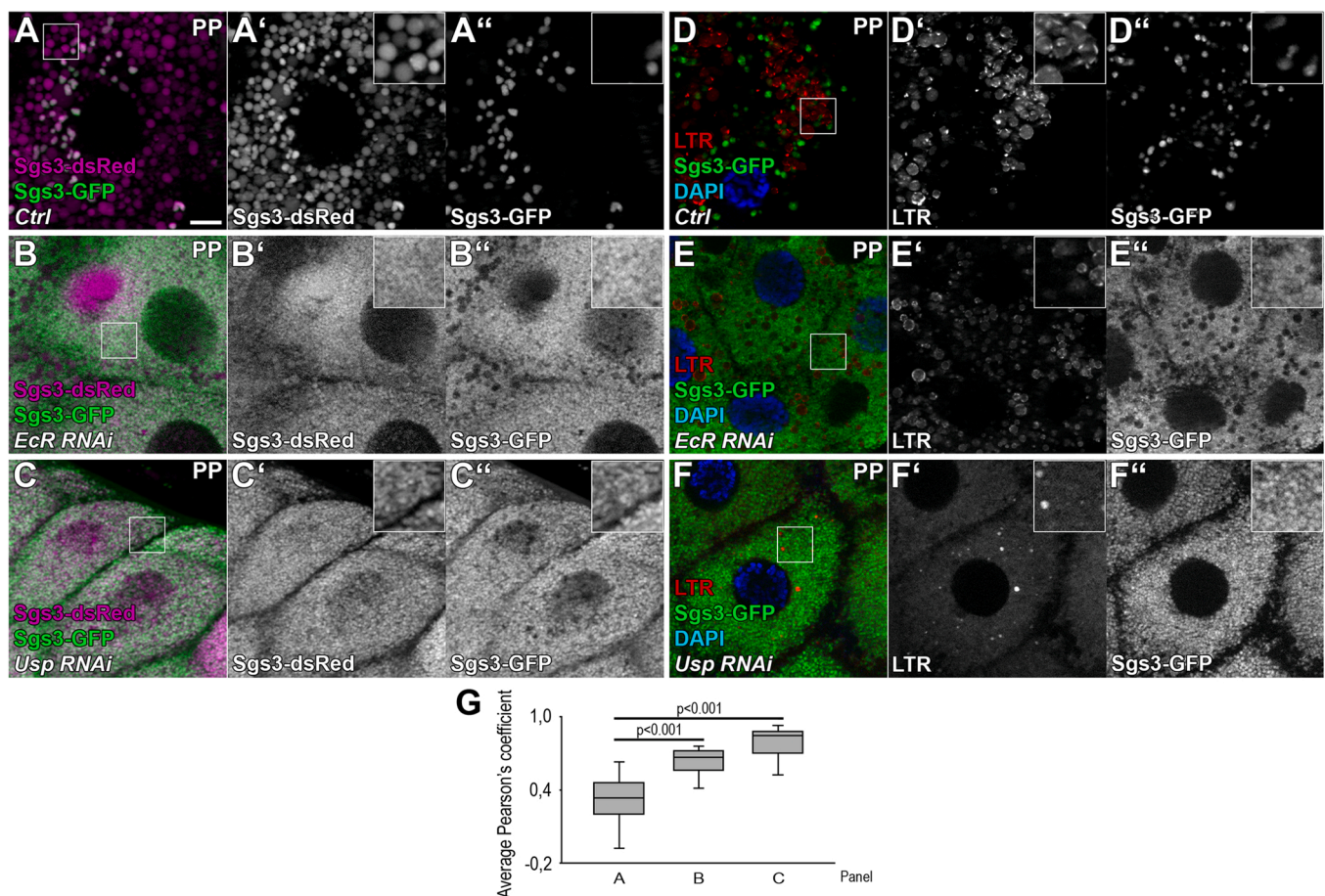
Fig. 1. EcR and Usp are necessary for glue granule acidification. A-–C: Most secretory granules lose the GFP but not dsRed signal due to acidification in control prepupal salivary gland cells expressing Sgs3-GFP and Sgs3-dsRed (A). In contrast, most granules of EcR or Usp RNAi cells retain the Sgs3-GFP signal (B-C). D–F: Several Lysotracker Red positive and GFP-negative structures and just a few GFP-only granules can be observed in control salivary gland cells of prepupae expressing Sgs3-GFP (D). Most of the GFP signal remains detectable in the secretory granules in EcR or Usp RNAi cells. (E-F). The insets show Sgs3-dsRed/LTR and Sgs3-GFP channels and are enlarged from the boxed areas of the corresponding main panels. G: Quantification of the data shown in panels A-C, 30 cells were investigated from 5 independent salivary glands (6 cells/salivary gland) and every experiment was repeated at least twice. The box plot shows the data ranging between lower and upper quartiles; the medians are indicated as a horizontal black line in the boxes. The numbers above the lines show p values, and error bars denote SE. Scale bar in panel A, 20 μm (A-F). Ctrl: control, PP: prepupa, LTR: Lysotracker Red.
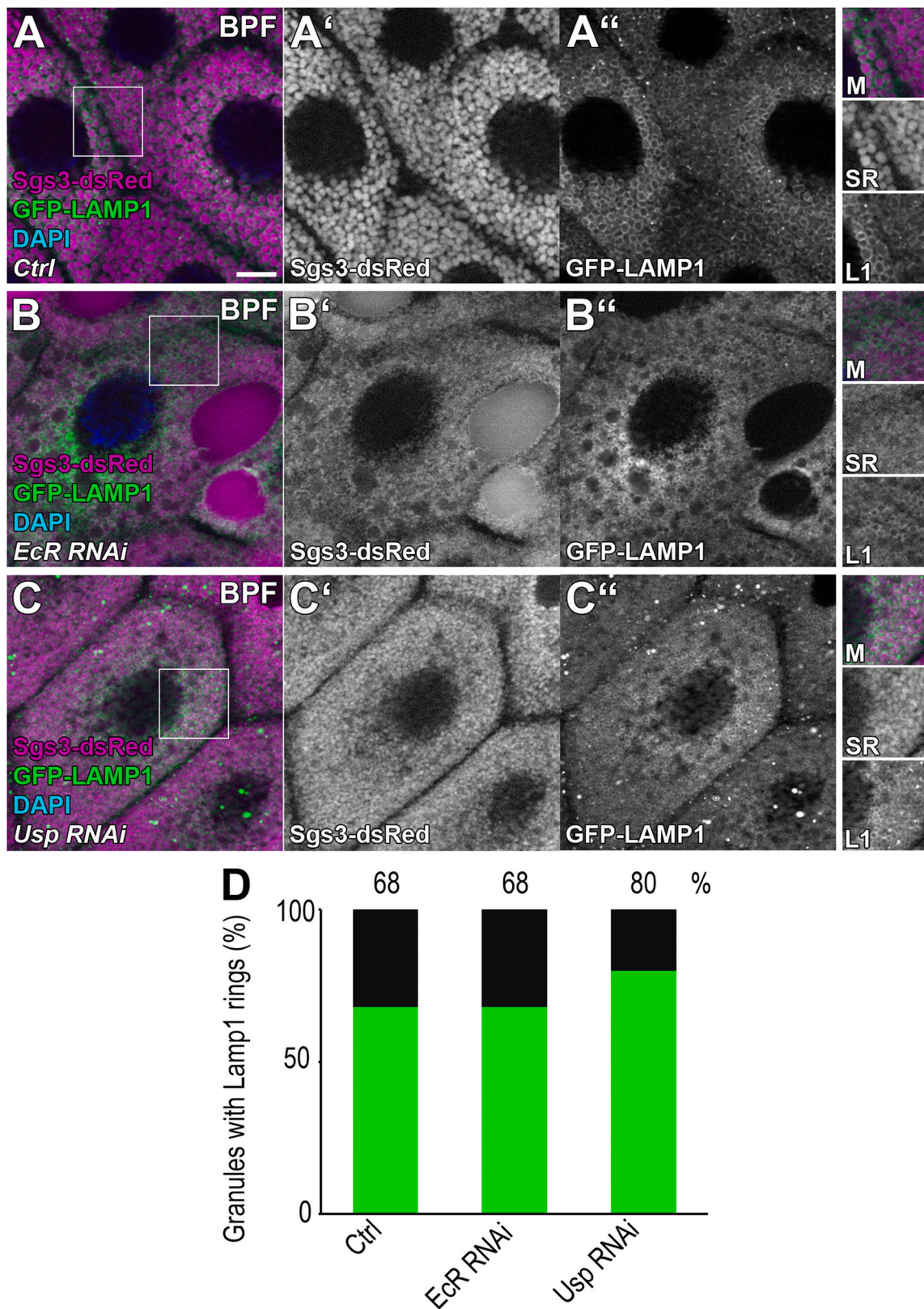
Fig. 2. EcR and Usp are dispensable for secretory granule-lysosome fusion. A–C: GFP-Lamp1 rings can be detected around the Sgs3-dsRed positive glue granules shortly before puparium formation in control larval salivary gland cells (A), as well as in EcR (B) or Usp (C) RNAi cells. D: Quantification of the data shown in panels A–D. 100 granules were investigated (10 granules/cell) from 5 independent salivary glands (2 cells/salivary gland). Every experiment was repeated at least twice. The insets show merged images (top), Sgs3-dsRed (middle) and GFP-LAMP1 (bottom) channels and are enlarged from the boxed areas of the corresponding main panels. Scale bar in panel A, 20 μm (A-C). Ctrl: control, BPF: before puparium formation, M: Merged, SR: Sgs3-dsRed, L1: GFP-LAMP1.

salivary glands, whilst the dsRed signal remains detectable (Fig. 1A, G). The dsRed positive structures may be either mature granules or granules undergoing degradation (crinosomes). We found that upon silencing of EcR, most of the GFP signal remained detectable in glue granules, indicating that the granule contents were not secreted from the prepupal salivary gland cells (Fig. 1B, G). This suggests that acidification of the granules cannot take place normally if EcR is knocked down. We also used this system for investigating the knockdown of Usp, which resulted in a similar phenotype to that of EcR RNAi (Fig. 1C, G). Interestingly, a lot of smaller than control granules were detected both in Usp RNAi and EcR RNAi glands (Fig. 1A–C).

Next Lysotracker Red staining was carried out on prepupal salivary glands that expressed Sgs3-GFP and EcR or Usp RNAi-s. We observed diminished GFP signal in controls, while large Lysotracker Red positive structures (Fig. 1D), likely mature granules or crinosomes, appeared suggesting that acidification took place normally. In contrast, both EcR and Usp knockdowns decreased the ratio of acidic granules concomitant with more granules remaining positive for GFP (Fig. 1E–F).

3.2. Ecdysone receptor and Ultraspiracle are dispensable for lysosome-secretory granule fusion

The crosstalk of the secretory system with the endolysosomal compartment is required for secretory granule maturation (Ma and Brill, 2021; Ma et al., 2020). Thus, we investigated whether the decreased acidification of granules upon loss of EcR or Usp is a consequence of an endosome/lysosome-secretory granule fusion defect. For this, we used *Sgs3-dsRed, UAS-GFP-Lamp1; fkhGal4* flies (Csizmadia et al., 2018; Pulipparacharuvil et al., 2005). Secretory granules fuse with endosomes or lysosomes shortly before pupariation, which can be detected as GFP-Lamp1 positive circles surrounding the dsRed positive glue granules in control cells (Fig. 2A, D). We found that secretory granule-lysosome fusion proceeds normally in both EcR and Usp RNAi cells, as GFP positive circles were seen around the dsRed positive granules (Fig. 2B–D).

3.3. Ecdysone receptor and Ultraspiracle dependent acidification is important for glue granule maturation

Acidification has an important role in secretory granule maturation in the *Drosophila melanogaster* salivary glands (Syed et al., 2022). This led us to investigate whether EcR and Usp are required for glue granule maturation. We performed ultrastructural analysis of prepupal salivary glands undergoing EcR or Usp RNAi. Glue could be observed in the lumen of control salivary glands, indicating that most of the granules had been already released and secreted. Only a few granule-like structures remained in the cytoplasm, which could represent mature granules or crinosomes (Fig. 3A). On the contrary, the EcR or Usp silenced cells contained a large amount of small immature granules (Fig. 3B, C). Some crinosomes and multivesicular bodies were also detected in the cytoplasm, which likely correspond to the few Lysotracker Red positive acidic structures. These data indicate that EcR and Usp dependent acidification is required for the maturation of secretory granules.

3.4. Acidification of secretory granules requires the B1 isoform of EcR

Based on the existence of different EcR isoforms with different functions, we decided to investigate which of the EcR isoforms may be responsible for the acidification of glue granules using EcR-A and -B1 isoform specific RNAi lines. First, we used the *Sgs3-dsRed* and *Sgs3-GFP* reporters in prepupal salivary glands and found that depletion of EcR-B1, but not EcR-A, causes an acidification defect (Fig. 4A-C, K). The potential antagonism of EcR isoforms gave us motivation to test the effect of EcR-A overexpression. Strikingly, we found that most of the GFP signal was still detectable in the prepupal salivary gland cells in these animals (Fig. 4D, K), indicating that EcR-B1 mediated acidification is suppressed by EcR-A overexpression. In contrast, acidification took place in glands overexpressing the EcR-B1 isoform (Fig. 4E, K). To test whether EcR-B1 is key for acidification, we examined EcR-B1 overexpression in an earlier stage (wandering 3rd stage larvae) in which granules are not yet acidified. Indeed, we found more dsRed positive-only granules in EcR-B1 overexpressing cells than in controls (Fig. 4F, G, L), further supporting that EcR-B1 induces, and EcR-A suppresses, the acidification of glue granules. This was also confirmed by Lysotracker Red staining (Fig. 4H–J).

3.5. Neither EcR isoforms are required for lysosome-secretory granule fusion

To confirm the lack of an effect of pan-EcR RNAi on secretory granule-lysosome fusion we also tested EcR isoforms, again using *Sgs3-dsRed, UAS-GFP-Lamp1* flies. Neither EcR-B1 knockdown, nor EcR-A overexpression had any effect on secretory granule-lysosome fusion as in all cases GFP circles were detected around the dsRed positive granules, similarly to controls (Fig. 5A–C, F). Since we previously observed premature acidification in EcR-B1 overexpressing cells, we tested
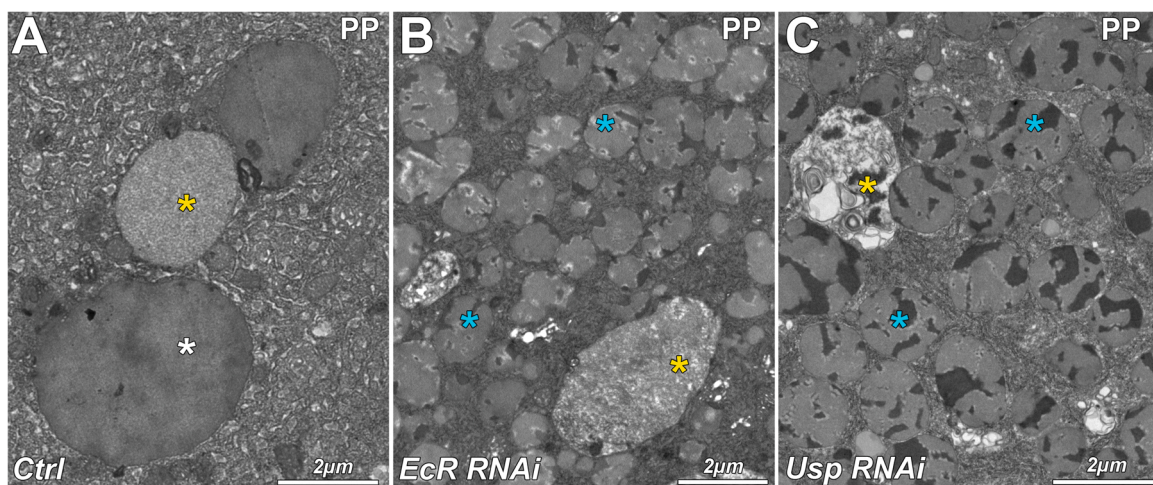

Fig. 3. EcR and Usp are required for glue granule maturation. A–C: Only a few mature electron-dense granules (white asterisk) and electron-lucent crinosomes with loose luminal content (yellow asterisk) can be found in the prepupal salivary gland cells of control animals (A). However, most secretory granules of EcR (B) or Usp (C) RNAi cells seem still immature at this stage as their content seems heterogenous and intact (cyan asterisks). Crinosomes could be also observed (yellow asterisks). Ctrl: control, PP: prepupa.
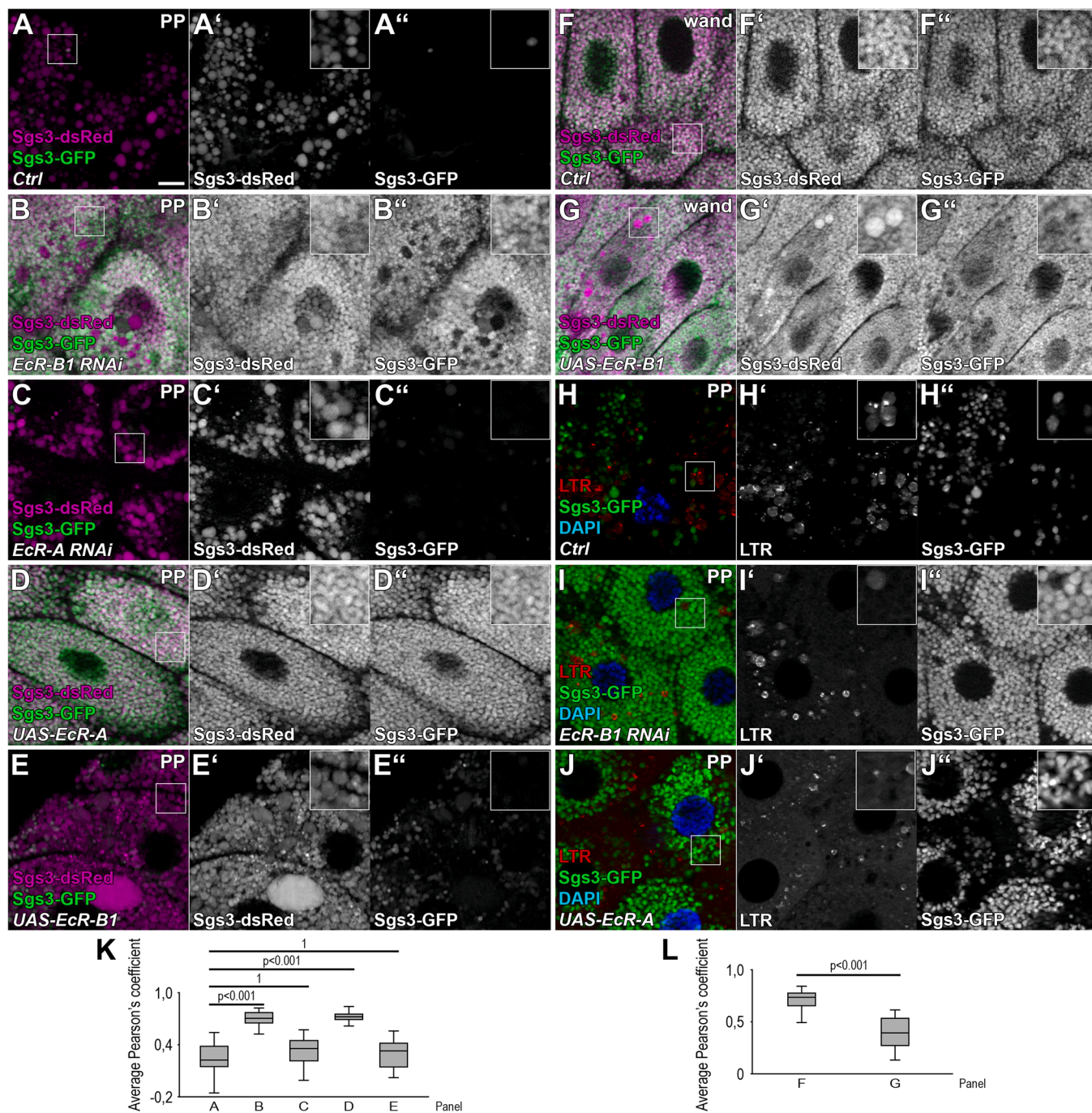
Fig. 4. EcR isoform specific regulation of glue granule acidification. A–E: Sgs3-GFP signal gradually disappears during acidification and only the Sgs3-dsRed signal remains visible in the granules of control prepupal salivary gland cells (A). In contrast, both GFP and dsRed signals can be detected in EcR-B1 RNAi cells (B). GFP signal is quenched in EcR-A knockdown cells, similar to controls (C). Overexpression of EcR-A results in the failure of acidification based on persisting Sgs3-GFP signal in the granules (D). GFP signal is quenched in most granules in EcR-B1 overexpressing cells (E). F–G: Both the Sgs3-GFP and Sgs3-dsRed signals are detectable in the secretory granules of salivary gland cells of control wandering (wand) 3rd instar larvae (F). Upon EcR-B1 overexpression, some granules lose their GFP fluorescence indicating premature acidification (G). H–J: GFP signal is lost from most granules, while Lysotracker Red positive structures simultaneously appear in the salivary gland cells of control prepupae, indicating ongoing maturation (H). However, most of the granules remain positive for GFP and fewer Lysotracker Red positive structures can be detected in EcR-B1 RNAi or EcR-A overexpressing cells, (I–J). The insets are enlarged from the boxed areas of the corresponding main panels and show Sgs3-dsRed/LTR and Sgs3-GFP channels. K-L: Quantification of colocalization data shown in panels A-E and F-G, respectively. 30 cells were investigated from 5 independent salivary glands (6 cells/salivary gland) and every experiment was repeated at least twice. The box plots show data ranging between lower and upper quartiles; the medians are indicated as a horizontal black line in the boxes. P values are also shown, and error bars denote SE. Scale bar in panel A, 20 μm (A-J). Ctrl: control, LTR: Lysotracker Red, PP: prepupa, wand: wandering larva.
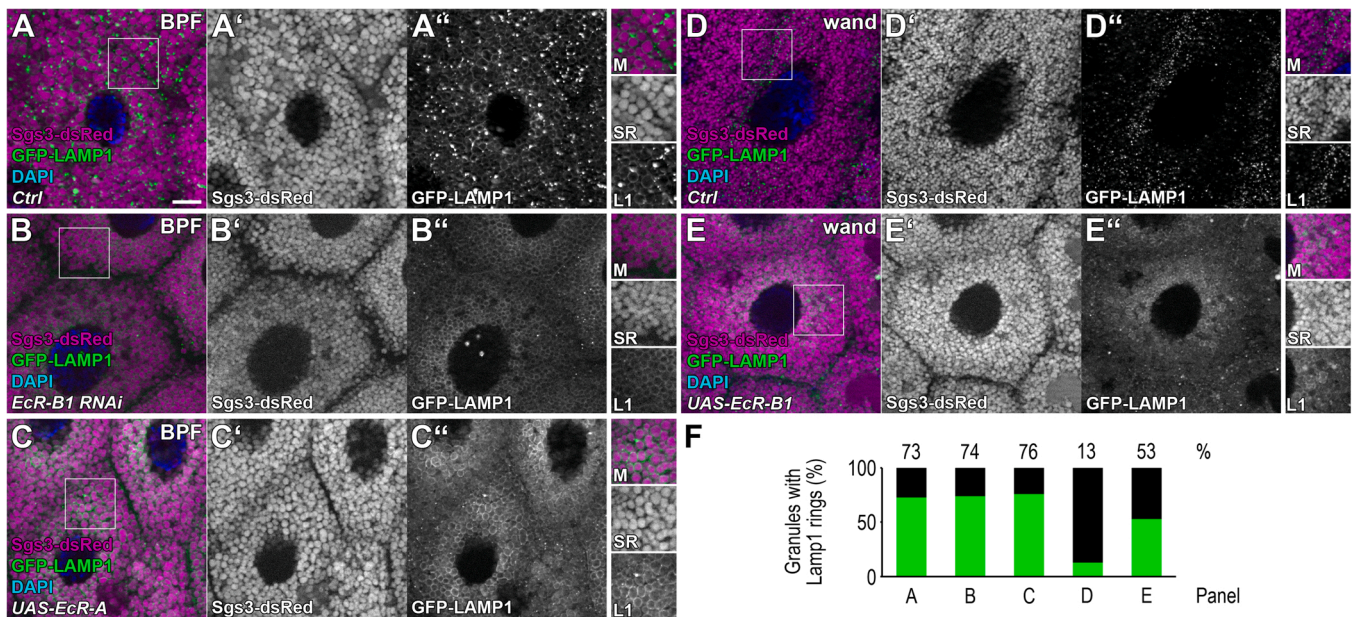
Fig. 5. EcR isoforms are dispensable for secretory granule-lysosome fusion. A–C: Sgs3-dsRed positive granules acquire GFP-LAMP1 in the salivary gland cells of larvae shortly before pupariation (A). GFP positive rings around the granules are also detected in EcR-B1 silenced or EcR-A overexpressing cells (B-C). D–E: DsRed positive granules lack GFP-Lamp1 in the salivary gland cells of control wandering larvae (D). EcR-B1 overexpression results in the early appearance of GFP-LAMP1 rings around the Sgs3-dsRed granules in wandering animals (E). F: Quantification of data shown in panels A-E. 100 granules were investigated (10 granules/cell) from 5 independent salivary glands (2 cells/salivary gland). Every experiment was repeated at least twice. The insets show merged images (top), Sgs3-dsRed (middle) and GFP-LAMP1 (bottom) channels and are enlarged from the boxed areas of the corresponding main panels. Scale bar in panel A, 20 μm (A-E). Ctrl: control, BPF: before puparium formation, wand: wandering larva, M: merge, SR: Sgs3-dsRed, L1: GFP-LAMP1.

whether this is a result of early fusion events between lysosomes and secretory granules. This was indeed the case, as GFP-Lamp1 rings were detected around dsRed positive granules, unlike in control cells in which no GFP rings were observed (Fig. 5D–F). EcR-B1 and A isoforms thus appear to be dispensable for lysosome-secretory granule fusion, while EcR-B1 overexpression could accelerate secretory granule-lysosome fusion.

3.6. EcR-B1 controls expression of the Vha55 V- ATPase subunit

It was suggested that ecdysone signaling is necessary for the expression of five V-ATPase subunits (Lim et al., 2020), which could potentially explain the degradation defects we observed in EcR-B1 RNAi or EcR-A overexpressing cells. Thus, we examined the expression of the EcR isoforms and the V-ATPase subunit Vha55 using immunohisto-chemistry with EcR-A, EcR-B1 and Vha55 antibodies in salivary glands shortly before puparium formation. In control cells EcR-B1 isoform can be found in the nucleus, and Vha55 can be detected in the cytoplasm of the cells (Fig. 6A). In EcR-B1 silenced cells not only EcR-B1 but also Vha55 disappeared, in contrast to EcR-A knockdown cells that resembled control cells (Fig. 6B-C). EcR-B1 overexpression had no obvious effect on Vha55 expression, while EcR-A overexpression caused a striking decrease in both EcR-B1 and Vha55 levels (Fig. 6D-E). These results indicate that knockdown of EcR-B1 or overexpression of EcR-A decreases EcR-B1 expression in the cells, which leads to decreased expression of Vha55, confirming the idea that appropriate level of the EcR-B1 isoform is needed for the expression of V-ATPase to promote glue granule acidification.

We also investigated the expression of the EcR-A isoform and found that a weak nuclear EcR-A signal can be observed in controls and EcR-B1 RNAi cells (Fig. S1A, B), and it was lost in EcR-A RNAi cells (Fig. S1C). The weak nuclear signal of EcR-A was also seen in EcR-B1 over-expressing cells, while the nuclear signal became more prominent upon EcR-A overexpression (Fig. S1D, E), as expected.

3.7. The EcR-B1 isoform is necessary for ultrastructural changes during glue granule maturation

Since silencing of EcR-B1 and overexpression of EcR-A resulted in defective acidification of glue granules, we hypothesized that these also affect granule maturation. Electron microscopy experiments were performed to test this. Unlike controls (Fig. 7A), most of the granules were still immature in both EcR-B1 RNAi and EcR-A overexpressing cells as their contents remained compact (Fig. 7B, C). These results show that EcR-B1 mediated acidification is important for the proper maturation of glue granules and that EcR-A antagonizes this process.

It was previously reported that EcR is necessary for the expression of lysosomal cathepsins in *Bombyx mori* (Gui et al., 2006; Lee et al., 2009; Yu et al., 2012). We thus investigated if silencing of the EcR-B1 isoform results in any alterations in the transport of lysosomal enzymes. We tested this by performing acid phosphatase cytochemistry on the sali-vary glands of larvae shortly before pupariation. The electron dense acid phosphatase reaction product can be detected in the glue granules of both control and EcR-B1 RNAi cells, indicating the ongoing transport of lysosomal enzymes towards the maturing granules (Fig. 7D, E). This is in line with our previous observations that EcR-B1 is dispensable for secretory granule-lysosome fusion (thus the transport of lysosomal en-zymes) in salivary gland cells.

4. Discussion

The larval salivary gland of *Drosophila melanogaster* is an excellent model to investigate the biogenesis, maturation and exocytosis of secretory granules, while many steps of these processes are still unclear (Biyasheva et al., 2001; Burgess et al., 2011; Costantino et al., 2008; Farkas and Sutáková, 1999; Kamalesh et al., 2021; Ma et al., 2020; Neuman et al., 2021). Glue granules contain special glycoproteins (the Sgs proteins), which are later secreted into the lumen of the salivary glands (Beckendorf and Kafatos, 1976; Biyasheva et al., 2001; Cos-tantino et al., 2008). Granules undergo a maturation process before
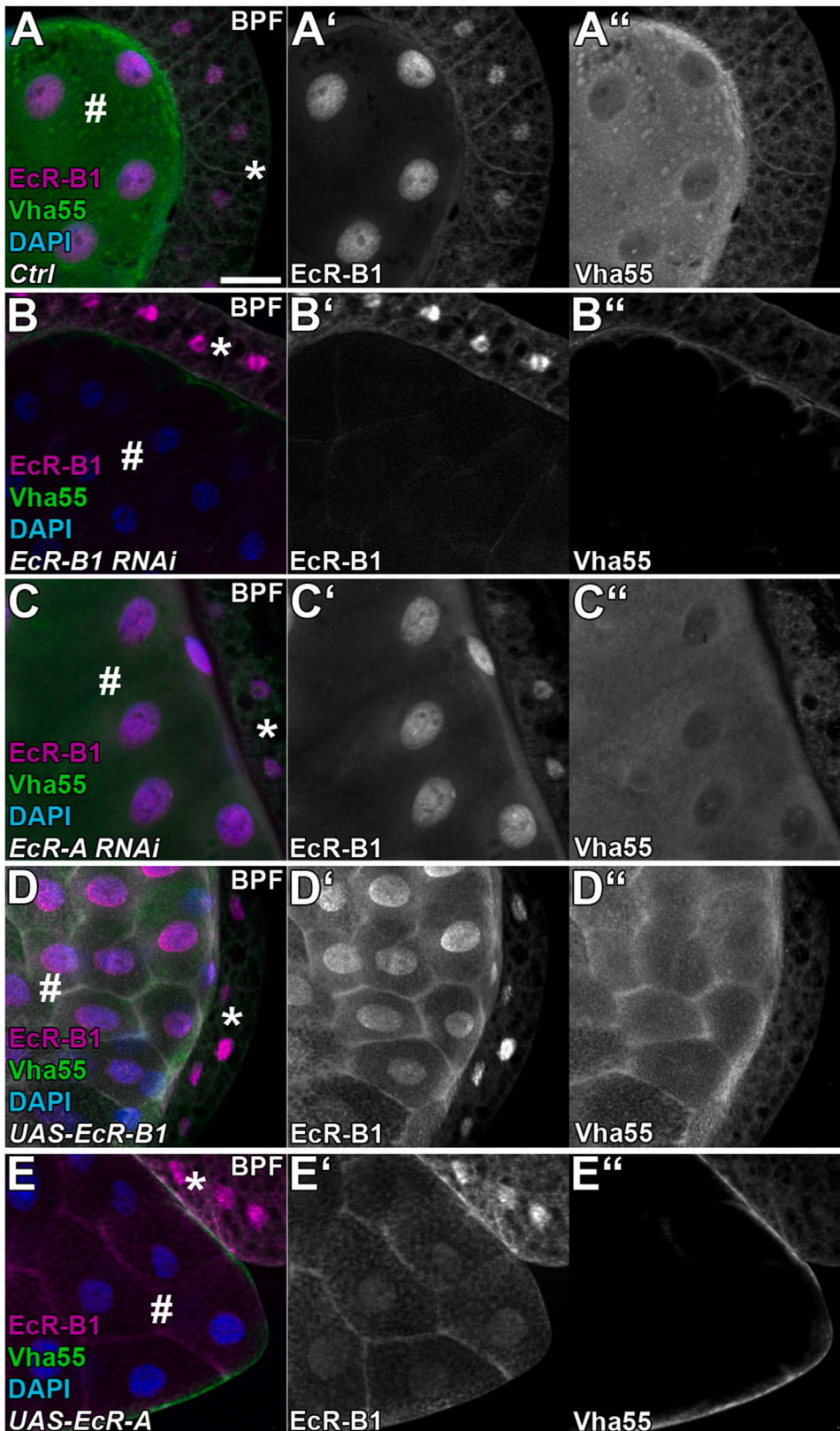
Fig. 6. EcR-B1 is necessary for expression of the vacuolar ATPase subunit Vha55. A–E: Nuclear EcR-B1 and cytosolic Vha55 is clearly seen in control salivary gland cells of larvae before puparium formation (A). Salivary gland specific EcR-B1 knockdown diminishes not only EcR-B1 but also Vha55 expression, which are still detectable in the neighboring fat cells that are unaffected by the RNAi (B). Nuclear EcR-B1 and cytosolic Vha55 can be still detected in EcR-A RNAi glands (C). Higher levels of both proteins are seen in cells overexpressing EcR-B1 (D). Importantly, EcR-A overexpression decreases both EcR-B1 and Vha55 levels (E). Scale bar in panel A, 50 μm (A-E). Ctrl: control, BPF: before puparium formation. Hash signs (#) mark salivary glands and asterisks indicate the attached fat bodies, the latter serving as staining control in all panels.

being secreted, which includes homotypic and endolysosomal fusion events, acidification, and calcium and chloride ion uptake (Farkas and Sutáková, 1999; Lane et al., 1972; Ma et al., 2020; Ma and Brill, 2021; Niemeyer and Schwarz, 2000; Syed et al., 2022).

The steroid hormone 20-hydroxyecdysone is an important regulator of *Drosophila* development (Baehrecke, 1996; Borst et al., 1974; Riddiford, 1993). This hormone can bind to the nuclear ecdysone receptor and its binding partner Ultraspiracle to mediate the transcription of multiple genes, and that ecdysone signaling is important for the secretion of the mucin content of the glue granules (Biyasheva et al., 2001;
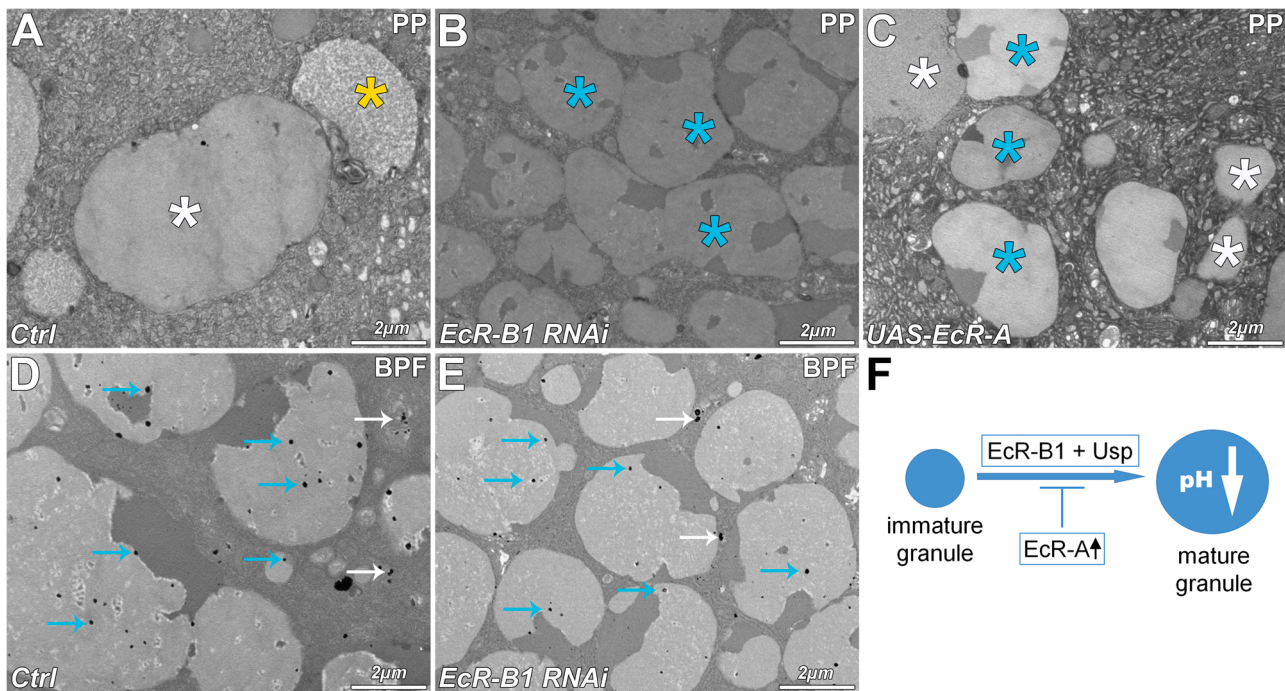
Fig. 7. EcR isoforms regulate the maturation of glue granules, and they are dispensable for the transport of acid phosphatase to granules. A–C: Electron-dense, compact, mature secretory granules (white asterisk) and electron-lucent crinosomes (yellow asterisk) are seen in salivary gland cells of control prepupae (A). Maturation of the granules is impaired in EcR-B1 RNAi (B) or EcR-A overexpressing (C) cells as most of the granules (cyan asterisks) seem immature based on their heterogenous content. **D-E:** Acid phosphatase reaction product (electron dense, black deposits) can be detected in the secretory granules (cyan arrows) and lysosomes (white arrows) in salivary gland cells of both control and EcR-B1 RNAi larvae shortly before pupation (D, E). **F:** Model of ecdysone signaling regulation of secretory granule maturation. EcR-B1 and Usp are required for glue secretory granule maturation by regulating acidification, which is antagonized by EcR-A. Ctrl: control, BPF: before puparium formation, PP: prepupa.

Koelle et al., 1991; Thomas et al., 1993; Yao et al., 1993). However, the potential role of ecdysone signaling in secretory granule maturation was still unclear.

In this study we investigated the possible role of ecdysone signaling in the maturation of glue secretory granules in the larval salivary gland of *Drosophila melanogaster*. We found that knockdown of all EcR isoforms or only of EcR-B1 inhibits granule acidification similar to Usp knockdown, and thus the maturation of secretory granules is impaired. This acidification failure is not a consequence of a lack of lysosomal fusion events, as neither EcR nor Usp knockdown inhibits the fusion of lysosomes with secretory granules. Interestingly, these RNAi experiments did not prevent glue granule biogenesis, which is also an ecdysone-dependent process, indicating partial loss-of-function (which allowed us to investigate the role of these factors in glue granule maturation). EcR-A and EcR-B1 were suggested to function in an antagonistic way (Mouillet et al., 2001; Truman et al., 1994). In line with this, we observed that overexpression of EcR-A leads to the decreased expression of EcR-B1, leading to inhibited secretory granule maturation.

As neither EcR-B1 nor Usp RNAi had any effect on secretory granule-lysosome fusion or lysosomal enzyme transport, we suggest that the expression of genes required for acidification is dependent on isoform specific EcR signaling. It is known that ecdysone signaling is necessary for the expression and assembly of V-ATPase subunits in *Drosophila* and other insects (Dai et al., 2020; Gui et al., 2006; Lee et al., 2009; Lim et al., 2020; Yu et al., 2012). Accordingly, we observed decreased Vha55 expression in EcR-B1 RNAi or EcR-A overexpressing cells. This pinpoints the importance of the EcR-B1 mediated signaling in late stages of secretory granule maturation.

Taken together, our data identified a new role for ecdysone signaling in secretory granule maturation: glue granule acidification and the concomitant ultrastructural changes associated with their maturation depends on the heterodimer EcR-B1/Usp steroid receptor, and this process is inhibited by EcR-A overexpression (Fig. 7F).

CRediT authorship contribution statement

Csizmadia: Conceptualization, Validation, Project administration. Low: Conceptualization, Writing – review & editing, Supervision, Project administration. Lorincz: Conceptualization, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration. Nagy: Conceptualization, Investigation, Formal analysis, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration. Szenci: Investigation, Writing – review & editing. Boda: Investigation, Formal analysis, Writing – original draft, Writing – review & editing. Al-Lami: Investigation. Juhasz: Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that there is no conflict of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ejcb.2022.151279.

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