The role of ubiquitin in the secretory granule crinophagic degradation in *Drosophila* and Examination of developmental program-independent secretory granule degradation in *Drosophila*

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Background

After the burst of secretion, the unnecessary or damaged secretory granules instead of their exocytosis remain in the cytoplasm and directly fuse with late endosomes or lysosomes (crinophagy) for the fast degradation and recycling of the secretory cargo (Smith and Farquhar 1966, Csizmadia et al. 2018, Szenci, Csizmadia and Juhász 2023). During our work with the PD OTKA funding, we have answered the interesting question that, what may be the molecular background that determines secretory granule fate for degradation. The salivary gland cells of the fruit flies can exactly recognize which of the unreleased or low-quality secretory granules to degrade.

In our experiments we have identified ubiquitin on the surface of glue granules in the late larval salivary gland cells of *Drosophila*, when the developmentally programmed crinophagy is naturally activated in these cells (Csizmadia et al. 2018). Therefore, we made a genetic screen, which involved the main components of the ubiquitination system (E3 ubiquitin ligases and deubiquitinating enzymes), and we have discovered new regulators of crinophagy: the E3 ubiquitin ligase Cnot4 and the deubiquitinating enzyme Usp7, which have equivalent role in the mechanism of glue granule-lysosome fusion based on our loss of function analyses.

Importantly, we have also discovered that, these enzymes are recruited to the surface of glue granules, when the developmentally programmed crinophagy is activated in the salivary gland cells. Moreover, we examined the possible type of the earlier identified ubiquitin on glue granules, and we showed with immunohistochemical analysis that, the K63-linked polyubiquitin that is presented on glue granules and may be involved in their crinophagic degradation. Interestingly, the K63-linked polyubiquitin has equivalent role in several vesicular trafficking pathways and also in other types of autophagic processes (Dósa and Csizmadia 2022).

It is important to note that, our manuscript containing data about the role of ubiquitin in crinophagy is under preparation for publication.

Unexpectedly, we have discovered a special glue granule crinophagic degradation process, which operates too early in the larval development, independently of the developmental program, when the retrograde transport from endosome-to-TGN is perturbed in the cells. Interestingly, hindering of endosome-to-TGN retrograde transport in these cells causes abnormally small glue granules which are not able to fuse with each other (Ma et al. 2020). We continued our examinations, because the new data were very interesting, and this topic seemed suitable for rapid publication. In our paper with the PD OTKA funding, we showed that loss of function of the SNARE genes *syntaxin 16* (*syx16*) and *synaptobrevin* (*syb*), the genes of the small GTPase Rab6 and the GARP tethering complex members Vps53 and Scattered (Vps54) all involved in retrograde transport caused intense early degradation of immature glue granules via crinophagy independently of the developmental program. Moreover, silencing of these genes also provoked secretory failure and accelerated crinophagy during larval development (Csizmadia et al. 2022).

On the other hand, we have discovered Rab26 and the ecdysone receptor isoform specific regulation of secretory granule maturation and acidification in the larval salivary gland of *Drosophila* (Nagy et al. 2022, Boda et al. 2023).

Importantly, our new observations strongly contribute to the better understanding of the molecular background of targeting unnecessary or low-grade secretory granules for crinophagic degradation and may help to find an answer how this process is activated in the larval salivary gland cells of *Drosophila* (Csizmadia et al. 2022).

Results 1.: The role of ubiquitin in the secretory granule crinophagic degradation in *Drosophila*

In our first experiment we have identified developmentally induced ubiquitin recruitment to the surface of glue granules overexpressing GFP-tagged ubiquitin (Ub) specifically in the late larval salivary gland cells of *Drosophila* with fluorescent microscopy and biochemical analysis. Importantly, endogenous ubiquitin was also detected by us on the surface of glue granules, when the developmentally programmed crinophagy is activated in these cells (**Figure 1. A-C** and **H**). It is important to note that, ubiquitin is recruited to the surface of glue granules before their crinophagic degradation (**Figure 1. E**).

In the next part of our results (Results 2.), we show the newly discovered developmental program-independent crinophagic degradation of glue granules (Csizmadia et al. 2022). The absence of the endosome-to-TGN retrograde traffic (*rab6* RNAi) the unmatured small glue granule primordia accumulate in the cytoplasm and rapidly fuse with late endosomes or lysosomes instead of their homotypic fusion (Csizmadia et al. 2022). Interestingly, we have discovered that, ubiquitin may not only regulate the developmental program-dependent, but also necessary for the developmental program-independent glue granule degradation too. We have identified Ub positive, abnormally small glue granules in the *rab6* silenced larval salivary gland cells compared to the control (**Figure 1. A** and **D**).

On the other hand, we examined the connection between glue granule ubiquitin positivity and their designation for degradation. Importantly, the actin filaments recruited to the cytoplasmic surface of glue granules during the exocytosis (Rousso, Schejter and Shilo 2016). Therefore, we examined the colocalization of actin (exocytotic glue granules) with LyfeAct and ubiquitin (glue granules marked for degradation) with GFP-Ub. We did not find any colocalization between these factors on the surface of glue granules. Therefore, it is possible that, Ub designates glue granules for degradation instead of the exocytosis (**Figure 1. G**).

Moreover, w examined the presented Ub on the surface of glue granules and identified K63-linked polyubiquitin on these granules with linkage specific antibody (**Figure 1. F**).

Taken together, ubiquitin is recruited to the membrane of glue granules from the cytoplasm, when developmental program-dependent or -independent crinophagy is activated in the effected cells. This Ub may be mainly K63-linked, and this molecular sign may direct glue granules for degradation instead of the exocytosis.

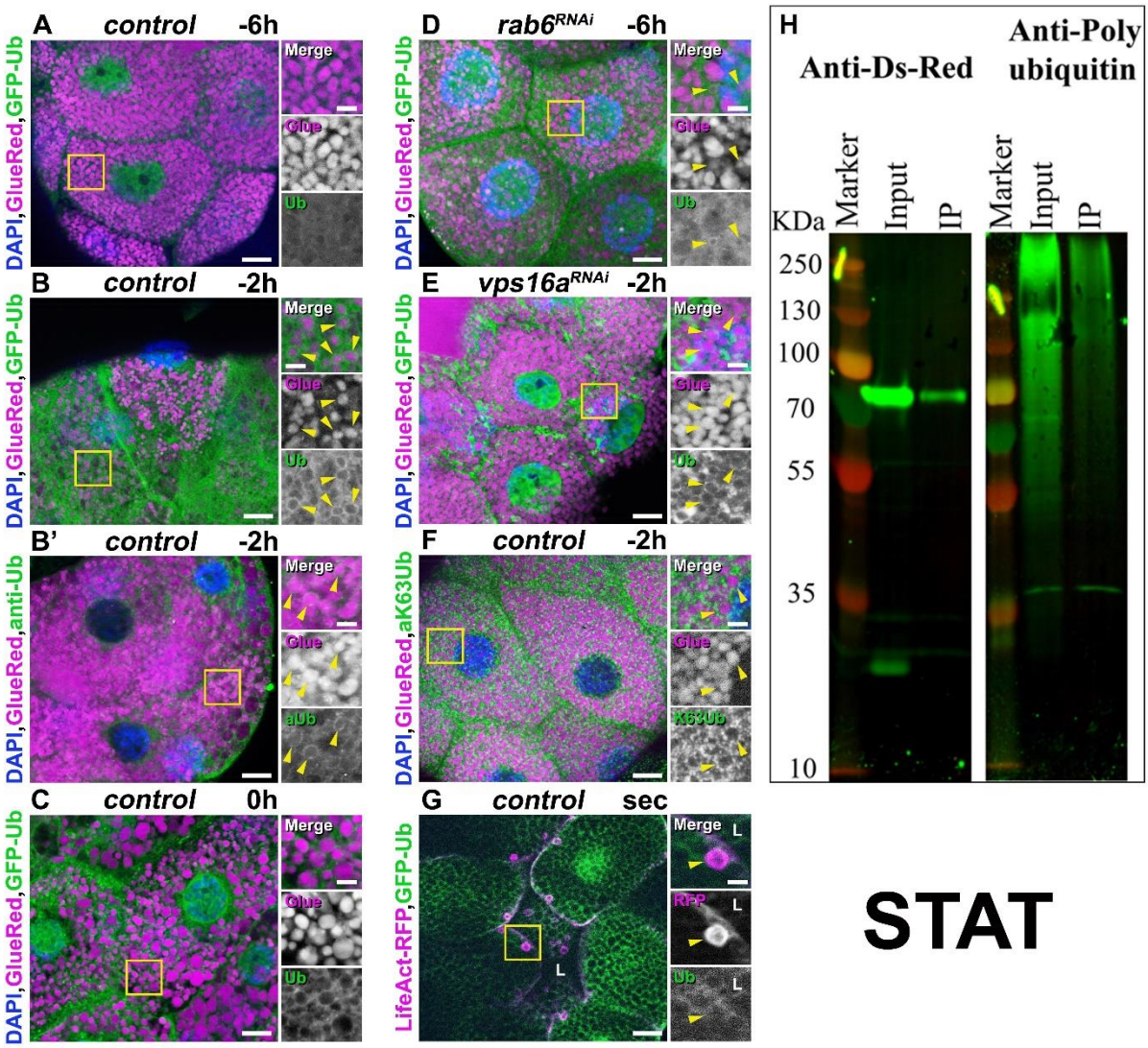

Figure 1 Ubiquitin directs glue granules for crinophagic degradation instead of their exocytosis and time course observation of ubiquitin localization in *Drosophila* larval and prepupal salivary gland cells. (A-C) Salivary glands from animals expressing Glue-DsRed and GFP tagged ubiquitin. (A) Salivary gland cells from wandering larvae (-6h RPF) contain plenty DsRed positive glue granules, and the GFP tagged ubiquitin appears in the cytoplasm and in the nucleus. (B) 2h before puparium formation (-2h RPF), GFP-ubiquitin localizes on the membrane of many glue granules in the salivary gland cells (yellow arrowheads). (B') The endogenous ubiquitin was also detected by ubiquitin specific antibody on the surface of glue granules in the salivary gland cells at the -2h RPF developmental stage (yellow arrowheads). (C) Ubiquitin is disappeared from the surface of most of glue granules/crinosomes in the prepupal salivary glands (0h RPF). Silencing of the gene of Rab6 causes early ubiquitination of the membrane components of glue granules. (D) GFP-ubiquitin can already be detected on the membrane of small glue containing vesicles in the salivary gland cells from the wandering animals, when *rab6* gene is silenced (yellow arrowheads). The *vps16a* RNAi does not perturb the ubiquitin positivity of the membrane of glue containing secretory granules in the late larval salivary gland cells (-2h RPF). (E) GFP-ubiquitin is still localized to the surface of glue granules in the *vps16a* silenced salivary gland cells from the late larval period (yellow arrowheads).
Detection of the type of the ubiquitin chain on glue containing secretory granules. (F) The K63-linked polyubiquitin localizes to the membrane of glue granules, which was detected by K63-linkage specific antibody at the -2h RPF developmental stage (yellow

arrowheads). Ubiquitin does not direct glue granules for exocytosis. (G) GFP-ubiquitin does not localize to the surface of the secreting, actin (Life-act) positive glue granules in the salivary gland cells (yellow arrowheads). (H) Biochemical analysis of the presentation of ubiquitin on the surface of glue granules. (I) Statistics. The boxed regions in panels A–G are shown enlarged on the right side of each panel. Magenta and green channels of merged images are also shown separately as indicated. Scale bars 20µm, insets 5µm.

In our further experiments we searched genes, which regulates the mechanism of ubiquitination and have a role in secretory granule acidification and crinophagic degradation. We made genetic screens, which effect the genes of E3 ubiquitin ligases and deubiquitinating enzymes. We find that, silencing of the gene of Cnot4 E3 ligase shows strong glue granule acidification, glue granule-lysosome fusion and glue granule normal ubiquitination defects (**Figure 2. B, B' and B"**) compared to the control cells (**Figure 2. A-A"**). Moreover, Cnot4 is presented on the surface of glue granules, when the developmentally induced crinophagy is active in the salivary gland cells (**Figure 2. D**). Besides that, we have identified the equivalent role of Usp7 deubiquitinating enzyme in glue granule acidification and granule to lysosome fusion (**Figure 2. C** and **C'**). Importantly, ubiquitin remains for a longer time on glue granules in *usp7* RNAi cells compared to the control, which phenomenon also indicates the role of this enzyme in glue granule degradation (**Figure 2. A"** and **C"**). Furthermore, Usp7 is also presented on the surface of glue granules or crinosomes, when the developmentally programmed crinophagy is activated in the late larval salivary gland cells (**Figure 2. E**).

Importantly, *usp7* RNAi also prevents the developmental program-independent early glue granule degradation in salivary gland. In these cells we simultaneously silenced *usp7* - which is required for crinophagy, and *syx16* lack the huge, only DsRed-positive acidic structures present in only *syx16* RNAi-treated cells (Csizmadia et al. 2022).

Taken together, ubiquitin is required for the glue containing secretory granule designation for crinophagic degradation, may regulate developmental program-dependent and -independent glue granule degradation, and this ubiquitin sign is modulated by Cnot4 E3 ubiquitin ligase and Usp7 deubiquitinating enzyme.

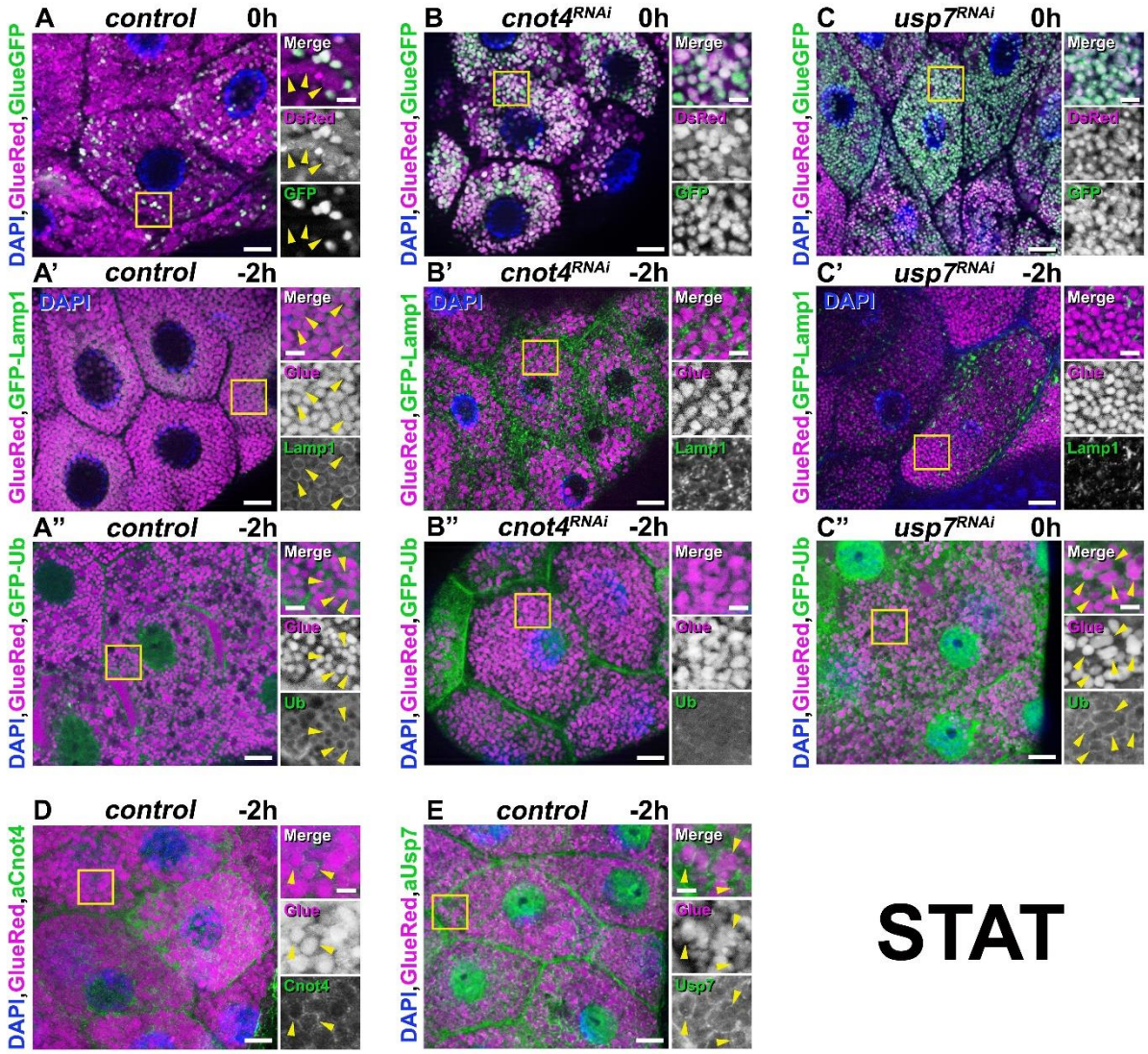

Figure 2 The newly identified regulators of crinophagy. (A-A") Normal designation and degradation of large, glue containing secretory granules in the salivary gland cells of prepupal animals (A), and in the -2h APF late larvae (A' and A"). Salivary glands from prepupal animals (0h APF) co-expressing Glue-GFP/Glue-DsRed (GlueFlux) reporters show normal glue granule acidification state in accordance with this developmental stage together with an RNAi construct for one of the genes involved in ubiquitination and deubiquitination system. (A–C) Glue granule degradation in white prepupal (0 h) salivary glands. (A) The majority of glue granules lack GFP signal in control cells. (B and C) Loss of Cnot4 and Usp7 function leads to persisting GFP signal at the same developmental stage upon salivary gland-specific knockdown of Cnot4 (B) and Usp7 (C). (A'-C') Glue granule fusion with late endosomes and lysosomes at −2 h. (A') GFP-Lamp1 is seen as rings (arrowheads) around DsRed-positive glue granules, indicating ongoing crinophagy in control gland cells. (B' and C') In contrast, no rings are seen and small GFP-Lamp1–positive lysosomes often accumulate near Glue-Red granules in Cnot4 and Usp7 RNAi cells because a block of fusion. (A") Ubiquitin is presented on glue granules at −2 h, but not in Cnot4 RNAi cells (B"). Ubiquitin is still presented on glue granules in white prepupe animals, when ssp7 is silenced in the salivary gland cells (C"). Cnot4 (D) and Usp7 (E) localize to the surface of glue granules. (F) Statistics. The boxed regions in panels A-E are shown enlarged on the right side of each panel. Green and magenta channels of merged images are also shown separately as indicated. Scale bars 20µm, insets 5µm.

Results 2.: Examination of developmental program-independent secretory granule degradation in *Drosophila*

By our other experiments we showed that, glue granule formation, maturation and degradation are developmentally programmed, consecutive mechanisms in *Drosophila* salivary gland cells. Moreover, we have identified proteins, which have a role in the retrograde transport from endosome-to-TGN and we showed that, these factors are required for glue granule maturation and prevent early granule acidification and glue degradation (**Figure 3. A** and **B**). These results indicate that in the salivary gland cells of *Drosophila*, failure of the endosome-to-TGN retrograde trafficking causes strong defects in secretory granule formation and maturation. In addition, these abnormally accumulated, immature granules are prematurely diverted to the acidification and degradation pathway independently of the developmental program. Furthermore, we clearly showed that, in the absence of the endosome-to-TGN retrograde traffic, immature and small glue granules are degraded via crinophagy in *Drosophila* salivary gland cells (**Figure 3. C)**. These results strongly indicate that, deficiencies in the retrograde transport mechanisms cause formation of abnormally small glue granules which are eventually degraded via crinophagy at the wandering developmental stage preceding (and thus independently from) the normal developmental program (Csizmadia et al. 2022).

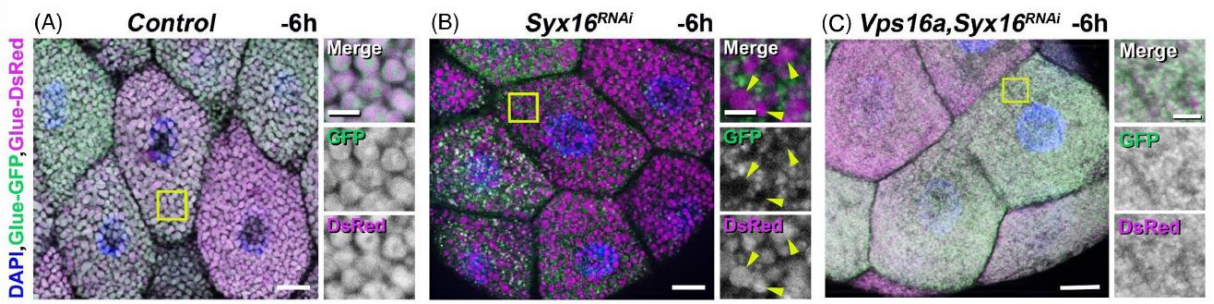

Figure 3 Loss of function of genes involved in the endosome-to-TGN retrograde transport and secretory granule maturation leads to early acidification and breakdown of immature glue granules in *Drosophila* salivary gland cells at the wandering larval stage of development. (A–C) Degradation of very small glue granules via crinophagy in the salivary gland cells of wandering L3 (-6 h RPF) animals co-expressing Glue-GFP/Glue-DsRed (GlueFlux) reporters together with an RNAi construct for one of the gene involved in retrograde transport, secretory granule maturation (B), and crinophagy (C). (A) Control wandering L3 stage (-6 h RPF) larval salivary gland cells normally enclose large (3–3.5 µm), intact (GFP- and DsRed- double positive) glue-containing secretory granules. Compared to the control, salivary gland cells containing the RNAi construct for the SNARE-protein Syx16 (B). Salivary gland cells simultaneously silenced for Vps16a - which is required for crinophagy - (Csizmadia et al. 2018), and Syx16 lack the huge, only DsRed-positive acidic structures present in only Syx16 RNAi-treated cells (B). Under double RNAi conditions, cells were filled mostly with small, immature, and intact glue granules in the wandering animals (C). The boxed regions in panels A-C are shown enlarged on the right side of each panel. Green and magenta channels of merged images are also shown separately as indicated. Bars: 20 µm (A–C), 5 µm (A–C right insets)

In our further experiments we showed the strong cooperation of developmental program-dependent and the newly identified developmental program independent crinophagy in *Drosophila* larval salivary gland cells. This phenomenon suggests that when the endosome-to-TGN retrograde trafficking pathway is damaged in the *Drosophila* salivary gland cells,

immature glue granules are degraded in a cooperative way: firstly, premature crinophagy then by the normal, developmentally programmed crinophagy process (Csizmadia et al. 2022).

Interestingly, we also showed that, the endosome-to-TGN retrograde transport deficiency causes strong secretory defect in salivary gland from the late larval period of *Drosophila*. This result strongly support the role of the endosome-to-TGN retrograde transport mechanism in normal secretory process in the larval salivary gland cells of *Drosophila* (Csizmadia et al. 2022).

Furthermore, our experiments show that, the developmental program-independent glue granule degradation is launched early in Vps53-silenced salivary gland cells of *Drosophila* independently of the secretory defect. This observation points that, disruption of the endosome-to-TGN retrograde transport causes early defects in glue granule homotypic fusion, and the accumulated granule primordia fuse preferably with lysosomes (**Figure 4 A** and **B**). This process takes place even during the initial steps of glue granule formation, independently from and preceding the developmental program and manifestation of the secretory defect (Csizmadia et al. 2022).

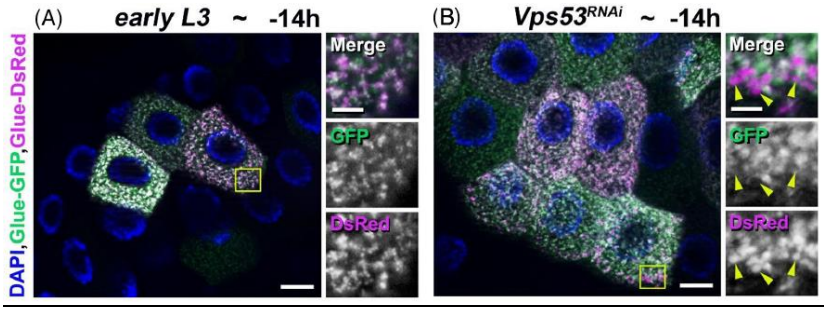

Figure 4 In the absence of Vps53, secretory granule degradation is launched early, independently from the normal developmental program in *Drosophila* salivary gland cells compared to the control. (A, B) Glue granule primordia in salivary gland cells co-expressing the Glue-GFP and Glue-DsRed (GlueFlux) reporters. (A) Early L3 stage (-14 h RPF) salivary gland cells contain very small (0.1–0.5 µm), intact (both GFP- and DsRed-positive) pre-secretory vesicles (primordia) in the control cells. (B) Early L3 stage (-14 h RPF) salivary gland cells contain very small (0.1–0.5 µm), mostly intact (GFP and DsRed-positive) presecretory vesicles (primordia) and larger (1.5–2.5 µm), acidic structures in the *vps53* silenced cells. The boxed regions in panels A and B are shown enlarged on the right side of each panel. Green and magenta channels of merged images are also shown separately as indicated. Bars: 20 µm (A, B), 5 µm (A, B right insets)

Finally, we have discovered that, the R type SNARE protein, Synaptobrevin (Syb) may have a role in glue granule maturation and granule-to-granule fusion, based on our localization data of Syb in the salivary gland cells. This result indicates that, Syb might be a part of the membrane fusion apparat, which coordinates granule-to-granule fusion in *Drosophila* larval salivary gland cells during the development (Csizmadia et al. 2022).

Moreover, we find that, this secretory granule membrane protein, which required for exocytosis is removed from the glue granule membrane, when these granules are degraded by crinophagy. We plan continue the examination of this interesting topic for a potential new publication.

In summary, our new observations with the PD OTKA grant strongly contribute to the better understanding of the molecular background of targeting unnecessary or low-grade secretory granules for developmental program-dependent and -independent crinophagic degradation and strongly help to find answers how this process is activated in the larval salivary gland cells of *Drosophila.* Thank you for this grant.

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